BBA 75874

THE EFFECT OF GLYCEROL, ETHANOL AND DIMETHYLSULPHOXIDE ON RAT LIVER LYSOSOMES

DONALD LEE

Division of Cryobiology, Clinical Research Centre, Watford Road, Harrow, Middlesex, HA1 3UJ (Great Britain)

(Received October 7th, 1971)

SUMMARY

Homogenates of rat liver prepared in either sucrose or β-glycerophosphate were exposed, for 30 min at 0°, to concentrations of glycerol between 5 and 28 % (v/v). After this treatment, and in spite of the fact that the concentration of sucrose or β-glycerophosphate was always maintained at 0.25 M, the latency of β-glucuronidase was shown to have fallen progressively as the concentration of glycerol increased.

The latency of this enzyme also decreased when homogenates prepared in sucrose were exposed to increasing concentrations of dimethylsulphoxide. However, when the homogenates were prepared in β-glycerophosphate, the latency of β-glucuronidase showed only a slight fall on exposure to 10 % dimethylsulphoxide but a progressive decrease at higher concentrations.

On exposure to concentrations of ethanol between 5 and 25 % (v/v), the latency of β-glucuronidase did not decrease until the concentration of ethanol was greater than about 15 %.

The rat liver lysosomal membrane was found to be freely permeable to dimethylsulphoxide and ethanol. However, glycerol penetrated the membrane much less readily and it is possible that rat liver lysosomes are disrupted after exposure to glycerol as a result of the transient osmotic pressure gradients which occur when the concentration of glycerol outside the lysosomes is reduced.

INTRODUCTION

The presence of dimethylsulphoxide in homogenates of rat liver has been shown[1] to affect the permeability of the lysosomal membrane in a manner which is dependent both on the concentration of dimethylsulphoxide present and on the nature of the non-penetrating solute used in preparation of the homogenate. It was therefore of interest to study the effect of glycerol, the other widely used cryoprotective compound[2,3], and to compare the response with glycerol to that obtained for ethanol, a compound which has shown little or no cryoprotective properties towards nucleated cells[4] and erythrocytes[5].

This paper shows that the latency of β-glucuronidase (β-D-glucuronide glucuronohydrolase, EC 3.2.1.31) in homogenates of rat liver fell progressively after exposure to increasing concentrations of glycerol, irrespective of whether the homogenates

were prepared with sucrose or β-glycerophosphate. In contrast, ethanol had little or no effect on the latency of β-glucuronidase until relatively high concentrations were used.

β-Glucuronidase has also been used as a marker enzyme to confirm the observation[1] that dimethylsulphoxide affects the permeability of the rat liver lysosomal membrane in a manner which is dependent on the non-penetrating solute used in preparation of the homogenate.

MATERIALS AND METHODS

Sucrose, glycerol and ethanol, of Analar grade, and dimethylsulphoxide of laboratory reagent grade were obtained from Hopkin and Williams (Chadwell Heath, Essex). Sodium β-glycerophosphate (Grade 1), the anhydrous salt of phenolphthalein glucuronic acid and Triton X-100 were from Sigma (London, SW6).

Homogenisation of rat liver

After death by cervical dislocation, the livers were removed from 150–250 g August strain rats, weighed, placed in a chilled beaker, chopped into small pieces and suspended (2.5 ml/g tissue) in ice-cold solutions of 0.25 M sucrose or 0.25 M β-glycerophosphate (pH 7.0). The tissue was then homogenised in a Potter–Elvehjem homogeniser with a Teflon pestle and a clearance of 0.2 mm.

Exposure of lysosomes to varying concentrations of glycerol, ethanol or dimethylsulphoxide

Because of the high viscosity of concentrated glycerol solutions, the more dilute solutions were prepared by weighing samples of the Analar material. Volumes of glycerol were calculated using a specific gravity of 1.259 g/ml, and the following procedure was carried out at 0°. Samples of homogenates prepared in sucrose or β-glycerophosphate were diluted with equal volumes of similar solutions containing various concentrations of glycerol, ethanol or dimethylsulphoxide. After 30 min, the homogenates were diluted with 4 vol. of 0.25 M sucrose and samples were assayed for β-glucuronidase activity[6] at 37° in reaction mixtures which contained either ethanol or dimethylsulphoxide at a concentration of 2.5 % (v/v), or glycerol at a concentration of 2.8 %(v/v). These concentrations were obtained by making appropriate additions to the substrate solutions, where necessary.

In all determinations of total activity, Triton X-100 was present in the assay mixtures at a concentration of 0.05 % and latency was defined as the percentage of the total enzyme activity unmasked by the presence of the Triton X-100.

Assessment of the permeability of the rat liver lysosomal membrane to glycerol, ethanol and dimethylsulphoxide

In the above procedure the concentration of sucrose or β-glycerophosphate was always maintained at 0.25 M during the study of the effect of the cryoprotective compounds. In these experiments the cryoprotective compounds were substituted for sucrose and the following procedure was carried out at 0°. Samples of homogenates prepared in sucrose were diluted with 4 vol. of a 0.25 M solution of the compound under examination. At timed intervals thereafter, these diluted solutions were further diluted with an equal volume of 0.5 M sucrose. The free and total activities of β-glucuronidase were then determined[6] at 37°.

RESULTS

In order to assess the effect of the residual glycerol, ethanol and dimethylsulphoxide during determination of the latency of β-glucuronidase, samples of homogenates were assayed both in the presence and in the absence of these substances. The latency values obtained are shown in Table I and the only instance where there was a significant difference in latency was in the presence of glycerol when β-glycerophosphate was used as non-penetrating solute. This difference in latency was due to an increase in total activity and a similar 'activation' of bovine heart β-glucuronidase by glycerol has been reported[7].

Fig. 1 shows that the latency of β-glucuronidase in homogenates of rat liver decreased progressively after exposure to increasing concentrations of glycerol, and that the decrease followed the same course irrespective of whether the homogenates

TABLE I

Additive	*Osmotic protectant (0.25 M)*	*Mean latency ± S.E. (n = 4)*	
		In the presence of additive	*In the absence of additive*
Glycerol (2.8%)	Sucrose	84.0 ± 3.0	84.5 ± 3.4
	β-Glycerophosphate	72.1 ± 3.0*	60.8 ± 2.4*
Ethanol (2.5%)	Sucrose	80.1 ± 1.7	75.4 ± 1.6
	β-Glycerophosphate	84.2 ± 3.3	78.8 ± 1.6
Dimethylsulphoxide (2.5%)	Sucrose	82.1 ± 1.7	79.1 ± 1.5
	β-Glycerophosphate	82.2 ± 2.0	87.2 ± 1.7

* Significantly different at the level $0.02 < P < 0.05$.

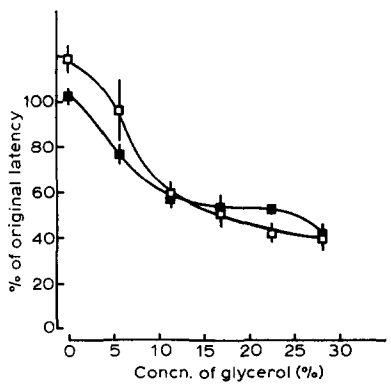

Fig. 1. Percentage of the latency of β-glucuronidase remaining after exposure of homogenates to different concentrations of glycerol. ■, homogenates prepared with 0.25 M sucrose; □, homogenates prepared with 0.25 M β-glycerophosphate. In all figures, points represent the mean of the latency obtained in four different determinations and the vertical lines represent the standard errors of the mean although, in some cases, these were too small to be shown.

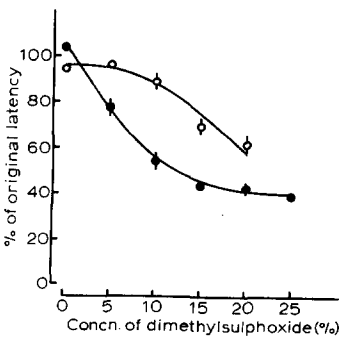

Fig. 2. Percentage of the latency of β-glucuronidase remaining after exposure of homogenates to different concentrations of dimethylsulphoxide. ●, homogenates prepared with 0.25 M sucrose; ○, homogenates prepared with 0.25 M β-glycerophosphate.

were prepared in sucrose or β-glycerophosphate. This observation differed from that obtained with dimethylsulphoxide when acid phosphatase was used as marker enzyme[1] and for that reason the effect of dimethylsulphoxide was re-investigated using β-glucuronidase as marker. The results obtained are shown in Fig. 2 where it can be seen that the effect of dimethylsulphoxide is dependent on the non-penetrating solute used in preparation of the homogenate.

After exposure to increasing concentrations of ethanol, the latency of β-glucuronidase did not decrease until the concentration of ethanol was greater than about 15 % (Fig. 3). Similar results were obtained with homogenates prepared in sucrose or β-glycerophosphate when acid phosphatase was used to assess lysosomal integrity; in these experiments, β-glycerophosphate was used as substrate for the acid phosphatase assay[1].

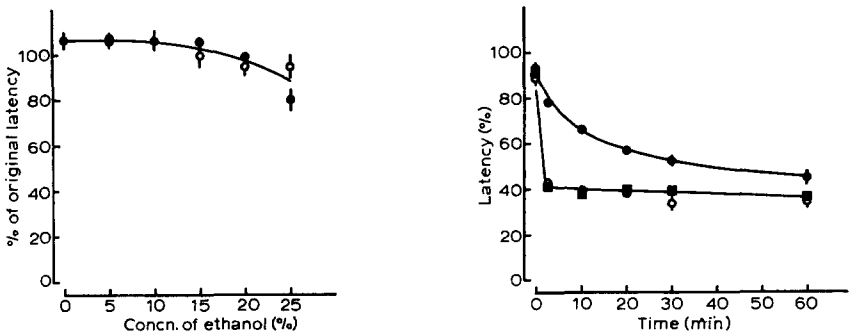

Fig. 3. Percentage of the latency of β-glucuronidase remaining after exposure of homogenates to different concentrations of ethanol. ●, homogenates prepared with 0.25 M sucrose; ○, homogenates prepared with 0.25 M β-glycerophosphate.

Fig. 4. Latency of β-glucuronidase remaining after dilution of homogenates with glycerol (●), ethanol (■) or dimethylsulphoxide (○).

The fact that the decrease in latency obtained after exposure to glycerol was similar in the presence of both non-penetrating solutes meant that one could not exclude the possibility that the effect was due to transient osmotic pressure gradients. For this reason, a study of the relative permeability of the rat liver lysosomal membrane to glycerol, ethanol and dimethylsulphoxide was undertaken and the results are shown in Fig. 4. The latency of β-glucuronidase decreased from control values of about 90 %, to only 40 %, 2.5 min after dilution of an homogenate with 0.25 M dimethylsulphoxide or 0.25 M ethanol. However, 2.5 min after dilution with 0.25 M glycerol, the latency of β-glucuronidase had decreased only slightly and after an hour it had not fallen to the value obtained with dimethylsulphoxide or ethanol. Even at 25° 60 % of the β-glucuronidase activity in an homogenate remained latent 2.5 min after dilution with glycerol and as 92 % of the activity of this enzyme was originally latent, it appears that glycerol traverses the rat lysosomal membrane much less readily than ethanol or dimethylsulphoxide.

DISCUSSION

Extension of previous studies on the effect of dimethylsulphoxide on rat liver lysosomes[1] was complicated by the fact that the other main cryoprotective compound,

glycerol, is a product of the action of acid phosphatase on β-glycerophosphate. For that reason, it was desirable to use the enzyme β-glucuronidase in the studies reported above.

The decrease in latency of β-glucuronidase observed when homogenates prepared in sucrose were exposed to increasing concentrations of glycerol (Fig. 1) was similar to the decrease in latency of both acid phosphatase[1] and β-glucuronidase (Fig. 2) found when homogenates prepared in sucrose were exposed to increasing concentrations of dimethylsulphoxide.

However, a common response to both cryoprotective agents was not observed when β-glycerophosphate was used in preparation of the homogenate. The presence of dimethylsulphoxide had little effect on the latency of acid phosphatase or β-glucuronidase (Fig. 2) until its concentration was greater than 10% although there was a continuous decline in the latency of β-glucuronidase on exposure to increasing concentrations of glycerol.

The similar response to glycerol in the presence of both non-penetrating solutes could indicate that the lysosomal membrane had been rendered permeable to these substances by the presence of glycerol. But, one could not exclude the possibility that the effects were caused by transient osmotic pressure gradients resulting from dilution of the glycerol. These gradients would be greatest on dilution of the homogenates after the 30-min exposure period and would result from water entering the lysosomes more rapidly than glycerol leaves.

The possibility of large osmotic pressure gradients seemed unlikely since glycerol was known to penetrate the membranes of rat liver[8,9] and *Tetrahymena pyriformis*[10] lysosomes more rapidly than hexoses and appeared to enter the lysosomes of the protozoan as rapidly as dimethylsulphoxide. Nevertheless, the permeabilty of the rat liver lysosomal membrane to glycerol was investigated using shorter time intervals than had been used previously and it can be seen from Fig. 4 that the latency of β-glucuronidase decreased quite slowly after dilution of an homogenate with 0.25 M glycerol. In contrast, the latency reached a low (and stable) value within 2.5 min of dilution with dimethylsulphoxide or ethanol and it would appear that glycerol penetrates relatively slowly into rat liver lysosomes.

In the light of these observations on the permeability of the rat liver lysosomal membrane it appears that the effect shown in Fig. 1 is, in fact, the consequence of transient osmotic pressure gradients.

Problems caused by the relatively low permeability of glycerol have also been encountered with whole cells[11] and tissues[12] and have been overcome by slow dilution of the glycerol. However, if the permeability of a cell membrane to glycerol is greater than the permeability of its lysosomal membranes, then it is possible that the lysosomes within a cell could be disrupted even when slow dilution is used and such disruption would probably be lethal.

Previous reports have indicated that rapidly dividing cells have a smaller proportion of their acid hydrolases within lysosomes than do non-dividing cells (see ref. 13) but this cannot be taken as evidence that cells are able to tolerate release of acid hydrolases into the cytosol. The reported degeneration of the Golgi apparatus during metaphase[14] and the inability of regenerating rat liver to package β-glucuronidase into lysosomes[15] suggests that newly synthesised lysosomal enzymes are probably held back in the endoplasmic reticulum during mitosis. Such an accumulation ob-

viously does not damage the cell and one should probably consider two kinds of latency *in vivo*; the latency of lysosomal acid hydrolases, which is retained during homogenisation, and the latency of the hydrolases within the endoplasmic reticulum which is destroyed. Evidence in support of the concept of latency of lysosomal enzymes within the endoplasmic reticulum derives from the recent observation[16] that the 'microsomal membrane' is impermeable to anions with a molecular weight as low as 90.

The effect of ethanol on the lysosomal membrane has not previously been studied using high concentrations of ethanol although conflicting results have been obtained using a concentration of only 1 %[17,18]. The results presented here show that high concentrations of ethanol have little effect on the rat liver lysosomal membrane but it is difficult to correlate this with its poor cryoprotective properties since any direct effect of glycerol on the lysosomal membrane is masked by its low permeability.

The lysosomal membrane has also been shown to be damaged during chronic alcohol intoxication[19] but the results presented above indicate that this is not the result of a direct effect of ethanol on the lysosomal membrane although the effect could stem from some action of ethanol on the biosynthesis of lipoprotein membranes.

In conclusion it may be useful to point out that present studies have given another example of how the properties of lysosomes are dependent on the cell from which they are derived. The fact that glycerol penetrates the membrane of *T. pyriformis* lysosomes as readily as dimethylsulphoxide, together with the observation that the lysosomes from this protozoan are less stable to temperature than those from rat liver[20], would seem to indicate that the protozoan lysosomes possess a more 'open' but less stable structure than those of rat liver.

ACKNOWLEDGMENT

It is a pleasure to thank Miss Jacqueline Osborne for excellent technical assistance.

REFERENCES

1 D. LEE, *Biochim. Biophys. Acta*, 233 (1971) 619.
2 A. U. SMITH, *Biological Effects of Freezing and Supercooling*, E. Arnold London, 1961.
3 H. T. MERYMAN, in H. T. MERYMAN, *Cryobiology*, Academic Press, New York, 1966, p. 1.
4 O. VOS AND M. C. A. C. KAALEN, *Cryobiology*, 1 (1965) 249.
5 J. E. LOVELOCK, *Biochem. J.*, 56 (1954) 265.
6 R. GIANETTO AND C. DE DUVE, *Biochem. J.*, 59 (1955) 433.
7 D. ROMEO, N. STAGNI AND M. C. PUGLIARELLO, *Experientia*, 23 (1967) 247.
8 J. BERTHET, L. BERTHET, F. APPELMANS AND C. DE DUVE, *Biochem. J.*, 50 (1951) 182.
9 J. B. LLOYD, *Biochem. J.*, 115 (1969) 703.
10 D. LEE, *Biochim. Biophys. Acta*, 211 (1970) 550.
11 H. A. SLOVITER, *Lancet*, 1 (1951) 823.
12 C. E. HUGGINS, *J. Physiol.*, 145 (1959) 54P.
13 D. LEE, *J. Cell Physiol.*, 76 (1970) 17.
14 A. KIMURA AND T. ONOE, *Gann*, 61 (1970) 517.
15 J. L. VAN LANCKER, in W. H. FISHMAN, *Metabolic Conjugation and Metabolic Hydrolysis*, Vol 1, Academic Press, New York, 1970, p. 391.
16 R. NILSSON, E. PETTERSSON AND G. DALLNER, *Febs. Lett.*, 15 (1971) 85.
17 J. T. DINGLE, *Biochem. J.*, 70 (1961) 509.
18 G. WEISSMANN, G. SESSA AND V. BEVANS, *Ann. N.Y.Acad. Sci.*, 141 (1967) 326.
19 L. SAMOCHOWIEC, Z. STEPLEWSKI AND J. WOJCICKI, *Acta Biol. Med. German.*, 18 (1967) 625.
20 D. LEE, *J. Protozool.*, 18 (1971) 173.