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EFFECT OF INCREASING CONCENTRATIONS OF SALT ON THE LYSOSOMES OF RAT LIVER AND *TETRAHYMENA PYRIFORMIS*

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SUMMARY

When cells are cooled below their freezing point there will be an increase in the concentration of solutes surrounding the subcellular organelles and, for that reason, we have studied the effect of different concentrations of a model solute, KCl, on the lysosomes of rat liver and *Tetrahymena pyriformis*.

The latency of acid phosphatase and β-glucuronidase in homogenates of rat liver fell progressively after exposure to increasing concentrations of KCl and, although the sedimentable acid phosphatase also declined, the decrease was less marked.

After exposure of homogenates of *T. pyriformis* to increasing concentrations of KCl, there was no decrease in the sedimentability of acid phosphatase although a progressive decline in the latency of the enzyme was demonstrated.

The rat liver lysosomal membrane was shown to be only slightly permeable to KCl although the lysosomes of *T. pyriformis* were much more permeable to this salt. It seems possible that the disruptive effect of high concentrations of KCl is due to transient osmotic pressure gradients which may arise when the concentration is reduced by dilution.

INTRODUCTION

Providing supercooling does not occur, any lowering of the temperature of tissues or suspensions of cells below the freezing point results in the formation of ice crystals. However, the distribution of these crystals either within the cell or in the extracellular fluid depends on the cooling rate and on the permeability of the cell membrane to water[1]. Nevertheless, the formation of ice will result in an increase in the solute concentration in the fluid remaining within the cell, irrespective of whether ice crystals occur intracellularly or extracellularly.

Lovelock[2,3] has studied the effect of salt on the erythrocyte membrane but little or no work has been done on the effect of solutes on subcellular organelles. We therefore decided to study the effect of increasing solute concentrations on the lysosomes of rat liver and *Tetrahymena pyriformis*. Lysosomes were chosen for the study since they are known to be damaged by freezing and thawing[4-6], and cells are likely to be damaged irreversibly if their lysosomes are disrupted and the hydrolytic enzymes released into the cytoplasm.

A large part of the complement of low molecular weight anions within any cell is probably provided by phosphorylated compounds[7], however, efforts to determine the composition will depend on the time taken to arrest metabolic reactions[8–10], and the exact composition may also vary from cell to cell. For these reasons we opted to avoid the use of phosphorylated compounds in these studies and have investigated the effect on lysosomes of exposure to increasing concentrations of KCl.

A preliminary account of some of these results has already appeared[11].

MATERIALS AND METHODS

Sucrose and KCl of Analar grade were obtained from Hopkin and Williams Ltd, Chadwell Heath, Essex, while sodium β-glycerophosphate (Grade 1), the anhydrous salt of phenolphthalein glucuronic acid and Triton X-100 were from Sigma (London) Ltd. Kingston-upon-Thames, Surrey. Tryptone and yeast extract were purchased from Oxoid Ltd, London SE1, and bovine serum albumin (Fraction V) was supplied by Armour Pharmaceutical Ltd, Eastbourne, Sussex.

Homogenisation of rat liver

After death by cervical dislocation the livers were removed from August strain rats (150–250 g), weighed, placed in a chilled beaker, chopped into small pieces, suspended in ice-cold solutions of 0.25 M sucrose (1.0 ml/g tissue) and homogenised in a Potter–Elvehjem homogeniser with a teflon pestle.

Growth and homogenisation of T. pyriformis

T. pyriformis, strain S, was grown in the dark in 1 % tryptone–0.05 % yeast extract, harvested 4 days after inoculation and homogenised in the manner described previously[12].

Exposure of rat liver homogenates to increasing concentrations of KCl

All exposures were conducted at 0 °C. Samples of homogenates were diluted with equal volumes of 0.25 M sucrose containing various concentrations of KCl and after 30 min were further diluted with 9 vol. of 0.25 M sucrose. The latency and sedimentability of acid phosphatase (orthophosphoric monoester phosphohydrolase EC 3.1.3.2) and β-glucuronidase (β-D-glucuronide glucuronohydrolase, EC 3.2.1.31) was then determined in the manner described below.

Exposure of homogenates of T. pyriformis to increasing concentrations of KCl

All exposures were conducted at 0 °C in the manner described for rat liver homogenates. The latency and sedimentability of acid phosphatase was determined using the methods described below.

Determination of latency of acid phosphatase

Assays were conducted in triplicate at pH 4.75 using β-glycerophosphate (0.05 M) as substrate[12]. Rat liver homogenates were assayed at 30 °C although homogenates of *T. pyriformis* had to be assayed at 0 °C because of instability of the lysosomes above 10 °C[13]. Latency was defined as the percentage of the total activity which was unmasked by incorporation of 0.05 % Triton X-100 in the reaction mixture.

Determination of sedimentability of acid phosphatase

A sample of diluted homogenate was subjected to centrifugation (300000 g·min) at 0 °C and the supernatant removed for determination of non-sedimentable acid phosphatase activity (in the presence of 0.05 % Triton X-100) using the conditions described above. The pellet was resuspended in ice-cold 0.25 M sucrose and the sedimentable acid phosphatase activity determined using this resuspended material. In this procedure it was possible to use a temperature of 30 °C for the assay of fractions from both rat liver and *T. pyriformis* since all lysosomes are disrupted by the Triton X-100 present in the assay mixtures.

Determination of latency of β-glucuronidase

The activity of this enzyme was determined both in the presence and in the absence of 0.05 % Triton X-100 using the method of Gianetto and de Duve[5] and latency was defined as above.

Assessment of the permeability of rat liver and T. pyriformis lysosomal membranes to 0.15 M KCl.

Samples of homogenates at 0 °C were added to 4 vol. of ice-cold 0.15 M KCl. At timed intervals thereafter aliquots were removed and diluted with an equal volume of 0.5 M sucrose. The latency of acid phosphatase was then determined using the method described above.

Estimation of protein concentration

The method of Lowry *et al.*[14] was used with bovine serum albumin as standard.

RESULTS

In order to determine the effect of residual KCl on the latency of acid phosphatase and β-glucuronidase, samples of homogenates were assayed in the absence and in the presence of KCl at the appropriate concentrations. The values obtained for latency and sedimentability are shown in Table I and, while in four instances there was no significant difference, the presence of KCl caused a small but statistically significant reduction in the sedimentability of acid phosphatase in homogenates of rat liver. The mean specific activities of the enzymes in control samples of homogenates are also shown in Table I and it is interesting to note that the values were not changed significantly by the presence of KCl in control samples of homogenates. After exposure to high concentrations of KCl the specific activities were also unaffected except in the case of acid phosphatase in homogenates of *T. pyriformis* (assayed at 0 °C) where the mean specific activities were about 75 % of the control value of 5.34 mI.U./mg protein.

The latency of acid phosphatase and β-glucuronidase showed a progressive decline when homogenates of rat liver were exposed to increasing concentrations of KCl (Fig. 1). A similar decline was obtained with homogenates prepared in 0.25 M β-glycerophosphate (J. Osborne and D. Lee, unpublished results). This decline in latency could have been due either to the release of acid phosphatase from the lysosomes or to an alteration in the permeability of the lysosomal membrane which allowed entry of the substrate to the lysosomes during the determination of 'free'

TABLE I

LATENCY, SEDIMENTABILITY AND SPECIFIC TOTAL ACTIVITY (I.U.) OF ACID PHOSPHATASE AND β-GLUCURONIDASE

Source of enzymes	*Enzyme studied and assay temperature*	*Mean of latency or sedimentability in homogenates prepared in 0.25 M sucrose ± S.E.; n = 4*	*Mean of latency or sedimentability in homogenates containing 0.25 M sucrose–0.15 M KCl (rat liver) or 0.25 M sucrose–0.25 M KCl (T. pyriformis) ± S.E.; n = 4*	*Mean specific activity in homogenates prepared in 0.25 M sucrose (mI.U./mg protein) ± S.E.; n = 4*	*Mean specific activity in homogenates containing 0.25 M sucrose–0.15 M KCl (rat liver) or 0.25 M sucrose–0.25 M KCl (T. pyriformis) ± S.E.; n = 4*
Rat liver	Latency of acid phosphatase (30 °C)	83.4 ± 1.1	83.5 ± 1.6	7.6 ± 1.4	8.3 ± 1.9
	Percentage of total acid phosphatase which is sedimentable (30 °C)	88.2 ± 1.1 *	76.5 ± 2.2 *	8.8 ± 1.6 **	7.1 ± 1.4 **
	Latency of β-glucuronidase (37 °C)	69.1 ± 3.2	68.0 ± 4.0	2.2 ± 0.2	2.4 ± 0.3
T. pyriformis	Latency of acid phosphatase (0 °C)	56.5 ± 2.4	55.3 ± 2.0	62.8 ± 3.5	53.4 ± 2.5
	Percentage of total acid phosphatase which is sedimentable (30 °C)	70.8 ± 4.0	71.9 ± 3.3	305.3 ± 36.5	273.0 ± 39.1

* $n = 6$; these means are significantly different at the level $P < 0.05$.
** $n = 6$, but the means are not significantly different.

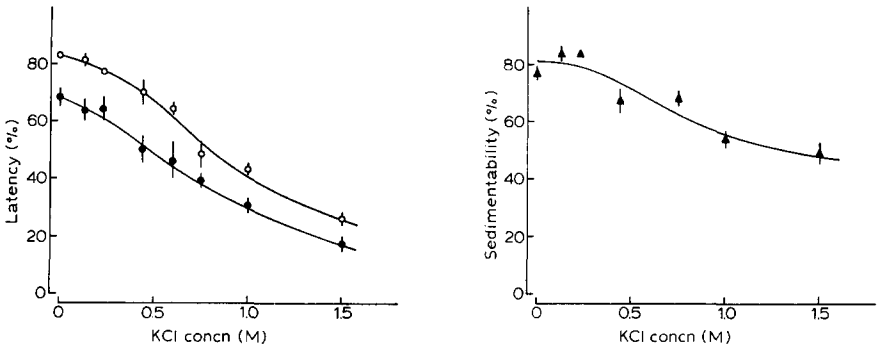

Fig. 1. The latency of acid phosphatase (○) and β-glucuronidase (●) remaining after exposure of rat liver homogenates to different concentrations of KCl. The points represent the mean of four determinations and, in all figures, the vertical lines represent the standard error of the means although, in some cases, these were too small to be shown.

Fig. 2. The percentage of total acid phosphatase which remains sedimentable after exposure of homogenates of rat liver to different concentrations of KCl. The points represent the mean of six determinations.

acid phosphatase activity. For that reason, we also used centrifugation to assess whether acid phosphatase was released from lysosomes after exposure to increasing concentrations of KCl. Fig. 2 also shows a decline in the sedimentability of acid phosphatase and, although the percentage sedimentabilities are higher than the corresponding values for latency, the difference is probably due to non-specific adsorption of acid phosphatase to sedimentable material[15].

A decline in latency of acid phosphatase was also found when homogenates of *T. pyriformis* were exposed to high concentrations of KCl. There was, however, no similar decrease in the sedimentability of this enzyme (Fig. 3). This may have been due to non-specific adsorption of acid phosphatase, nevertheless, we have been able to use this method to demonstrate a decline in sedimentability of acid phosphatase in homogenates of *T. pyriformis* incubated at temperatures between 10 and 30 °C (C. Allen and D. Lee, unpublished results).

The effect of increasing concentrations of KCl on rat liver lysosomes could result from a direct effect of salt on the lysosomal membrane or from transient osmotic pressure gradients which would arise on dilution of the homogenates with 0.25 M sucrose if KCl passed through the lysosomal membrane much more slowly than water. The early work on rat liver lysosomes showed the lysosomal membrane to be permeable to KCl and NaCl[4,15,16] and it seemed possible that the release of lysosomal enzymes after exposure to high concentrations of KCl could indeed result from transient osmotic pressure gradients. But, more recently, data have been published which indicate that rat liver lysosomes are impermeable to NaCl[17]. Consequently, we decided to reinvestigate the permeability of the rat liver lysosomal membrane. The results are presented in Fig. 4 and show that KCl enters rat liver lysosomes relatively slowly. The pH of the homogenates used was close to 7.0 and was therefore different from the value of 6.1 used in previous studies[15] but we have also obtained results similar to those presented above using homogenates in which the pH was adjusted to 6.1.

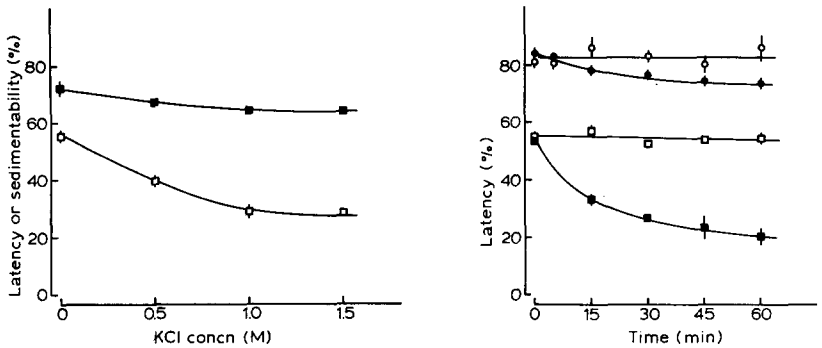

Fig. 3. The latency (□) and sedimentability (■) of acid phosphatase remaining after exposure of homogenates of *T. pyriformis* to different concentrations of KCl. The points represent the mean of four determinations.

Fig. 4. The latency of acid phosphatase remaining after dilution of homogenates with 4 vol. of 0.25 M sucrose (○, rat liver; □, *T. pyriformis*), or with 4 vol. of 0.15 M KCl (●, rat liver; ■, *T. pyriformis*).

Since uncharged molecules have been found to pass through the membrane of *T. pyriformis* lysosomes more readily than they traverse the rat liver lysosomal membrane[18,19], it seemed likely that KCl would pass more rapidly into the lysosomes of the protozoan and Fig. 4 shows that this is indeed so.

DISCUSSION

When cells are frozen, the subcellular organelles are subjected to high concentrations of the low molecular weight solutes which are normally present within the cell and, if the results obtained with KCl (Fig. 1) are representative of the response of rat liver lysosomes to other salts, then it is probable that the lysosomes in rat liver cells would be irreversibly damaged by freezing and thawing. However, the properties of lysosomes from different cells are not identical[13] and it is not surprising to find that the lysosomes of *T. pyriformis* respond differently to high concentrations of KCl (Fig. 3).

The early work of de Duve and his colleagues[4,15,16] showed that rat liver lysosomes were freely permeable to salts such as KCl, but the results of Lloyd[17], and those shown in Fig. 4 indicate that such salts pass through the rat liver lysosomal membrane slowly; the reason for the different findings is not known. However, accepting the fact that rat liver lysosomes are not readily permeable to KCl under normal circumstances the disruptive effect of high concentrations of KCl could be explained if the salt readily entered lysosomes under a high concentration gradient. In that case, the disruptive effect could be due to transient osmotic pressure gradients which would occur when the concentration of KCl was reduced by dilution. This mechanism also seems likely since the lysosomes of *T. pyriformis* are more permeable to KCl than those of rat liver and the latency of acid phosphatase in homogenates of the protozoan suffers less from exposure to high concentrations of KCl. Nevertheless, we cannot exclude the possibility that high concentrations of salt have a direct effect on the lysosomal membrane.

It is not easy to explain the fact that the latency of acid phosphatase in homogenates of *T. pyriformis* decreases on exposure to high concentrations of KCl while the sedimentability of this enzyme is virtually unaffected. Nevertheless, these results are in accord with those of Müller *et al.*[20] on the freezing of *T. pyriformis* lysosomes. One possible explanation is that the lysosomes of *T. pyriformis* are sufficiently damaged to allow access of substrate to lysosomal acid phosphatase during the assay procedures although they are not sufficiently damaged to allow release of this enzyme from the lysosomes.

The presence of cryoprotective compounds in tissues and suspensions of cells during freezing reduces the concentration of salts in the unfrozen solution which is in equilibrium with ice at any given temperature and this has been postulated as the mechanism of action of cryoprotective agents[3,21]. Since we have shown that lysosomes are damaged by exposure to high concentrations of salt it follows that the presence of cryoprotective agents could protect lysosomes against damage during freezing. However, cryoprotective agents such as glycerol and dimethylsuphoxide also have a deleterious effect on lysosomes[19,22]. The effect of glycerol seems to be the result of transient osmotic pressure gradients[19] while that of dimethylsulphoxide is dependent on the non-penetrating solute which is present, and is probably the consequence of changes in the permeability properties of the lysosomal membrane[22]. Hence, the effect of dimethylsulphoxide on the lysosomal system *in vivo* will depend on the nature and the composition of the low-molecular-weight non-penetrating solutes present within cells.

Conway[7] analysed the composition of low molecular weight anions which counterbalance the charge of the intracellular cations in muscle and found that phosphorylated compounds provided the bulk of the negative charge. Some of these substances have a role in muscular contraction but it seems possible that phosphorylated compounds provide 'osmotic protection' for lysosomes in most cells. However, the presence of a charged group in a molecule greatly reduces the ability of that compound to traverse the lysosomal membrane as compared with an uncharged molecule of similar molecular dimensions[18,23] and it may be that other low-molecular-weight anions and cations also contribute to the provision of an intracellular environment in which lysosomes remain stable. In that case, we would expect the *in vivo* response of lysosomes to increasing concentrations of dimethylsulphoxide to be more akin to that found with lysosomes prepared in β-glycerophosphate than those prepared in sucrose.

The content of phosphorylated compounds within a cell falls rapidly after the onset of ischaemia[8,9] and, if these compounds contribute largely to the 'osmotic protection' of lysosomes within cells, it is possible to understand why the ligation of a lobe of rat liver leads to a decrease in the latency of lysosomal enzymes within that lobe while there is no difference in the latency of acid phosphatase in a 'mitochondrial fraction' prepared in sucrose and bubbled with either oxygen or nitrogen[24]. Similarly, *post-mortem* changes within a cell could well become irreversible when the intracellular concentration of phosphorylated compounds has decreased to an extent where the lysosomes are disrupted.

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REFERENCES

1 P. Mazur, *Science*, 168 (1970) 939.
2 J. E. Lovelock, *Biochim. Biophys. Acta*, 10 (1953) 414.
3 J. E. Lovelock, *Biochim. Biophys. Acta*, 11 (1953) 28.
4 J. Berthet and C. de Duve, *Biochem. J.*, 50 (1951) 174.
5 R. Gianetto and C. de Duve, *Biochem. J.*, 59 (1955) 433.
6 O. Z. Sellinger, H. Beaufay, P. Jacques, A. Doyen and C. de Duve, *Biochem. J.*, 74 (1960) 450.
7 E. J. Conway, *Ir. J. Med. Sci.*, Series 6 (1950) 216.
8 O. H. Lowry, J. V. Passonneau, F. X. Hasselberger and D. V. Schulz, *J. Biol. Chem.*, 239 (1964) 18.
9 A. Wollenberger and A. Krause, *Am. J. Cardiol.*, 22 (1968) 349.
10 D. F. Swaab, *J. Neurochem.*, 18 (1971) 2085.
11 D. Lee and C. Allen, *Biochem. J.*, 128 (1972) 142 P.
12 D. Lee, *J. Cell Physiol.*, 76 (1970) 17.
13 D. Lee, *J. Protozool.*, 18 (1971) 173.
14 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
15 J. Berthet, L. Berthet, F. Appelmans and C. de Duve, *Biochem. J.*, 50 (1952) 182.
16 F. Appelmans and C. de Duve, *Biochem. J.*, 59 (1955) 426.
17 J. B. Lloyd, *Biochem. J.*, 121 (1971) 245.
18 D. Lee, *Biochim. Biophys. Acta*, 211 (1970) 550.
19 D. Lee, *Biochim. Biophys. Acta*, 266 (1972) 50.
20 M. Müller, P. Baudhuin and C. de Duve, *J. Cell Physiol.*, 68 (1966) 165.
21 J. Farrant and A. E. Woolgar, in G. E. W. Wolstenholme and M. O'Connor, *The Frozen Cell*, Churchill, London, 1970, p. 97.
22 D. Lee, *Biochim. Biophys. Acta*, 233 (1971) 619.
23 J. B. Lloyd, *Biochem. J.*, 115 (1969) 703.
24 C. de Duve and H. Beaufay, *Biochem. J.*, 73 (1959) 610.