The Osmotic Stability of Lysosomes from Adult and Foetal Guinea-Pig Liver Tissue

By JULIENNE M. TURNBULL* AND M. W. NEIL
*Department of Biochemistry, London Hospital Medical College,
London, E. 1*

(Received 5 August 1968)

1. Lysosome-rich fractions were obtained from foetal liver tissues as early as 35 days uterine age. Foetal lysosomes showed the same 'structure-linked latency' and acid hydrolytic potentiality characteristic of their adult counterparts. 2. The osmotic stability of lysosome-rich fraction from foetal guinea-pig liver tissue was greater than that of the corresponding adult lysosome fractions, *p*-nitrophenylphosphatase being used as marker enzyme. 3. The observation was confirmed by using β -glycerophosphatase and phenolphthalein β -glucuronidase as alternative marker enzymes. *p*-Nitrophenyl phosphate and β -glycerophosphate appear to act as substrates for the same enzyme. 4. By using *p*-nitrophenylphosphatase activity measurements it was shown that the osmotic stability of foetal lysosomal fractions decreased with increasing foetal age, but at no time achieved the degree of osmotic instability characteristic of adult lysosomal fractions. 5. The correlation of these findings with the intracellular environment of lysosomes is discussed.

Lysosomes were defined biochemically as granules containing acid hydrolases and exhibiting structure-linked latency (de Duve, 1955). Appelmans & de Duve (1955) first investigated the osmotic stability of granules from adult rat liver. They showed that adult lysosomes acted as osmometer systems when exposed to a series of graded osmotic shocks. Since these initial experiments, which led to the biochemical operational definition of lysosomes, much work has been carried out implicating lysosomes in a wide variety of functions in the economy of living organisms (de Duve & Wattiaux, 1966).

During experiments in this Laboratory to obtain formal evidence for the existence of lysosomes in guinea-pig foetal liver it was observed that the resistance of these granules to osmotic rupture appeared to be considerably greater than that of similar granules from adult liver tissue. In view of the well-known enzymological and other differences between adult and foetal tissues, further experiments were undertaken to examine this behaviour in more detail.

In the present work lysosome-rich fractions were obtained from adult guinea-pig liver tissue and from foetal tissue at different stages of development. It was found that the osmotic stability patterns of the granules in the foetal tissue fractions were

different at each stage of development and from that of the adult tissue granules. An attempt was made to correlate this observation with the cellular environment of the granules and their possible roles in cell physiology.

MATERIALS AND METHODS

Animals. Albino guinea pigs were used. All animals originated from The Research Institute (Animal Virus Diseases), Pirbright, Surrey.

Foetal guinea pigs of known age were obtained from controlled matings. Time of onset of oestrus in adult females was predicted by the results of vaginal swabbing over several oestrous cycles. The male was introduced when oestrus was imminent and removed 48 hr. later. The females were killed at known intervals after mating and the crown-rump lengths of the fetuses measured. The mean crown-rump length for each litter from 36 females was plotted against foetal age over the range 27–63 days. This reference curve was used to estimate the ages of fetuses from randomly mated females from the same stock.

Chemicals. β -Glycerophosphate was laboratory-reagent grade (British Drug Houses Ltd., Poole, Dorset) containing less than 3% of the α -isomer. Phenolphthalein mono- β -glucuronide was obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.). Sucrose was Tate and Lyle Co. Ltd. (Liverpool) caster sugar. Triton X-100 was obtained from Lennig Chemicals Ltd. (London, W.C. 1). *p*-Nitrophenyl phosphate was prepared as described by Neil & Horner (1964).

Tissue fractions. (a) Adult preparations. Liver tissue homogenates, prepared in ice-cold 0.25 M-sucrose by

*Present address: Department of Biochemistry, Charing Cross Hospital Medical School, London, W.C. 2.

standard procedures, were centrifuged (5000g-min. average field, $g_{\max}$. 1000; de Duve & Berthet, 1953) to separate the cytoplasmic extract from nuclei and cell debris. Lysosome-rich fractions were prepared from the cytoplasmic extracts in 0.25M-sucrose solution at 2° by further centrifugation (100000g-min. average field, $g_{\max}$. 30000) and washed twice with ice-cold 0.25M-sucrose solution.

(b) Foetal preparations. A precision-bore glass tube containing foetal liver suspended in ice-cold 0.25M-sucrose (1 g. of liver to 3 ml. of suspension medium) was passed once up and down, over a period of 7–10 sec., while a close-fitting Teflon pestle rotated in the tube at 500 rev./min. The lysosome-rich fractions were then prepared in the same way as for adult guinea-pig liver preparations.

The suitability of both adult and foetal fractionation procedures was assessed by phase-contrast microscopy and by enzymic analysis. Our major consideration in developing a method for obtaining adult and foetal lysosomes was that a constant proportion of the lysosomal granule population must be obtained from each preparation; this was of special importance with the developing foetal liver, where the tissue was undergoing rapid change. Our aim therefore was not a maximum quantitative recovery of sedimentable phosphatases from the original homogenate, but to obtain fractions rich in granules with lysosomal properties, with minimum contamination by sedimentable phosphatases of nuclear or microsomal origin. In the 30000g fraction we found: (1) a constant percentage of sedimentable activity, which was about 75% of that in the whole homogenate; (2) a constant proportion of the sedimentable activity was 'bound' or lysosomal activity, and was assayable only after the granular phosphatases had been 'released' by mechanical, detergent or osmotic activation.

In two experiments on 45-day foetal guinea-pig livers $g_{\max}$. was doubled; the total sedimentable activity increased by 4% and 19% respectively. However, two washes of the large-granule fraction resulted in preparations with marginally lower free activities. This suggested an increase in small-granule (or microsomal) contamination. Several variations on sedimentation conditions failed to produce a preparation that showed significant improvement on the 30000g fraction. This procedure was therefore adopted for foetal tissues.

Enzyme assay procedures. The marker enzymes chosen to test lysosomal integrity were acid *p*-nitrophenylphosphatase, β -glycerophosphatase and phenolphthalein β -glucuronidase. *p*-Nitrophenylphosphatase activity was determined by mixing *p*-nitrophenyl phosphate (2.5 mM) substrate with the enzyme preparation in the presence of a buffer, which was either sodium acetate, pH 5.0 (5 mM), or sodium maleate, pH 5.8 (5 mM or 50 mM). Concentrations given are those obtained in a final total assay volume of 2 ml. The assay was conducted at 3° over an 80–120 min. period. The reaction was stopped by the addition of 5 or 10 ml. of 0.04M-NaOH, and the extinction of the *p*-nitrophenol liberated was measured at 415 m μ with a Unicam SP.1400 spectrophotometer. β -Glycerophosphatase and phenolphthalein β -glucuronidase activities were measured at 3° and pH 5.0 (Gianetto & de Duve, 1955). The terms 'free' and 'total' activities of lysosomal enzymes are used in the senses defined by de Duve & Berthet (1953). 'Bound' activity is defined as total activity minus free activity. Units of enzyme activity are defined as μ moles of substrate

hydrolysed/min./g. of fresh tissue in the conditions of the experiment. Total lysosomal enzyme activity, representing 100% release of assayable enzyme, was achieved by homogenization of lysosome-rich preparations in an MSE high-speed homogenizer or assay in the presence of Triton X-100.

Osmotic activation system. Stock solutions, made up of sodium acetate buffer, pH 5.0 at 3°, sucrose and water, were so designed that 1.5 ml. of buffer-sucrose stock solutions with added lysosomal preparation and substrate, in a final total volume of 2 ml., resulted in an acetate buffer concentration of 5 mM and sucrose concentrations in the range 0.0125–0.025 M. (The buffer system maintained pH 4.85–5.05 at 3° throughout the assay period. There was no significant change in enzyme activity over this pH range.) The stock solutions were stored at –14°. The pH of the stock solutions at 3° was checked at intervals and adjusted when necessary. Experiments with sodium maleate buffer, pH 5.8, were designed similarly.

The total osmolality in any solution was taken as the sum of the osmolar contributions attributable to known added electrolytes at the pH used and to sucrose.

RESULTS

Basis for comparison of different lysosome-rich fractions. The initial free activities of lysosome-rich preparations varied appreciably from experiment to experiment. For example, the values for the free *p*-nitrophenylphosphatase activity (at pH 5.0 and 3°) of liver preparations from 12 male guinea pigs were 6.7–12.9% of the corresponding total enzyme activities, with a mean value of 9.5%. Corresponding values for eight foetal liver preparations were 8.7–14.4% with a mean value of 10.2%. To compare osmotic activation of different preparations it was essential (a) to establish that the intact particles in a preparation were uniformly representative of the parent population of particles and (b) to establish a common basis for the comparisons.

As the contamination of the 30000g fraction with non-lysosomal sedimentable phosphatases such as glucose 6-phosphatase was known to be constant under standardized conditions, the variations in initial free activities mentioned above were almost certainly due to differing degrees of mechanical activation during preparation. Experiments were designed to establish that the intact particles remaining were a representative part of a population of granules, homogeneous in their response to osmotic activation, i.e. that the remaining granules were not a 'selected' group. Osmotic activation of the intact particles remaining after deliberate mechanical activation of single preparations was studied. Lysosome-rich fractions from both adult and foetal tissues were suspended in 0.25M-sucrose solution and treated for different times at 3° in an MSE high-speed homogenizer. The partially activated preparations were subsequently tested in the osmotic activation system. The osmotic

Fig. 1. Partial mechanical activation of a lysosome-rich fraction followed by osmotic activation (adult male guinea-pig liver): (a) free enzyme activity plotted as percentage of total enzyme activity; (b) enzyme activity released by osmotic activation plotted as percentage of initial residual bound activity. Assays were carried out in 5 mM-sodium maleate buffer, pH 5.8, at 3° for 120 min., with *p*-nitrophenyl phosphate as substrate. Osmotic activation was carried out as described in the Materials and Methods section. For homogenizer activation samples of the preparation were activated in the homogenizer running at half speed for: ○, 0 min.; ●, 1/2 min.; △, 1 min.

activation curves obtained from homogenized fractions from an adult guinea-pig liver preparation are shown in Fig. 1. Fig. 1(a) shows the free activity at different osmolarities as a percentage of total enzyme activity. In Fig. 1(b) enzyme activity released by osmotic activation is plotted as a percentage of the initial residual bound activity. The near coincidence of the curves, reduced to a common basis for comparison, was accepted as satisfactory evidence to establish (a) above. Similar results were obtained with foetal preparations.

Comparative osmotic activation curves from adult and foetal guinea-pig liver large-granule preparations. (a) *p*-Nitrophenylphosphatase activity. The responses of a series of adult male guinea-pig liver large-granule preparations to graded osmotic

Fig. 2. Osmotic activation curves from adult and foetal guinea-pig liver lysosome-rich preparations. Assays were carried out in 5 mM-sodium acetate buffer, pH 5.1, at 3° for 80 min., with *p*-nitrophenyl phosphate as substrate. Osmotic activation was carried out as described in the Materials and Methods section. The results for adult liver preparations (●) were obtained from six guinea pigs (age range 18–20 weeks) and are plotted as mean and range at each osmolarity. The foetal results were obtained from seven foetuses (age range 37–68 days) of which only three are shown for clarity: ○, 37 days; ■, 52 days; △, term.

activation are shown in Fig. 2. The ages of the animals used in this series were in the range 8–62 weeks and the mean total *p*-nitrophenylphosphatase activity was 0.13 unit (range 0.07–0.18) at pH 5.0 and 3°. There was no trend in total enzyme activities with increasing age of animal. Also shown in Fig. 2 are the osmotic activation curves for lysosome-rich liver preparations from foetuses of different ages. The ages of the seven foetuses used ranged from 37 days to parturition (about 68 days). For clarity, only three foetal curves are shown in Fig. 2. The total enzyme activity increased with increasing age of foetus from 0.03 unit (at pH 5.0 and 3°) at 37 days to about 0.1 unit at term. This observation is in accord with the results of Flexner & Flexner (1949), who showed a similar increase in β -glycerophosphatase activity with age of foetus.

The results summarized in Fig. 2 demonstrate that the adult and foetal lysosome-rich granule preparations respond differently to osmotic activation. The foetal preparations show a considerably higher stability in media of low osmolarity than do the adult particles, and there is no overlap between the two populations. Further, without exception, the foetal particles decrease in osmotic stability, within the foetal population range studied, with increasing age of foetus.

The possibility that both the differentiation within the foetal population and the difference between the results for adult and foetal preparations

Fig. 3. Osmotic activation curves from a foetal guinea-pig liver lysosome-rich preparation with three substrates. Foetal age was 67 days. Assays were carried out in 5mm-sodium acetate buffer, pH 5.1, at 3° for 100 min., with phenolphthalein β -glucuronide (Δ), β -glycerophosphate ($\circ$) or *p*-nitrophenyl phosphate ($\blacktriangle$) as substrate. Osmotic activation was carried out as described in the Materials and Methods section.

might have been caused by enhancement of total foetal enzyme activities by Triton X-100 (which was used for estimation of total activities in these experiments) was eliminated by control experiments. Total activities were obtained by treatment of the preparation for 10 min. in the MSE high-speed homogenizer. The same preparation was treated with Triton X-100 (0.1%), and a combination of the treatments was used.

The detergent-treated and homogenized preparations gave similar 'total' values for enzyme activity in both adult and foetal preparations.

(b) Other lysosomal enzyme activities. Similar experiments to those described above were carried out with β -glycerophosphate and phenolphthalein β -glucuronide as substrates. The higher stability of foetal particles in media of low osmolarity was again evident and the decrease in stability of foetal particles with increasing age of foetus was also observed with phenolphthalein β -glucuronide as substrate. Comparison of the osmotic activation curves for these two substrates suggested that the difference between adult and foetal particles with phenolphthalein β -glucuronide as substrate was not as great as the corresponding difference with *p*-nitrophenyl phosphate or β -glycerophosphate as substrate. Osmotic activation curves were therefore obtained in identical conditions for the same foetal lysosome-rich particle preparation with the three substrates. The results (Fig. 3) show that phenolphthalein β -glucuronidase activity is released more readily in hypo-osmotic solutions than either *p*-nitrophenyl phosphatase or β -glycerophosphatase

activity. The coincidence of the osmotic activation curves for the last two enzyme activities suggests that for foetal liver, as for adult guinea-pig liver (Neil & Horner, 1964), *p*-nitrophenyl phosphate and β -glycerophosphate are hydrolysed by the same lysosomal enzyme, or this result could be interpreted as evidence that the enzymes hydrolysing these two substrates are found in the same population of granules.

DISCUSSION

Foetal granules were obtained from livers as early as 34 days of the total guinea-pig gestation period of approx. 65 days. The foetal lysosome-enriched mitochondrial preparations show the characteristic release of three acid hydrolases in response to graded osmotic shocks. In this response to a changing osmolar environment foetal lysosomal granules exhibit two of the main characteristics of adult lysosomes, namely the presence of a collection of acid hydrolases and structure-linked latency of these granular enzymes. The 'structure-linked latency' phenomena, however, appear to be different in foetal preparations.

During the present work it was observed that 'total' enzyme activity of foetal preparations obtained by water activation were consistently 10–18% lower than the values obtained by treatment with detergent or by high-speed homogenization. This suggests that foetal lysosomes may be heterogeneous in their response to osmotic activation. There may be a significant proportion of lysosomal enzymes in a sedimentable but 'pre-lysosomal' stage. In this context it may be noted that in Fig. 2 the foetal curves do not tend towards the 100% mark. Does this represent a compartment of enzyme activity not osmotically activatable? Again, the greatest stability to osmotic shock is shown by lysosomal populations from the earliest gestation stages. There is a progressive decrease in their osmotic stability characteristics, but at all times the curves for adult and foetal preparations are distinctly different from one another. Two hypotheses may be advanced to explain the difference in the 'structure-linked latency' phenomena. Differences in the stability of lysosomes from different tissues when incubated at room temperature have been reported by Wynn & Iqbal (1965). These workers suggest that the stability of the particles may be a reflection of the properties of the lipoprotein membranes. Lipid analyses of lysosomal membranes have been attempted by Wynn, Iqbal & Davies (1967) and Thinès-Sempoux (1967); the latter worker found that the composition of rat liver lysosomal membranes was similar to that of plasma membrane as prepared by Emmelot, Bos, Benedetti & Rumke (1964). The osmotic stability of foetal

liver lysosomal preparations may similarly be explained by suggesting a difference in the composition of the membranes. A changing pattern in the 'plasma-type' membranes from livers throughout foetal development may occur until the adult membrane type is achieved.

Another speculation is that the intracellular environment in which the lysosomes are formed is different in foetal life. Some attempts were made to estimate the intracellular osmolality of cells, and to compare adult and foetal liver cell populations in this respect (Turnbull, 1965). Intracellular K^+ concentration was chosen as an index of the osmolality of the intracellular fluid. Initial experiments indicated that the foetal liver has a higher K^+ concentration when compared with that of the adult liver cell. The intracellular K^+ concentration of foetal liver cells varied from 221 m-equiv./l. of intracellular water to 161 m-equiv./l. of intracellular water at term. The adult values were 163 m-equiv./l. of intracellular water. The corollary was drawn that the environment of the foetal lysosomes was hyperosmotic in comparison with the environment of adult liver lysosomes. It is assumed that the intralysosomal osmolar characteristics would be the same as those of the fluid surrounding them as there is no apparent apparatus for maintaining differential ionic gradients. It therefore seems strange that the foetal lysosomal population shows greater stability to osmotic shock, when its own intralysosomal environment is grossly hyperosmotic when compared with the adult intralysosomal environment. Several pieces of evidence suggest therefore that the membrane characteristics of these intracellular particles may indeed be very different.

It is apparent that foetal lysosomes play a significant role in the economy of the developing liver cell, as they are found in the earliest phases of liver biogenesis. Foetal liver tissue is haematopoietic and therefore some means of transport of iron across liver

cells to the haemoglobin-synthesizing site is called for. Foetal lysosomes could be concerned with the concentration and transport of iron. [Adult lysosomes from various tissues are known to contain iron (Desai & Tappel, 1965).] The presence of ferritin granules in lysosome-like particles has also been reported. Foetal liver cells are actively dividing through a large part of the gestation period in rodents. Lysosomes have been implicated in the mitotic process by many workers, including Dougherty (1964), Gahan & Maple (1966) and Robbins & Gonatas (1964); therefore the presence of a lysosomal population in foetal liver is not unexpected. Further work is needed to clarify the relationship of these membrane-bound particles with their cellular environment in foetal and adult liver tissue.

REFERENCES

- Appelmans, F. & de Duve, C. (1955). *Biochem. J.* **59**, 426.
 de Duve, C. (1955). *Exp. Cell Res. Suppl.* **7**, p. 169.
 de Duve, C. & Berthet, J. (1953). *Nature, Lond.*, **172**, 1142.
 de Duve, C. & Wattiaux, R. (1966). *Annu. Rev. Physiol.* **28**, 435.
 Desai, I. D. & Tappel, A. L. (1965). *Proc. Soc. exp. Biol., N.Y.*, **118**, 496.
 Dougherty, W. J. (1964). *J. Cell Biol.* **23**, 25a.
 Emmelot, P., Bos, C. J., Benedetti, E. L. & Rumke, Ph. (1964). *Biochim. biophys. Acta*, **90**, 126.
 Flexner, J. B. & Flexner, L. B. (1949). *J. cell. comp. Physiol.* **31**, 311.
 Gahan, P. B. & Maple, A. J. (1966). *J. exp. Bot.* **17**, 151.
 Gianetto, R. & de Duve, C. (1955). *Biochem. J.* **59**, 433.
 Neil, M. W. & Horner, M. W. (1964). *Biochem. J.* **92**, 217.
 Robbins, E. & Gonatas, W. H. (1964). *J. Cell Biol.* **21**, 429.
 Thinès-Sempoux, D. (1967). *Biochem. J.* **105**, 21p.
 Turnbull, J. M. (1965). Ph.D. Thesis: University of London.
 Wynn, C. H. & Iqbal, M. (1965). *Biochem. J.* **98**, 10p.
 Wynn, C. H., Iqbal, M. & Davies, I. ab I. (1967). *Biochem. J.* **105**, 19p.