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The Temperature-Dependence of the Loss of Latency of Lysosomal Enzymes

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1. When Triton-filled lysosomes from rat liver are incubated for up to 50min at 37°C, pH7.4, in 0.25M-sucrose, no loss of latency of *N*-acetyl-β-glucosaminidase or *p*-nitrophenyl phosphatase occurs unless the incubated lysosomes are cooled to approx. 15°C. 2. It is suggested that a phase change takes place in the incubated lysosomal membranes on cooling; it starts at approx. 15°C and probably is not complete at 0°C. 3. Incubation of the lysosomes causes an increased potential for loss of latency of the lysosomal enzymes. This potential is not fully expressed at elevated temperature (e.g. 37°C), but is expressed on cooling. 4. The increase at elevated temperature in potential for loss of latency exhibits biphasic kinetics, with an initial rapid phase followed by a slower phase, which is linear with respect to time. The extra loss of latency resulting from the rapid phase in proportional to the temperature of the incubation. 5. Arrhenius plots of the increase is potential for loss of latency during the slow phase for *N*-acetyl-β-glucosaminidase and *p*-nitrophenyl phosphatase exhibit marked deviations from linearity beginning at approx. 15°C. This suggests that the increase in potential for loss of latency is affected by a phase change that occurs around this temperature. 6. Activation energies for the increase in potential for loss of latency at and above 22°C are 53.1±5.4kJ/mol (12.7±1.3kcal/mol) for *N*-acetyl-β-glucosaminidase and 45.2±7.5kJ/mol (10.8±1.8kcal/mol) for *p*-nitrophenyl phosphatase. It is postulated that these energies reflect enzymic action, the products of which cause loss of latency to occur on cooling.

Much interest has focused on possible stabilizers and labilizers of lysosomal membranes (de Duve *et al.*, 1962; Weissmann, 1969; Ignarro, 1975), but very little is actually known about the molecular mechanisms involved in the maintenance or loss of latency of lysosomal enzymes. As a possible means towards gaining an understanding of the molecular basis of the loss of latency of lysosomal enzymes, we have characterized the temperature-dependence of this loss at neutral pH of some acid hydrolases of Triton-filled lysosomes in the presence of osmotic protection (0.25M-sucrose).

Experimental

Preparation of Triton-filled lysosomes

For a typical experiment four male Sprague–Dawley rats (Charles River Breeding Laboratories, Wilmington, MA, U.S.A.) weighing 200–300g were injected intraperitoneally with 1ml of a 25% (w/w) solution of Triton WR-1339 in 0.9% NaCl. For maximum yield of Triton-filled lysosomes it was essential that the solution of Triton WR-1339 was freshly prepared. At 4 days after injection the rats were stunned and decapitated. The livers were perfused with ice-cold 0.25M-sucrose/0.003M-$MgCl_2$/0.02M-Tris/HCl, pH7.2. All subsequent steps were performed at 0–4°C. The perfused livers were extracted in the above buffer by homogenization for 2s in a Sorvall Omni-Mixer, followed by three up-and-down strokes in a Thomas Teflon-pestle homogenizer. Triton-filled lysosomes were then prepared essentially as described by Weglicki *et al.* (1974). The 20000*g* pellet was suspended in 35ml of extraction buffer and 10ml was layered on each of three gradients consisting of 9ml of 0.94M-sucrose overlaid with 10ml of 0.82M-sucrose (unbuffered). The material at the 0.82M-/0.25M-sucrose interface after centrifugation for 2h at 90000*g* in a swinging-bucket rotor was collected, diluted 10-fold with extraction buffer and centrifuged at 70000*g* for 30min. When *N*-acetyl-β-glucosaminidase was to be assayed, the

Abbreviation used: Mops, 4-morpholinepropanesulphonic acid.

lysosomes pelleted thus were resuspended to a concentration of approx. 1 mg of protein/ml by three up-and-down strokes in a Thomas Teflon-pestle homogenizer in 30–35 ml of buffer containing 0.25 M-sucrose and 0.04 M-Mops, adjusted at 22°C to pH 7.4 with NaOH. Protein was quantified by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. When *p*-nitrophenyl phosphatase was to be assayed, the lysosomes were suspended in approximately twice this amount of the Mops/sucrose buffer. The lysosomal suspension was then divided into several portions for incubation at various temperatures.

Incubation of Triton-filled lysosomes

Incubations were performed in either a Precision Scientific Shaker Bath (Fisher Scientific, Medford, MA, U.S.A.) or a Forma Scientific Refrigerated Bath (Forma Scientific, Marietta, OH, U.S.A.). After being placed in the incubation bath, samples were shaken for at least 5 min to ensure uniform equilibration of temperature. At timed intervals samples were removed and were either returned to the ice (or another incubation bath) before assay or were added directly to assay medium at 37°C.

The temperature of the lysosomal suspension during incubation was measured with a probe-equipped laboratory thermometer from Bailey Instruments Co., Saddle Brook, NJ, U.S.A.

Enzyme assays and determination of percentage free activity

N-Acetyl-β-glucosaminidase was assayed by the method of Woollen *et al.* (1961), with 0.0036 M-*p*-nitrophenyl *N*-acetyl-β-glucosaminide as substrate. A 0.1 ml sample of the lysosomal suspension was assayed at 37°C in 1 ml of assay medium (0.05 M-sodium citrate/0.05 M-citric acid, pH 4.5, with 1 mg of bovine serum albumin/ml) containing either 0.25 M-sucrose or 0.1% Triton X-100. After 3 min, the reaction was stopped by addition of 0.5 ml of 25% (w/v) trichloroacetic acid. After centrifuging at 1300*g* for 10 min to remove precipitated protein, 1 ml of supernatant was added to 1.5 ml of 2 M-NH_3 adjusted to pH 10.7 with HCl. Percentage free activity was calculated as: 100 × activity in 0.25 M-sucrose/activity in 0.1% Triton X-100.

p-Nitrophenyl phosphatase was assayed with 0.005 M-*p*-nitrophenyl phosphate as substrate in assay medium (0.1 M-sodium acetate/0.1 M-acetic acid, pH 5.0) containing either 0.25 M-sucrose or 0.1% Triton X-100. A 0.1 ml sample of the lysosomal suspension was assayed in 1 ml of assay medium at 37°C for 3 min, after which the reaction was stopped and the colour developed as described for *N*-acetyl-β-glucosaminidase. Percentage free activity was calculated as described above.

β-Glucuronidase was assayed with 0.001 M-phenolphthalein glucuronide as substrate in assay medium (0.1 M-sodium acetate/0.1 M-acetic acid, pH 5.0, with 1 mg of bovine serum albumin/ml) containing 0.01% Triton X-100. A 0.1 ml sample of the lysosomal suspension was assayed in 1 ml of assay medium at 37°C for 15 min, after which the reaction was stopped and the colour developed as described by Gianetto & de Duve (1955).

Analysis of data

Least-squares regression analysis was used to determine best-fit lines for kinetic data and for Arrhenius plots (Steel & Torrie, 1960). To estimate significance of differences between slopes of lines, confidence intervals were constructed about the mean values being compared (Steel & Torrie, 1960).

Results

Triton-filled lysosomes maintained at pH 7.4 at 0°C displayed an increase in free activity of both *N*-acetyl-β-glucosaminidase and *p*-nitrophenyl phosphatase that was linear with respect to time for up to 28 h (Fig. 1).

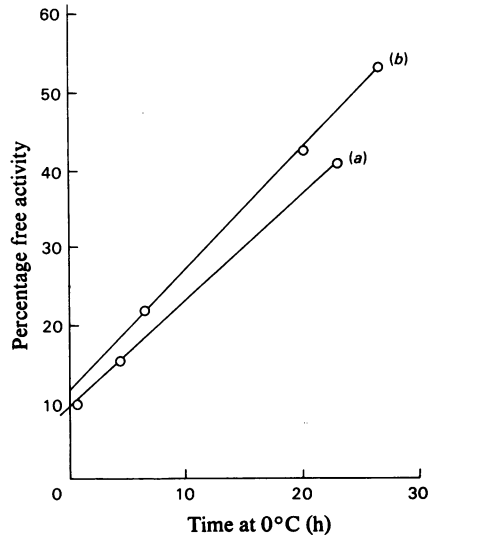

Fig. 1. *Rate of increase in percentage free activity at 0°C*
Triton-filled lysosomes were maintained on ice for up to 28 h. Samples were assayed for percentage free activity at the time intervals indicated. Lines (*a*) and (*b*) represent single experimental determinations of the rate of increase in free activity of *N*-acetyl-β-glucosaminidase and *p*-nitrophenyl phosphatase respectively. The mean ± S.E.M. for 21 determinations of the increase in percentage free activity of *N*-acetyl-β-glucosaminidase per 10 min at 0°C was 0.22 ± 0.01%. That for six determinations of the increase in percentage free activity of *p*-nitrophenyl phosphatase per 10 min at 0°C was 0.21 ± 0.02%.

When the Triton-filled lysosomes were incubated at pH 7.4 at 37°C and were assayed immediately at 37°C without intermittent cooling, there was no increase in free activity observed for either enzyme after incubation for as long as 50min (Figs. 2*a* and 2*b*). However, if the lysosomes were incubated at 37°C and then cooled to 0°C before assay, a marked increase in free activity was observed (Figs. 2*a* and 2*b*). (Total activity in the presence of Triton X-100 remained constant both during the incubation at 37°C and after cooling to 0°C.)

To ascertain that the loss of latency took place on cooling and not during the rewarming that occurred before assay at 37°C, Triton-filled lysosomes were incubated for 50min at 37°C. An assay was performed immediately at the end of the incubation to determine the percentage free activity before cooling. The remainder of the sample was placed on ice for 45min, after which assays were performed both at 37°C for 3min and at 0°C for 40min. Before cooling, percentage free activity of *N*-acetyl-β-glucosaminidase was 9.3–11.3 (range of duplicates). After cooling, percentage free activity was 55.0–57.1 when the assay was at 37°C and was 59.5–60.7 when the assay was at 0°C. Thus the loss of latency occurred on cooling, not on rewarming.

The effect of cooling on the release of soluble enzymes as determined for *N*-acetyl-β-glucosaminidase and β-glucuronidase is shown in Table 1. The extent to which these enzymes became solubilized after incubation at 37°C was greatly enhanced by cooling to 0°C. This is further evidence that effects

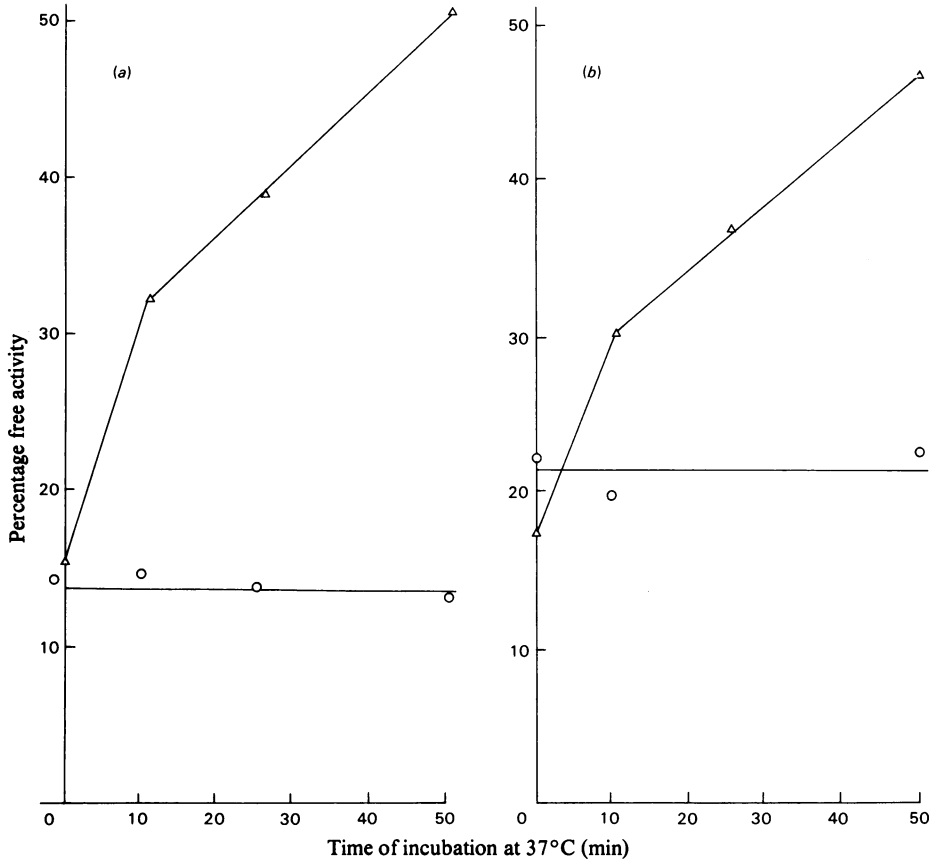

Fig. 2. *Rate of increase in percentage free activity of (a) N-acetyl-β-glucosaminidase and (b) p-nitrophenyl phosphatase caused by incubation at 37°C*

Triton-filled lysosomes were incubated at 37°C. Shown are percentage free activities found when samples were removed at the indicated times and added immediately to assay medium at 37°C (○) or placed on ice for at least 45min, after which they were assayed at 37°C (△).

Table 1. *Effect of cooling on release of soluble enzymes after incubation at 37°C*

Triton-filled lysosomes were incubated at 37°C. At the indicated times, samples were removed and either centrifuged immediately at approx. 24°C or placed on ice for 15min before centrifuging at 4°C. Centrifugation was at 90000*g* for 10min. An unsedimented sample was assayed for total activity. Total and soluble activities were measured in the presence of 0.1% Triton X-100 (*N*-acetyl-β-glucosaminidase) or 0.01% Triton (β-glucuronidase). Results are given as ranges of duplicates.

	Time of incubation at 37°C (min)	Percentage of soluble activity	
		After cooling to 24°C	After cooling to 0°C
N-Acetyl-β-glucosaminidase	0	—	2.8–2.9
	10	3.7–3.8	15.8–17.1
	50	8.6–8.7	33.7–33.9
	100	11.7–14.8	52.9–53.2
β-Glucuronidase	0	—	5.5–5.8
	10	8.1–8.4	25.8–27.2
	50	13.9–14.5	46.2–48.3
	100	17.7–20.5	66.2–68.3

Table 2. *Effect of cooling on loss of latency of lysosomes from rats not injected with Triton WR-1339*

A partially purified lysosomal fraction was prepared from livers of untreated rats (not injected with Triton WR-1339). Livers were homogenized in 0.33M-sucrose/0.001M-EDTA/0.01M-Mops, pH7.2. The homogenate was centrifuged at 3300*g* for 10min. The sediment was discarded and the supernatant was centrifuged at 16000*g* for 20min. The sediment was washed six times by resuspension followed by centrifugation at 16000*g* for 10min. The first two washes were done with the buffer used for homogenization; the second two were with the homogenization buffer to which 0.3M-KCl had been added; the last two were with the homogenization buffer. The final sediment was resuspended in the homogenization buffer. Purification over the homogenate was approx. 15-fold for *N*-acetyl-β-glucosaminidase. During incubation at 37°C, samples were removed at the indicated times and either assayed immediately at 37°C or placed on ice for exactly 15min before assay at 37°C. Results are given as ranges of duplicates.

Time of incubation at 37°C (min)	Percentage free activity	
	Without cooling	After 15min at 0°C
0	14.2–15.3	15.0–15.3
30	18.3–18.6	27.4–27.4
120	20.8–22.9	38.3–40.4

observed after cooling are due to the cooling itself, since no rewarming was involved in obtaining the solubilized enzyme.

We wished to ascertain that the effect of cooling was not unique to Triton-filled lysosomes. We therefore measured the loss of latency occurring after incubation at 37°C of a lysosomal fraction prepared from normal rat liver (i.e. the rats had not been injected with Triton WR-1339). The results (Table 2) show that loss of latency was greatly enhanced by cooling.

The temperature to which the lysosomes must be cooled to elicit the cold-induced increase in free activity was determined for *N*-acetyl-β-glucosaminidase. Fig. 3 depicts two experiments in which Triton-filled lysosomes that had been incubated for 50min at 37°C were subjected to cold stress for an additional 15min at temperatures from 37°C to 0°C. The threshold of the cold-induced increase in free activity occurred at approx. 15°C. When the lysosomes had been incubated for only 10min at 37°C, the curve obtained by plotting increase in percentage free activity versus temperature as in Fig. 3 had the same shape as that in Fig. 3, but the overall increase in percentage free activity was smaller. These results suggest that, starting at approx. 15°C, a phase change occurs in the structural lipids of the lysosomal membrane.

The rate of the cold-induced increase in free activity at various temperatures is shown in Fig. 4. The temperature to which the lysosomes were cooled did not seem to affect the rate of increase in free activity as much as it affected the total increase in free activity that occurred after cooling. If the extent of loss of latency is taken as an index of the extent of phase change, then it appears that the phase change takes place over a fairly broad temperature range and is probably not complete at 0°C.

These results indicate that incubation at 37°C at neutral pH alters the Triton-filled lysosomes by inducing an increased potential for loss of latency of the lysosomal enzymes However, this potential is not expressed until the lysosomes have been subjected to cooling. The method that we used to measure this potential for loss of latency is illustrated schematically in Fig. 5. During incubation at elevated temperature

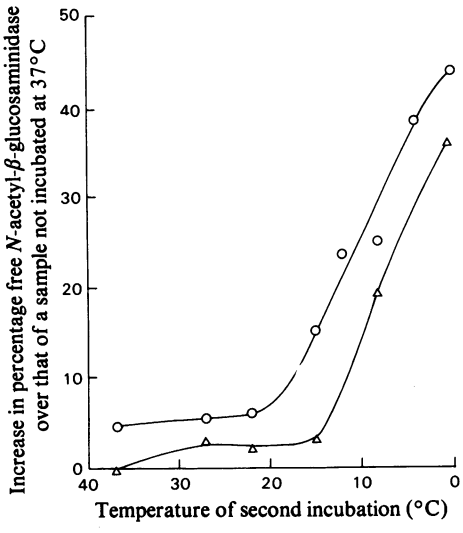

Fig. 3. *Temperature-dependence of loss of latency after incubation at 37°C*

Samples of Triton-filled lysosomes which had been on ice were assayed at 37°C to determine percentage free *N*-acetyl-β-glucosaminidase at zero time of incubation. The remainder of the suspension was then incubated at 37°C for 50min, after which the incubated suspension was placed in a constant-temperature bath at a given temperature as indicated on the abscissa, or on ice, for an additional 15min. Percentage free activity was then determined by assaying at 37°C. The ordinate shows the increase in percentage free activity over that of the non-incubated sample. ○ and △ shows results from two separate experiments.

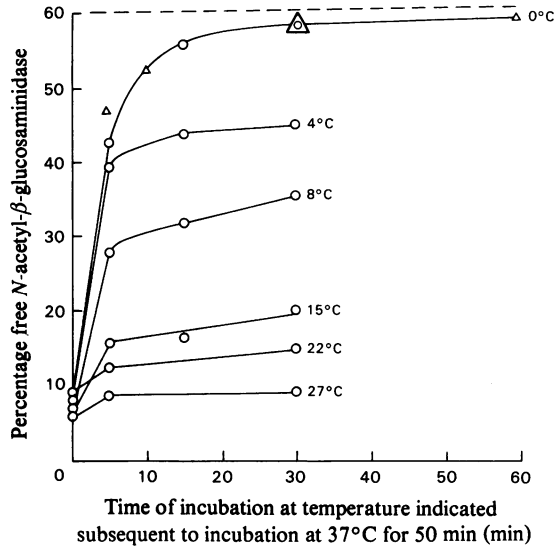

Fig. 4. *Rate of loss of latency at various temperatures after incubation at 37°C*

Triton-filled lysosomes were incubated at 37°C for 50min. A sample was assayed immediately to determine percentage free *N*-acetyl-β-glucosaminidase. Other samples were placed in constant-temperature baths at the temperatures shown, or on ice, for a second incubation. During this second incubation, at various time intervals as indicated on the abscissa, samples were removed for assay of percentage free activity. ○ and △ represent separate experiments. The dashed line has a slope equal to the rate of increase in percentage free activity of a sample maintained at 0°C throughout, and is included to illustrate the extent to which the rate of increase in free activity of the incubated sample, after cooling to 0°C, returned to that of a non-incubated sample.

(e.g. 37°C) samples were removed and returned to an ice bath. After a variable length of time, but at least 45min, all the timed samples were assayed at once (usually 2–3h after the end of the incubation). Thus increase in free activity was measured relative to the increase in free activity in a sample that remained at 0°C throughout (the zero-time sample). To calculate the actual loss of latency resulting from incubation at an elevated temperature, the increase in percentage free activity that would have occurred in a sample maintained at 0°C for the same amount of time was added to the measured increase in percentage free activity over the zero-time sample. It was assumed that, by 45min after return to 0°C, the rate of increase in free activity had become equal to that of lysosomes maintained at 0°C throughout. This assumption was made on the basis of the results for the samples cooled to 0°C illustrated in Fig. 4. The dashed line represents the rate of increase in percentage free activity at 0°C, which had an average value of 0.21%/10min for these two experiments. We also measured the rates of increase in percentage free activity occurring between 2 and 16h after return to 0°C for samples incubated at various temperatures for various lengths of time. These rates are shown in Table 3 and indicate that, even when extensive damage had occurred, the final rate of increase in free activity after return of an incubated sample to 0°C was not greatly different from that of a sample maintained at 0°C throughout. To ascertain that results were not substantially affected by any differences in lengths of time that samples were kept at 0°C after incubation at an elevated temperature, in some experiments we measured the increase in free activity that had occurred after an exact amount of time at 0°C. When samples were assayed exactly 45 min after return to 0°C after incubation at 37°C, no significant differences in rate of increase of free activity were found com-

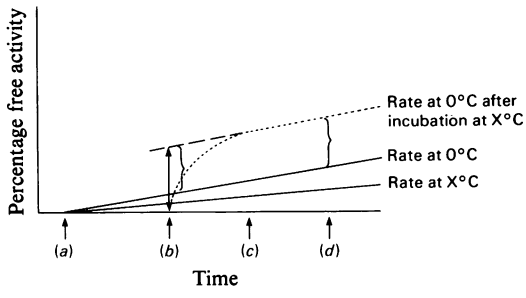

Fig. 5. *Determination of increase in percentage free activity resulting from incubation at elevated temperature*
This is a schematic representation of a situation in which a sample is incubated at an elevated temperature, X°C (e.g. 37°C), starting at time (*a*) and ending at time (*b*), whereupon the sample is returned to 0°C before assay. An increase in free activity occurs on return to 0°C as indicated by the broken line. After a certain length of time, the rate of increase in free activity of the incubated sample becomes equal to that of a sample maintained at 0°C throughout; the point in time at which this occurs is indicated by (*c*). The rate after time (*c*) is extrapolated (dashed line) back through time (*b*) to obtain the actual increase in free activity caused by the incubation at elevated temperature, indicated by the double-headed arrow at point (*b*). Both samples, the incubated one and the one maintained at 0°C throughout, are assayed at some later time (*d*). The difference in percentage free activity between the two is indicated by the bracket at (*d*) and also at (*b*). The value of this difference must be added to the amount of increase in percentage free activity that occurred in the sample maintained at 0°C between time points (*a*) and (*b*), in order to calculate the actual increase in percentage free activity that occurred as a result of incubation at elevated temperature.

pared with results obtained after variable lengths of time (at least 45 min) at 0°C.

As illustrated in Fig. 5, the total increase in percentage free activity, measured after incubation at elevated temperature plus an additional incubation at 0°C, is equal to any increase in percentage free activity that occurred at elevated temperature plus the increase that occurred on cooling to 0°C. As illustrated in Fig. 2, the increase in percentage free activity while the lysosomes were maintained at 37°C was zero. Thus, for incubation at 37°C, none of the potential for loss of latency was expressed unless the lysosomes were cooled before assay. We have defined the potential at a given temperature for loss of latency as being equal to the increase in percentage free activity that occurs at that temperature plus the increase in percentage free activity that occurs on cooling the lysosomes to 0°C for a period of time long enough for the rate of increase in free activity to return to that of a sample maintained at 0°C throughout. At temperatures lower than 37°C some of this potential may be expressed before cooling, and by definition the potential for loss of latency at 0°C is equal to the expressed loss of latency.

Table 3. *Final rate of increase in percentage free activity after cooling to 0°C subsequent to incubation at elevated temperature*
Triton-filled lysosomes were incubated at the temperatures indicated. Samples were removed at the times indicated and placed on ice. Percentage free activities were assayed at 2h after the end of the incubation and again at 16 or 14.5h. The final column shows the rate of increase in percentage free activity between the two assays.

(*a*) *N*-Acetyl-β-glucosaminidase

Time of incubation (min)	Percentage free activity: At 2h after incubation*	Percentage free activity: At 16h after incubation†	Increase in percentage free activity/10min†
0	14.7–15.6	32.8–36.8	0.179–0.230
10 at 37°C	30.7–30.9	51.6–57.3	0.216–0.277
50 at 37°C	55.1–57.6	71.0–74.2	0.140–0.199

(*b*) *p*-Nitrophenyl phosphatase

Time of incubation (min)	Percentage free activity: At 2h after incubation*	Percentage free activity: At 14.5h after incubation*	Increase in percentage free activity/10min*
0	22.3–22.4	43.0–45.0	0.237–0.261
50 at 27°C	41.3–41.3	59.5–59.8	0.209–0.213
60 at 4°C	22.2–22.2	40.0–42.0	0.193–0.228

* Range of duplicates.
† Range of triplicates.

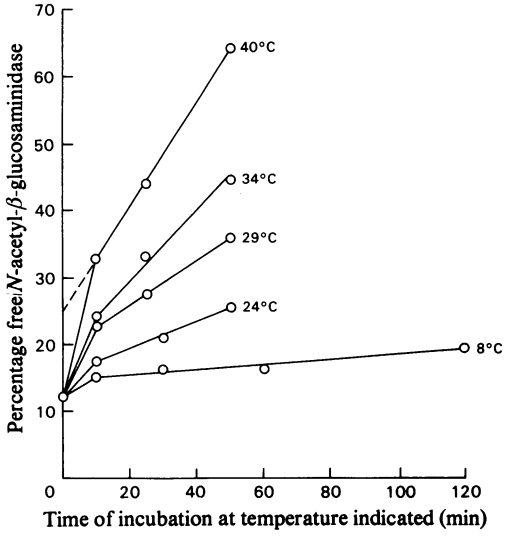

Fig. 6. *Temperature-dependence of the rate of increase in the potential for loss of latency of N-acetyl-β-glucosaminidase*

Triton-filled lysosomes were incubated at the temperatures indicated. At the times indicated, samples were removed and placed on ice for at least 45min, after which they were assayed for percentage free activity of *N*-acetyl-β-glucosaminidase. These are the results of one experiment. See Fig. 8 for explanation of the dashed line.

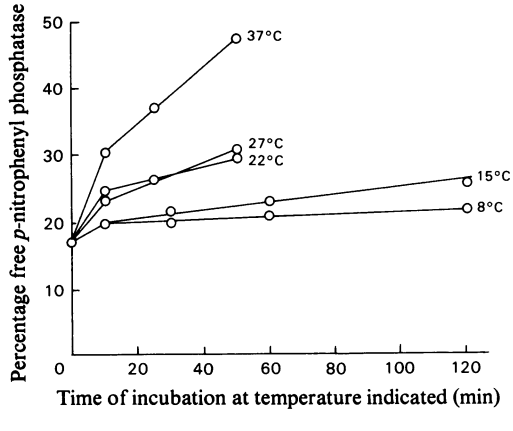

Fig. 7. *Temperature-dependence of the rate of increase in the potential for loss of latency of p-nitrophenyl phosphatase*

Triton-filled lysosomes were incubated as described for Fig. 6, but were assayed for percentage free activity of *p*-nitrophenyl phosphatase. These are results of one experiment.

Table 4. *Percentage free N-acetyl-β-glucosaminidase after short periods of incubation at* 37°C

Triton-filled lysosomes were incubated at 37°C. At the times indicated, samples were removed and placed on ice for at least 45min, after which they were assayed for percentage free activity of *N*-acetyl-β-glucosaminidase. The temperature of the suspension was measured as described in the Experimental section. Results are given as ranges of duplicates.

Time of incubation (min)	Percentage free activity	Temperature of lysosomal suspension (°C)
0	18.3–20.5	0
1	25.4–29.5	29
2	29.0–29.4	35
3	—	37
5	30.1–34.7	37

The increase in potential for loss of latency during incubation at various temperatures, as measured by the increase in percentage free activity after a second incubation at 0°C, is illustrated in Figs. 6 and 7. These curves have been corrected, as described, for the increase in percentage free activity of each enzyme at 0°C. The increase in potential for loss of latency appears to occur in two stages: an initial rapid phase followed by a slower phase. The rapid phase seems to be finished by the end of the first 10min. The rate between 10 and 50min can be regarded as being linear with respect to time at temperatures as high as 40°C. The rate during the rapid phase is not linear with respect to time, and most of the resulting loss of latency appears to occur on incubation for the time necessary for the temperature of the lysosomal suspension to reach that of the incubation bath, as shown in Table 4.

The latency lost as a result of the rapid phase can be calculated by extrapolating the line for the rate of loss resulting from the slow phase back to zero time (t_0) (see the dashed line in Fig. 6). The extra increase in percentage free activity resulting from the rapid phase is then equal to the extrapolated value minus the actual value for percentage free activity at t_0. For both *N*-acetyl-β-glucosaminidase and *p*-nitrophenyl phosphatase, this extra increase in free activity at a given temperature varied considerably from experiment to experiment, but was found to be roughly linearly proportional to the final temperature of the incubation, as shown in Figs. 8(*a*) and 8(*b*).

The rate of increase in potential for loss of latency during the slow phase was calculated from percentage free activities after incubation for 10min and longer. The rates so obtained at various temperatures were then used to construct Arrhenius plots of the increase in potential for loss of latency during the slow phase.

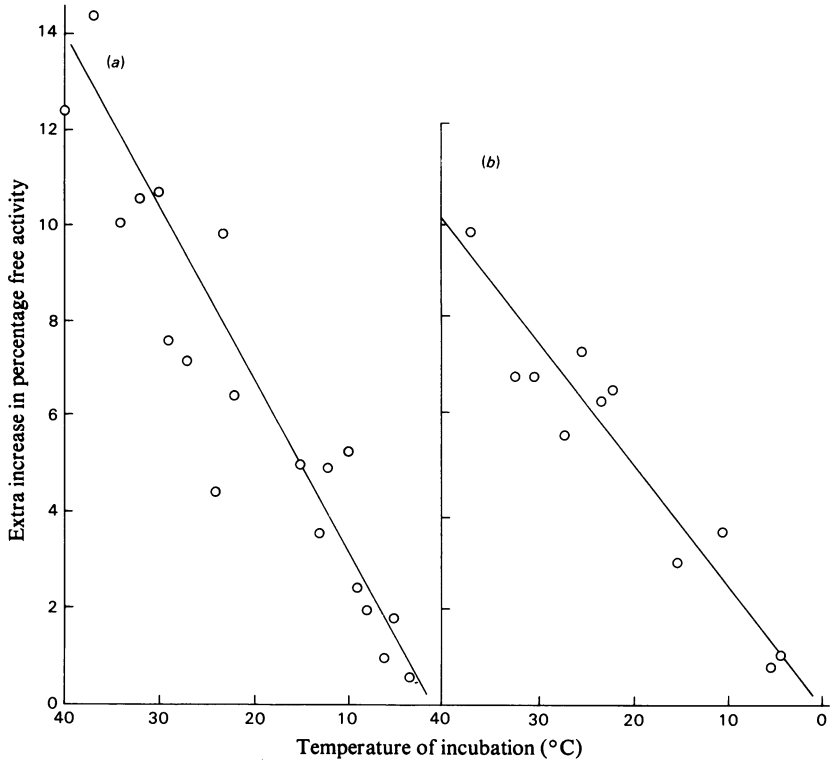

Fig. 8. *Temperature-dependence of the extra increase in potential for loss of latency*
Percentage free activities obtained after incubation at elevated temperature followed by a second incubation at 0°C were plotted against time (min) of incubation at elevated temperature as shown in Figs. 6 and 7. The rate constant for the slow phase of increase in potential for loss of latency was determined by least-squares regression analysis of points at 10min and longer. This best-fit line was then extrapolated to the ordinate. The extra increase in percentage free activity that resulted from the rapid phase was then calculated as being equal to the value of the intercept of the extrapolated line minus the actual value for percentage free activity of the non-incubated (zero-time) sample. Each point represents the average value from one or more experiments of the extra increase in free activity found after incubation at a given temperature. The lines, however, were calculated by least-squares regression analysis of all data points. The correlation coefficient based on all data points for the line for *N*-acetyl-β-glucosaminidase (*a*) is 0.67; that for the line for *p*-nitrophenyl phosphatase (*b*) is 0.85.

Arrhenius plots from individual experiments were combined so as to obtain a composite plot. To do this, an individual plot was shifted up or down the ordinate so that the value at 0°C ($10^4/T = 36.6$) coincided with the average value at 0°C obtained from all experiments. The average values for log(increase in potential for loss of latency/10min) obtained at various temperatures after this normalization are shown in the Arrhenius plots in Fig. 9.

For each plot in Fig. 9 the unbroken line is the best-fit line calculated by regression analysis of all (not the average) data points at and above 22°C. Beginning at approx. 15°C, both plots exhibit a marked deviation from linearity. This suggests that around this temperature a phase change occurs, which affects the rate of increase in potential for loss of latency. The activation energy, ±standard error of the estimate, for the increase in potential for loss of latency at and above 22°C is 53.1±5.4kJ/mol (12.7± 1.3 kcal/mol) for *N*-acetyl-β-glucosaminidase (based on 56 data points) and 45.2±7.5kJ/mol (10.8± 1.8kcal/mol) for *p*-nitrophenyl phosphatase (based on 19 data points). These values are not significantly different from one another ($P<0.5$).

Discussion

We have demonstrated that two stages can be resolved in the loss of latency of rat hepatic lysosomal enzymes resulting from incubation at elevated temperature (e.g. 37°C). In the first stage, which takes place at the elevated temperature, some perturbation

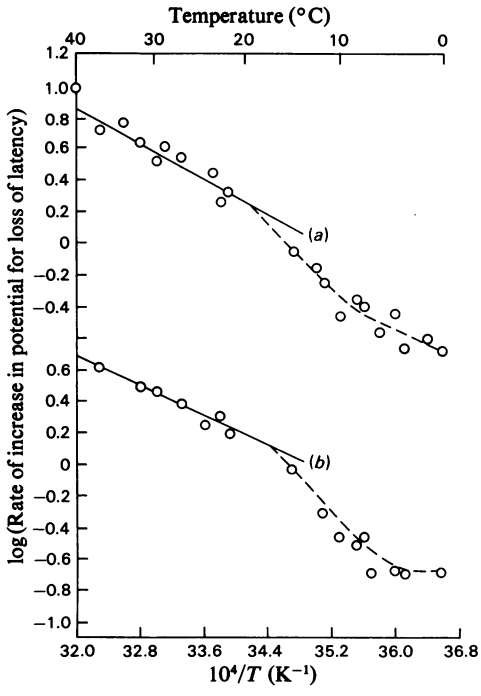

Fig. 9. *Arrhenius plots of the rate of increase in potential for loss of latency*

After incubation at a given temperature the rate constant for the slow phase of increase in potential for loss of latency was determined: the log of this rate was then calculated. The composite plot for each enzyme (*a*, *N*-acetyl-β-glucosaminidase; *b*, *p*-nitrophenyl phosphatase) was obtained by shifting the plots from individual experiments up or down the ordinate so that the values at 0°C coincided with the average value at 0°C obtained from all experiments. Each point on these plots represents the average value, after this normalization, of one or more experimental determination(s) of the log of the rate of increase in potential for loss of latency at a particular temperature.

of the membrane occurs, but latency is still not lost. In the second stage, which takes place on cooling to below approx. 15°C, latency is lost and the extent of loss is determined by the extent of damage incurred at elevated temperature. This is indirect evidence that a phase change occurs within the lysosomal membrane on cooling. The Arrhenius plots shown in Fig. 9 also suggest a phase change, since marked deviations from linearity occur, beginning at approx. 15°C. From the Arrhenius plots we have determined the activation energy for the increase in the potential for loss of latency, in other words the activation energy for the production of the damage that results in loss of latency on cooling. We are concerned both with the nature of this damage and with the mechanism by which it subsequently causes loss of latency.

It has long been recognized that the osmotic fragility of erythrocytes from several species undergoes a decrease as the temperature is elevated (see Aloni *et al.*, 1977). In other work, erythrocytes subjected to the action of phospholipase (*Clostridium welchii*) at 37°C did not exhibit an increase in permeability as long as the temperature was maintained at 37°C; however, on cooling, increases in permeability were observed (Macfarlane, 1950). Zymogen granules prepared at room temperature became leaky, as determined by release of amylase, on cooling to 0°C (Schramm *et al.*, 1967). This was attributed to a phase change occurring in the membrane lipids on cooling. Thus the phenomenon of an increase in permeability of a biological membrane taking place as a result of cooling is already well established. It has long been known that freezing and thawing causes a loss of latency of lysosomal enzymes (Berthet & de Duve, 1951). However, the loss of latency of lysosomal enzymes as a result of cooling at temperatures above 0°C does not seem to have been described. An increase in permeability of lysosomes to K^+ at 0°C compared with 37°C has been reported (Davidson & Song, 1975), and possible causes cited were a thermal transition in membrane lipids or a change in conformation, or aggregation, of membrane proteins.

At present we cannot distinguish between the following possibilities with regard to the existence of both a rapid and a slow phase of increase in potential for loss of latency: (*a*) there may be more than one population of lysosomes present that differ in their relative fragilities, or (*b*) all the lysosomes may undergo an initial rapid alteration that results in partial loss of latency. Most of the extra increase in free activity resulting from the rapid phase was obtained by incubation for only the time necessary for the temperature of the suspension to reach that of the incubation bath. One possibility would be that the extra increase results from a mechanical injury to the membrane caused by warming and cooling. It might be expected, however, that such injury would be inflicted mainly at temperatures within the range of the phase change, and therefore it would be predicted that the extra increase in free activity would level off at incubation temperatures above approx. 15°C. Present data do not indicate that this is the case (see Fig. 8).

An important question arising is: does a phase change occur to the same extent on cooling a sample that has been incubated at 37°C for 10min as on cooling a sample that has been incubated for 50min? If it does occur to the same extent, why is there a lower loss of latency in the sample incubated for the shorter time? If it does not occur to the same extent, why does incubation at 37°C cause an increase in the

extent of the phase change? In other words, there are two possibilities. (*a*) There is a phase change intrinsic to the lysosomal membrane; it occurs to the same extent on cooling no matter how long the membranes have been at 37°C. (*b*) There is little or no intrinsic phase change when undamaged membranes are cooled. However, as the membranes are incubated at 37°C, the damage produced causes increasing amounts of phase change, and therefore increasing loss of latency, to occur on cooling. To answer the question definitely about the extent of phase change occurring after various times of incubation, we need to obtain measurements of the phase change by methods that are independent of the loss of latency. We suggest, however, that the phase change indicated by the Arrhenius plots in Fig. 9 is intrinsic to the undamaged lysosome. These plots reflect the rate, at constant temperature before cooling, of production of membrane damage. If the products of this damage cause the phase change, then, to explain the departure from linearity in the Arrhenius plots, the rates at low temperatures should start at a normal value and then decrease with time, so that the overall measured rate would be lower than expected. This did not appear to be the case, as shown by the linear rates obtained at 0°C (Fig. 1).

One or more membrane component(s) must be lost or altered during the increase in potential for loss of latency. It has long been thought that loss of latency at elevated temperature results from enzymic digestion of the lysosomal membrane (Berthet *et al.*, 1951; Beaufay & de Duve, 1959). Beaufay & de Duve (1959) investigated the loss of latency resulting from treatment at pH7.4 with phospholipase (from *Clostridium welchii*) or trypsin. Both enzymes caused loss of latency of the lysosomal enzymes. These investigators found an activation energy of 79.5kJ/mol (19.0kcal/mol) for the autocatalytic loss of latency of acid phosphatase at pH5. It was suggested that such a low value for the activation energy indicated that the 'thermal release' of free activity was due to enzyme action rather than to denaturation. In addition, an activation energy of 72.0kJ/mol (17.2kcal/mol) was determined for the autolysis of protein at pH5 after disintegration of the lysosomes in a blender. We also suspect that the activation energies that we have found for the increase in potential for loss of latency of *N*-acetyl-β-glucosaminidase and *p*-nitrophenyl phosphatase at pH7.4 reflect enzymic mechanisms of damage. In fact, the temperature-dependence that we have found for the loss of latency of the lysosomal enzymes after incubation at 37°C is strikingly similar to the cold lysis observed for sheep erythrocytes after digestion with phospholipase (*Clostridium welchii*) at 37°C (Macfarlane, 1950). We have previously determined that incubation of Triton-filled lysosomes at pH5 causes the production of non-esterified fatty acids and lysophospholipids, which may have a detergent effect on the membrane structure (Weglicki *et al.*, 1974). Compared with results at pH5, we have found much smaller changes in lipid composition at pH7.4, but they do occur. For instance, in one determination we found increases in non-esterified fatty acids of 31nmol/mg of protein after incubation of Triton-filled lysosomes at 37°C, pH7.4, for 60min (R. C. Ruth, unpublished work). Studies of the effects of lysophosphatidylcholine on the haemolysis of erythrocytes seem pertinent to our findings for lysosomes; evidence has been obtained for the existence of principally different lysis mechanisms above and below 15°C for erythrocytes in sucrose-containing buffer (Weltzien *et al.*, 1976). These investigators suggest that the distribution of lysophosphatidylcholine in mixed lipid phases may be heterogeneous, depending on lipid composition and temperature. Thus local concentrations of lysophosphatidylcholine could be increased by a phase transition resulting in a less-fluid membrane. Applying this theory to lysosomes, with increasing amounts of lysophosphatidylcholine (such as could result from incubation at various temperatures), an intrinsic phase change could result in increasing amounts of damage to the membrane. This damage would be reflected in an increased loss of latency of the lysosomal enzymes.

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