THE PURIFICATION OF LYSOSOMES BY ISOPYCNIC ZONAL ULTRACENTRIFUGATION

Brenda Withers, I. ab I. Davies and C. H. Wynn

Department of Biological Chemistry, University of Manchester, Manchester 13.

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Since the discovery of the lysosomal particle (de Duve et al, 1955), various attempts have been made to obtain a preparation of this particle free from contaminating mitochondria and microsomes. Simple differential ultracentrifugation (de Duve et al, 1955) produces only an enrichment of lysosomes and the complex and lengthy method of Sawant, Shibko, Kumta and Tappel (1964) which combines differential and discontinuous density gradient ultracentrifugation has been unsuccessful in our laboratory in providing a sample of lysosomes free from mitochondria and microsomes. The best preparation of lysosomes has been obtained from density gradient ultracentrifugation by the method of Beaufay and Berthet (1963) but this method is on the analytical rather than the preparative scale and does not yield sufficient material for a further study of the chemistry and properties of lysosomes. The development of the zonal ultracentrifuge rotor has provided a valuable alternative method of fractionating sub-cellular components. A rate zonal preparation of lysosomes has been described by Corbett (see Anderson, 1967) in which the partial separation of lysosomes and mitochondria is achieved. The present report describes the preparation of large quantities of lysosomes by zonal isopycnic

ultracentrifugation.

A 12.5% (w/v) rat liver homogenate in ice-cold 0.25M sucrose containing 0.001M EDTA and adjusted to pH 7.0 was prepared using a tissue press and TRI-R stirrer rotating at 1000 rpm. for 30 secs. A Teflon pestle was used and the clearance was 0.006". The homogenate was centrifuged at 3,300 g. for 10 min. at 4° and the pellet was rehomogenised as before and centrifuged at 3,300 g. for 10 min. The combined supernatants were centrifuged at 33,000 g. for 20 min. The resulting pellet contained mitochondria, lysosomes and microsomes. The pellet was resuspended in 0.25M sucrose without added EDTA by gentle hand homogenisation in a glass-glass homogeniser so that 1 ml. of suspension contained the particles derived from 0.5 g. of initial wet weight of rat liver.

Preliminary experiments had shown that some separation of lysosomes and mitochondria was achieved when this suspension was centrifuged in a continuous linear sucrose gradient (0.5M-2.0M) in a SW 25 rotor in a Spinco Model L ultracentrifuge.

A B-XIV zonal rotor (M.S.E. Ltd., London) was loaded at 4,000 rpm. at 0° with 600 mls. of a linear gradient of sucrose from 0.5M-2.0M prepared using the method of Ayad, Bonsall and Hunt (1967). A cushion of 50 ml. of 2.2M sucrose was introduced at the periphery and then 25 ml. of the particulate suspension was fed into the centre of the rotor followed by 15 ml. of 0.2M sucrose to displace the sample from the core of the rotor. The rotor was accelerated and centrifuged at 46,500 g. (maximum) for 1 hr. and on deceleration the gradient removed by pumping 2.2M sucrose from the periphery. The effluent was monitored at 254 mμ and 10 ml. fractions were collected. The refractive index of samples was measured with an Abbe refractometer and the density calculated.

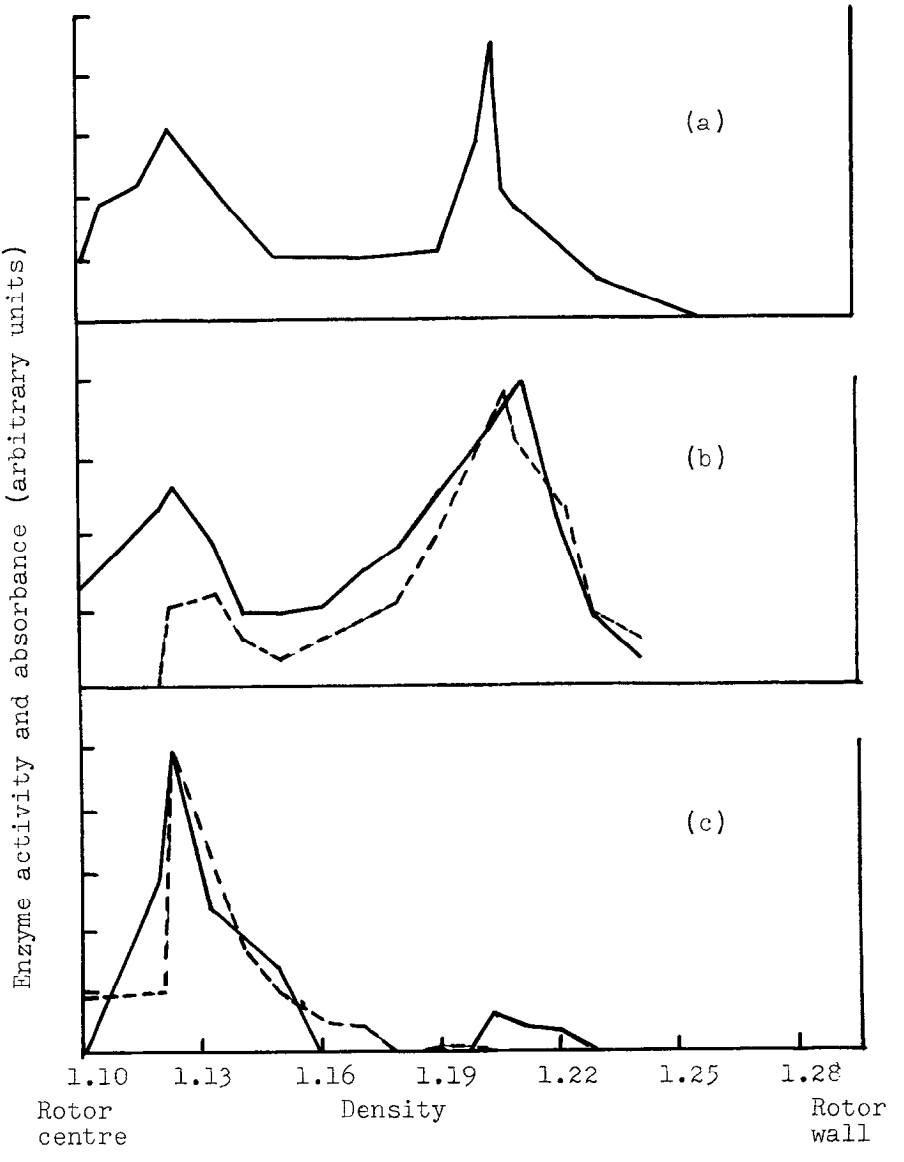

Fig. 1. Separation of impure rat liver lysosomes in B-XIV zonal rotor (see text for experimental details)

(a) Absorbance at 280 mu.
(b) —— , acid phosphatase activity.
---, arylsulphatases A & B activity.
(c) —— , arylsulphatase C activity
---, NADPH-specific cytochrome C reductase.

Fractions were assayed for protein (absorbance at 280 mμ), acid phosphatase (Torriani, 1960), arylsulphatases A & B (0.005M

nitrocatechol sulphate in 0.5M acetate, pH 5.2, see Dodgson, Spencer and Thomas, 1955), arylsulphatase C (0.005M p-nitrophenyl sulphate in 0.5M acetate, pH 8.0, see Dodgson, Spencer and Thomas, 1955) and NADPH-specific cytochrome C reductase (de Duve et al, 1955). The results of a typical experiment are shown in Fig. 1.

Two major protein peaks were observed at densities of 1.125 g/cm^3 and 1.2 g/cm^3. The lysosomal enzymes, acid phosphatase and arylsulphatases A & B were recovered in the broad peak in the region of 1.2 g/cm^3 density. Some of the activity of these enzymes was also present at density = 1.125 g/cm^3. The mitochondrial enzyme, NADPH-specific cytochrome C reductase, was recovered at density = 1.125 g/cm^3 and no detectable activity was present at density = 1.2 g/cm^3. The arylsulphatase C activity is a marker for microsomes but care is necessary in interpreting the data as arylsulphatases A & B also show some activity towards the substrate used for its assay viz. p-nitrophenyl sulphate. The arylsulphatase C activity is mainly located at density = 1.125 g/cm^3 with a minor peak at density = 1.2 g/cm^3. The activity of this minor peak was in agreement with that calculated from the arylsulphatases A & B activity using the relative affinity of these enzymes for the two substrates (Dodgson, Spencer and Thomas, 1955). This suggests that microsomes equilibrate at density = 1.125 g/cm^3.

The results were reproducible although minor variations did occur. The amount of lysosomal enzyme activity at density = 1.125 g/cm^3 varied quite considerably and in some experiments a very sharp band at the cushion was also seen. It is suggested that these peaks are a function of the resuspension technique since low activity at density = 1.125 g/cm^3 was accompanied by high activity at the cushion. Some of the material may remain

as aggregates after resuspension and sediment rapidly through the gradient. If the resuspension is too drastic, soluble enzyme may be found and remain in the region of density = 1.125 g/cm^3. These results are in good agreement with those of Beaufay & Berthet (1963) who showed that on isopycnic ultracentrifugation lysosomal enzymes were found at a density of approximately 1.22 g/cm^3. Corbett (see Anderson, 1967) has reported that lysosomes are found centripetal to the mitochondria but these results were obtained in a rate zonal ultracentrifugation.

Preliminary results suggest that at least 60% of the acid phosphatase activity in the peak at density 1.2 g/cm^3 was latent. Thus, on an enzymic basis, the preparation of lysosomal particles free from significant contamination by mitochondria and microsomes has been achieved.

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References

Anderson, N.G. (1967). in "Methods of Biochemical Analysis" (Glick, D. ed) 15, 271. Wiley, N.Y.
Ayad, S.R., Bonsall, R.W., Hunt, S. (1967). Anal. Biochem. (in press).
Beaufay, H. & Berthet, J. (1963). Biochem. Soc. Symp. 23, 66.
Dodgson, K.S., Spencer, B., Thomas, J. (1955). Biochem. J. 59, 29.
de Duve, C., Pressman, B.C., Gianetto, R., Wattiaux, R. and Appelmans, F. (1955). Biochem. J. 60, 604.
Sawant, P.L., Shibko, S., Kumta, U.S. & Tappel, A.L. (1964) Biochim. biophys. Acta, 85, 82.
Torriani, A. (1960). Biochim. biophys. Acta, 38, 460.