Biochem. J. (1978) **174**, 309–317
Printed in Great Britain

Heterogeneity of Lysosomes Originating from Rat Liver Parenchymal Cells

METABOLIC RELATIONSHIP OF SUBPOPULATIONS SEPARATED BY DENSITY-GRADIENT CENTRIFUGATION

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(*Received* 23 *January* 1978)

1. A crude lysosomal fraction obtained by differential centrifugation of a rat liver homogenate was subjected to zonal centrifugation in iso-osmotic self-generating gradients composed of modified colloidal silica (Percoll). Analysis of relevant marker-enzyme activities shows a continuous band of considerably purified lysosomal particles in the density range 1.04–1.11 g/ml. 2. A relationship between age and buoyant density of the parenchymal lysosomal subpopulations is indicated by the distribution of ^{125}I-labelled asialoglycoproteins in the heterogeneous lysosomes during the catabolism of the glycoprotein. The labelled asialoglycoprotein first appeared in lysosomal particles of low density, which with time progressively acquired a higher density. Furthermore, 30min after administration the ^{125}I-labelled asialocaeruloplasmin recovered in the light lysosomes was less degraded than the material recovered in the heavy lysosomes. 3. A lysosomal enzyme (arylsulphatase) was found to possess considerably higher isoelectric points in the heavy lysosomes than in the light lysosomes, which is consistent with a relationship between age and density of the lysosomes.

Lysosomes are cellular organelles in which the catabolic degradation of macromolecules occurs and the presence of a large number of different degradative enzymes in these particles is well documented. Lysosomes are, however, not homogeneous. Heterogeneities with regard to the density and the enzyme content of lysosomes from various tissues have been reported (for review see Davies, 1976). Little is known about the relationship between the different lysosomal subpopulations. Are they metabolically unrelated, and do they participate in separate catabolic processes, either in the same cell type or in different cell species? Or can they represent different stages of one common catabolic route?

Rat liver contains several cell types, e.g. hepatocytes and Kupffer cells, which show a quantitative difference in lysosomal enzyme content (Arborgh *et al.*, 1973; Berg & Boman, 1973; Munthe-Kaas *et al.*, 1976). The heterogeneity of rat liver lysosomes can thus at least partly be explained by the presence of different cell types. However, it cannot be excluded that lysosomes from the same cell type are heterogeneous.

To investigate the heterogeneity of lysosomes in a single cell type we have studied the degradation of asialocaeruloplasmin in rat liver. G. Ashwell, A. G. Morell and co-workers have demonstrated in a series of elegant studies that plasma glycoproteins treated with neuraminidase, thereby releasing terminal sialic acid residues and exposing the penultimate galactose units, are rapidly cleared from the circulation by the liver (for review see Ashwell & Morell, 1974). Hepatocytes and not Kupffer cells are involved in the catabolism of the glycoprotein. The initial event in this reaction is an interaction between the carbohydrate part of the glycoprotein and a receptor protein present on the surface of the cell (Pricer & Ashwell, 1971). The glycoprotein is subsequently internalized and degraded in lysosomal particles (Gregoriadis *et al.*, 1970).

Experimental

Materials

Caeruloplasmin was obtained from AB Kabi, Stockholm, Sweden, and neuraminidase from Calbiochem G.m.b.H., Frankfurt, Germany. [^{125}I]Iodide (carrier-free IMS 30), NaB^3H_4 and [^{3}H]acetic anhydride were products of The Radiochemical Centre

(Amersham, Bucks., U.K.). Galactose oxidase and lactoperoxidase were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and sialic acid was from E. Merck (Darmstadt, Germany). DEAE-cellulose (DE-20) was a product of Whatman Biochemicals (Springfield Mill, Maidstone, Kent, U.K.). The density-gradient medium Percoll, Sephadex and Sepharose gels were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Substrates based on 4-methylumbelliferone (4-methylumbelliferyl sulphate, 4-methylumbelliferyl β-D-galactopyranoside, 4-methylumbelliferyl β-D-glucuronide, 4-methylumbelliferyl 2-acetamido-2-deoxyglucopyranoside) were obtained from Koch–Light Laboratories (Colnbrook, Bucks., U.K.). The following substrates were purchased from Sigma: glucose 6-phosphate, β-glycerophosphate, AMP, *p*-iodonitrotetrazolium Violet, *p*-nitrophenyl *N*-acetyl-β-D-galactosaminide and haemoglobin. Sprague–Dawley rats (200–250g body wt.) were obtained from Anticimex (Stockholm, Sweden).

^{125}I-labelled asialocaeruloplasmin and [^{3}H]asialocaeruloplasmin were prepared as previously described (Morell *et al.*, 1966; Pertoft *et al.*, 1977). The specific radioactivities of the final products were 2.1 μCi/mg of protein and 0.06 μCi/mg of protein respectively.

Methods

Protein was determined by the method of Lowry *et al.* (1951), appropriate gradient mixtures being used as blanks and bovine serum albumin as a standard.

^{3}H was measured by a Packard model 2450 liquid-scintillation counter, with Insta-Gel (Packard Instrument Co., Downers Grove, IL, U.S.A.) as scintillation medium. ^{125}I was measured in a Searle model 1195 gamma counter.

The activities of the following enzymes were determined as markers for respective subcellular organelles: 5′-nucleotidase for plasma membranes (Bartlett, 1959; Morré, 1971); glucose 6-phosphatase for microsomal fraction (Bartlett, 1959; Morré, 1971); succinate–*p*-iodonitrotetrazolium Violet reductase for mitochondria (Morré, 1971); arylsulphatase (Sherman & Stanfield, 1967), β-*N*-acetylglucosaminidase (Bowers *et al.*, 1967), β-galactosidase (Robins *et al.*, 1968), acid phosphatase (Bowers *et al.*, 1967), β-glucuronidase (Allison & Sandelin, 1963; Robins *et al.*, 1968) and cathepsin D (method III of Barrett, 1972) for lysosomes.

Free isoelectric focusing was carried out as described by Lundahl & Hjertén (1973). Lysosomal fractions were isolated as outlined in Scheme 1 and gradient medium was removed by centrifugation (Pertoft & Laurent, 1977). The fractions were immediately covered with 1 ml of 0.1 M-sodium glycinate buffer, pH9.0, containing 0.2% of Triton X-100 and 0.1% of sialic acid (Edmond *et al.*, 1966). Disintegration of lysosomal particles was performed in an MSE ultrasonic disintegrator for 1 min at 150W, 20kHz/s, amplitude 4. The insoluble residues were removed by centrifugation at 100000*g* for 3h at 4°C. The soluble lysosomal proteins were fractionated by isoelectric focusing in an Ampholine gradient, pH10–3 (LKB, Stockholm, Sweden), containing 0.1% of sialic acid (Edmond *et al.*, 1966) in a rotating methylcellulose-coated electrophoresis tube for 24h at 10°C. Fractions (200 μl) were collected and samples were used for measurements of pH and enzyme activity.

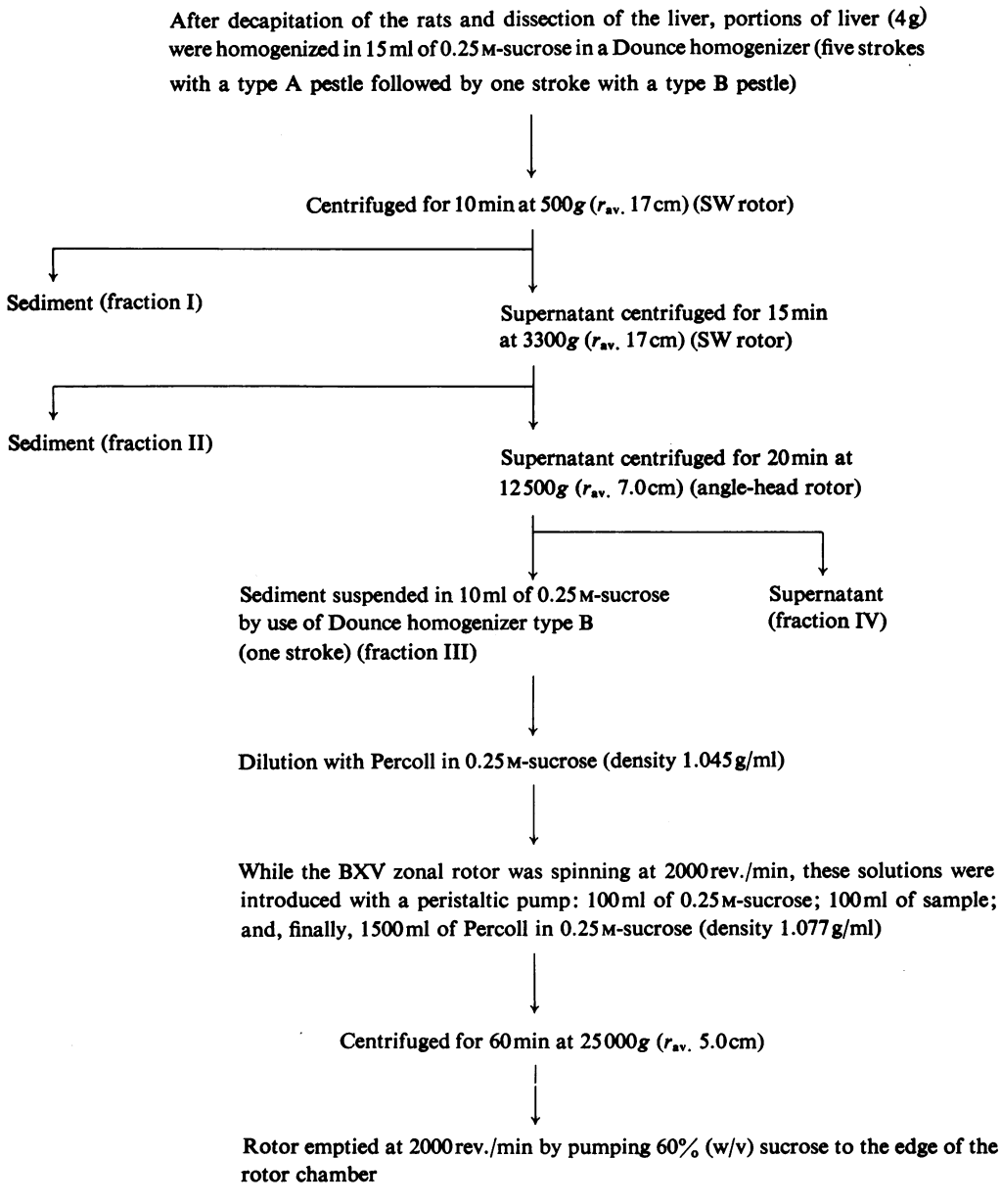
Results

Separation of heterogeneous lysosomal particles

Differential centrifugation. Sprague–Dawley rats (150g) were starved overnight before being killed. The livers were dissected out, homogenized and fractionated by differential centrifugation at 4°C as outlined in Scheme 1. Four fractions (I–IV) were collected and analysed for marker-enzyme activity (Table 1). Although all fractions contained substantial amounts of lysosomal enzyme activity, the highest specific activity of all lysosomal enzymes analysed was found, as expected, in fraction III. This fraction was therefore used in the further separation procedure.

Zonal centrifugation. Fraction III obtained from 20–25g of liver was suspended in 10ml of 0.25 M-sucrose by one stroke with a B pestle in a Dounce homogenizer and diluted to 100ml with iso-osmotic Percoll density-gradient medium to give a density of 1.045g/ml. Zonal centrifugation was performed in an MSE High-Speed 25 centrifuge as described in Scheme 1.

The distribution of protein and reference-enzyme activities in the fractions obtained after zonal centrifugation is shown in Fig. 1. The distribution of protein and enzyme activity versus fraction density was essentially unaltered when zonal runs were performed for a longer time (2h), indicating that the subcellular organelles had banded at their respective buoyant densities. However, 2h of centrifugation yielded a gradient of steeper shape, and consequently the distribution of protein and enzyme activities versus fraction number changed compared with that observed after the short-time centrifugation. Succinate–*p*-iodonitrotetrazolium Violet reductase, which indicates the presence of mitochondria, was present only in the three fractions of highest density and also, together with glucose 6-phosphatase and 5′-nucleotidase (marker enzymes for endoplasmic reticulum and plasma membranes respectively), in a few fractions of very low density (Fig. 1). In contrast, arylsulphatase, a lysosomal enzyme, showed



Scheme 1. *Flow sheet for the preparation of subcellular particles derived from rat liver*

activity throughout the gradient in the density range 1.04–1.11g/ml. Distribution patterns similar to that obtained for arylsulphatase in the zonal density gradient (Fig. 1) were also found for other lysosomal enzymes, i.e. acid phosphatase, β-galactosidase, cathepsin D and β-glucuronidase. Thus the zonal-centrifugation step results in a substantial purification of lysosomal particles and furthermore a considerable heterogeneity in the buoyant density of the particles is revealed.

That the density-gradient centrifugation really separates distinct lysosomal subpopulations was demonstrated in experiments where ^{125}I-labelled asialocaeruloplasmin-containing lysosomes (see

Table 1. *Distribution of reference enzymes after fractionation of the liver by centrifugation in* 0.25 M-*sucrose*
Fractionation was as described in Scheme 1, in which fractions I–IV are identified. Activity in the homogenate was taken as 100%. Values represent means of results of four separate experiments. Activity per mg of protein in the homogenate was taken as 1.00.

	Recovery (%)	Recovered amount (%)				Relative specific activity			
		I	II	III	IV	I	II	III	IV
Protein	103	55	6	8	31				
Enzymes									
Acid phosphatase	101	40	15	30	15	0.73	2.50	3.75	0.48
Arylsulphatase	100	25	15	51	8	0.45	2.50	6.37	0.26
β-Galactosidase	102	43	16	32	8	0.78	2.67	4.00	0.26
β-Glucuronidase	108	40	18	31	11	0.73	3.00	3.87	0.35
β-*N*-Acetylglucosaminidase	98	42	22	32	4	0.76	3.67	3.98	0.13
5′-Nucleotidase	103	70	8	8	14	1.27	1.33	1.00	0.45
Glucose 6-phosphatase	102	62	7	8	23	1.13	1.17	1.00	0.74
Succinate dehydrogenase	98	69	21	5	5	1.26	3.50	0.62	0.16

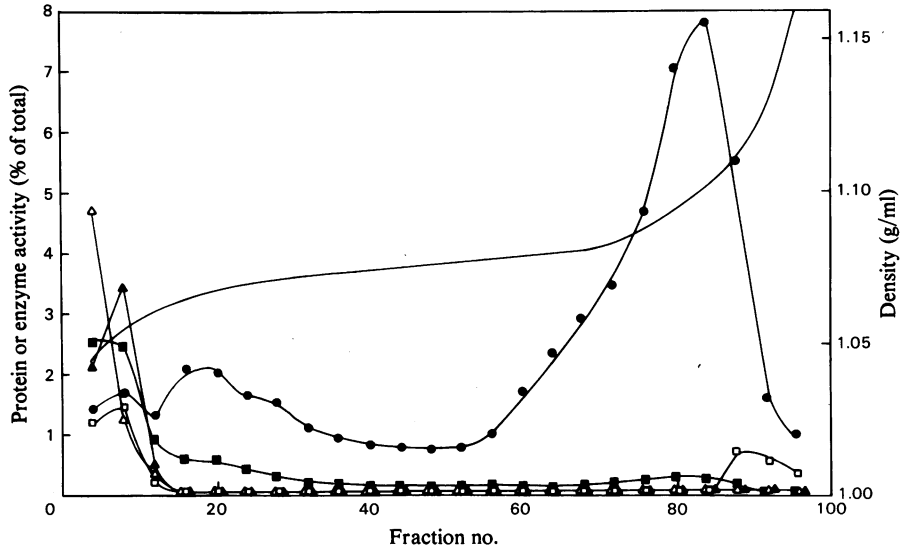


Fig. 1. *Distribution of protein and marker-enzyme activities in fractions obtained after zonal centrifugation of a crude rat liver lysosomal fraction*
After zonal centrifugation, carried out as described in Scheme 1, 96 fractions (about 18 ml each) were collected and density (——), protein (■) and activities of the enzymes 5′-nucleotidase (△), glucose 6-phosphatase (▲), succinate–*p*-Iodonitrotetrazolium Violet reductase (□) and arylsulphatase (●) were measured. The protein content and enzyme activities are expressed as percentages of the amount in the original homogenate.

below) of high and low density respectively were isolated and then thoroughly mixed with unlabelled lysosomes of opposite density, and subsequently re-centrifuged in density gradients (Fig. 2). The buoyant density of both types of labelled lysosomes originally observed remained after re-centrifugation.

Metabolic relationship of lysosomal subpopulations

The possibility that lysosomal particles of different densities can represent different stages of one common metabolic route was investigated by studying the distribution of ^{125}I-labelled asialocaeruloplasmin in the lysosomal subpopulations 1, 15, 30 and 60 min after administration of the labelled glycoprotein. In agreement with previous reports, the asialoglycoprotein was rapidly taken up by the liver, and at 1 min after injection 85% of the labelled material was recovered in a liver homogenate, although a major part of this material was not associated with fraction III after differential centrifugation (Table 2).

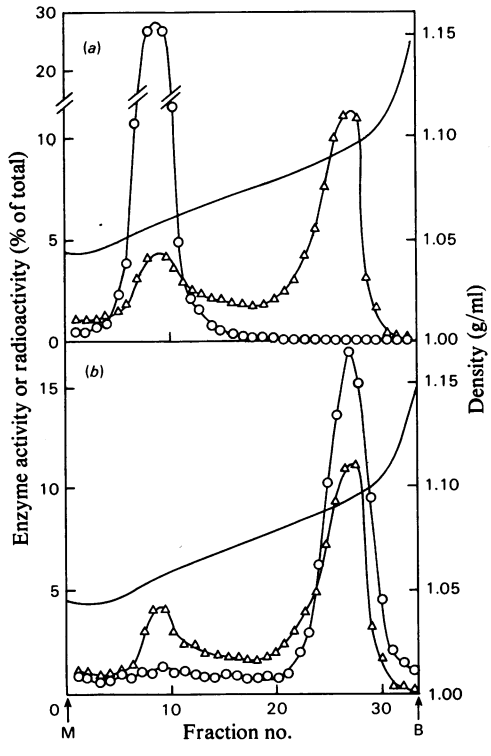


Fig. 2. *Re-centrifugation of ^{125}I-labelled particles in density gradients*

Lysosomal particles of different density were prepared in the following way. Fraction III, prepared as outlined in Scheme 1, was diluted to a final volume of 20ml with 0.25M-sucrose and layered on to 70ml of Percoll in 0.25M-sucrose (density 1.077g/ml) in 95ml tubes. Centrifugation was performed at 25000rev./min for 30min in an MSE angle rotor. After centrifugation the tubes were emptied by pumping 60% sucrose to the bottom of the tube, and fractions (about 3ml) were collected and analysed. Similarly ^{125}I-labelled particles were isolated from an animal which 30min earlier had received an injection of ^{125}I-labelled asialocaeruloplasmin. Labelled particles recovered at a density of (*a*) 1.09g/ml, or (*b*) 1.06g/ml were mixed with unlabelled particles of opposite density, suspended by one stroke in a Dounce homogenizer with a type A pestle and re-centrifuged in a density gradient as described above. Fractions were analysed for density (——), radioactivity (○) and acid phosphatase activity (△). The ordinate shows the percentage of the total activities and radioactivities applied on the gradients. M and B denote meniscus and bottom of the tube respectively.

Table 2. *Recovery of ^{125}I-labelled asialocaeruloplasmin during the isolation of rat liver lysosomes*

Animals were killed at different times after receiving an injection of ^{125}I-labelled asialocaeruloplasmin (2×10^7c.p.m.) and lysosomes were isolated as outlined in Scheme 1. Fraction III was obtained after differential centrifugation (see Scheme 1). Fraction 16–18 was the low-density fraction (1.06g/ml) and fraction 82–84 was the high-density fraction (1.09g/ml).

					Density-gradient centrifugation			
	Homogenate		Fraction III		Fraction 16–18		Fraction 82–84	
Time after injection (min)	$10^{-6}\times$ Radioactivity (c.p.m.)	% of injected radioactivity	$10^{-6}\times$ Radioactivity (c.p.m.)	% of radioactivity in the homogenate	$10^{-5}\times$ Radioactivity (c.p.m.)	% of radioactivity in the homogenate	$10^{-5}\times$ Radioactivity (c.p.m.)	% of radioactivity in the homogenate
1	17.5	85	2.7	15	1.9	1.1	0.05	0.02
15	14.6	71	6.4	44	2.8	1.9	0.7	0.5
30	12.1	59	4.9	41	1.4	1.1	1.2	1.0
60	6.8	33	3.3	48	0.7	1.0	2.1	3.1

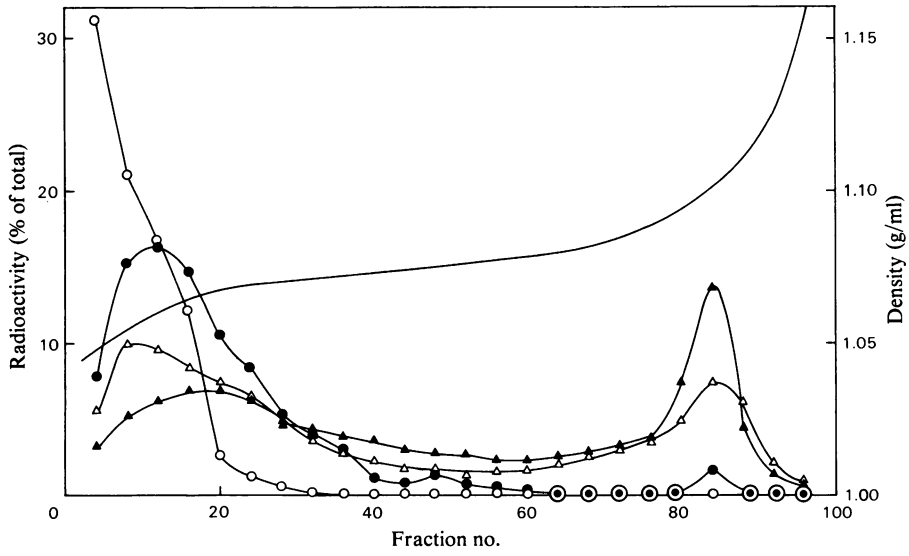
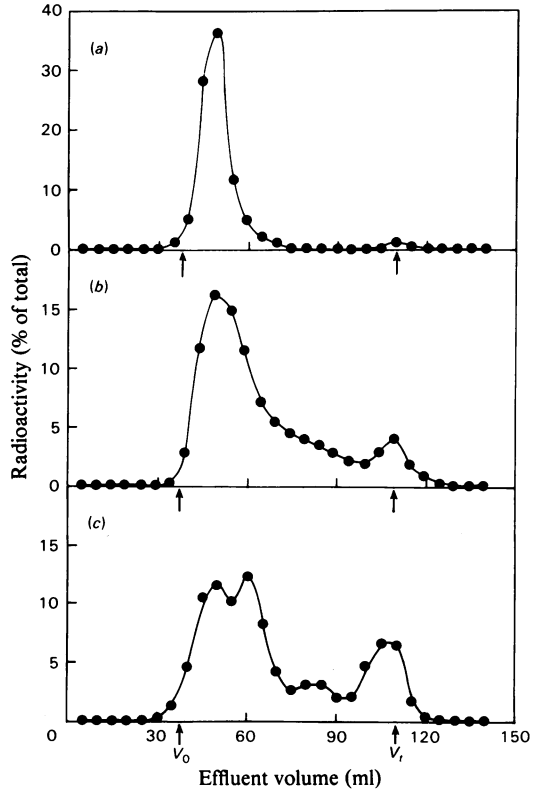


Fig. 3. *Distribution of ^{125}I-labelled asialocaeruloplasmin at different times after administration to the animal in lysosomal fractions obtained by zonal centrifugation*

Livers obtained from animals killed 1 (○), 15 (●), 30 (△) and 60 (▲) min after receiving the ^{125}I-labelled asialocaeruloplasmin were fractionated as outlined in Scheme 1. Material recovered in fraction III was subjected to zonal centrifugation (see the legend to Scheme 1) and fractions were analysed for density (——) and radioactivity. The ordinate shows the percentage of total radioactivity in fraction III.



When the ^{125}I-labelled asialocaeruloplasmin was left in the animals for longer times before they were killed the amount of label recovered in the liver homogenate declined. In these cases the amounts of radioactivity subsequently recovered in fraction III (Table 2) roughly paralleled the recovery of lysosomal enzyme in this fraction (Table 1).

Fig. 3 shows the distribution of ^{125}I-labelled material in subsequent zonal-density-gradient centri-

Fig. 4. *Gel chromatography of ^{125}I-labelled asialocaeruloplasmin and ^{125}I-labelled material recovered from lysosomes of different buoyant density*

^{125}I-labelled asialocaeruloplasmin was injected into a rat 30 min before the animal was killed. Rat liver lysosomes were isolated as outlined in Scheme 1. Subcellular organelles present in the zonal-density-gradient fractions were collected by centrifugation at 100000*g* in an angle rotor for 2 h (Pertoft & Laurent, 1977), lysed by sonication (see under 'Methods') and particulate material was removed by centrifugation for 1 h in a swing-out rotor. ^{125}I-labelled asialocaeruloplasmin (*a*; corresponding to 7.2×10^5 c.p.m.) and ^{125}I-labelled material recovered from zonal fractions of density 1.06 g/ml (*b*; corresponding to 1.7×10^4 c.p.m.) and of density 1.09 g/ml (*c*; corresponding to 1.5×10^4 c.p.m.) were applied to a column (1 cm × 80 cm) of Sepharose 6B and eluted at a rate of 2 ml/h with 6 M-guanidinium hydrochloride in 0.05 M-Tris/HCl, pH 8.5. Fractions (about 1.5 ml) were collected and analysed for radioactivity.

fugations. Except for the material obtained from the rat killed 1 min after receiving ^{125}I-labelled asialocaeruloplasmin, the radioactivity in the different samples was distributed similarly to a lysosomal marker enzyme (compare Figs. 3 and 1). However, the distribution of recovered radioactivity in the density gradient shifted progressively from fractions of low density to fractions of higher densities as a function of time between administration of labelled asialoglycoprotein and killing of the animal. Furthermore, the total amount of radioactivity present in fractions of high densities (fractions 82–84, of density 1.09 g/ml, was chosen for comparison; Fig. 3) increased in the material obtained 1, 15, 30 and 60 min after injection of ^{125}I-labelled asialocaeruloplasmin (Table 2). The total recovery of radioactivity in the gradient varied between 85 and 95% of the amount applied on the gradients.

A possible lysosomal nature of the subcellular particles containing the ^{125}I-labelled material was investigated by subjecting fractions of low density (fractions 16–18 in Fig. 3) to repeated freezing and thawing. The release of the activity of a lysosomal marker enzyme, acid phosphatase, and ^{125}I radioactivity occurred in a parallel fashion from all fractions isolated 15, 30 or 60 min after injection of ^{125}I-labelled asialocaeruloplasmin. In these cases more than 70% of the enzyme and ^{125}I radioactivity was released after five repeated freezings, suggesting that the labelled asialoglycoprotein was located in lysosomes. However, most of the ^{125}I-labelled asialocaeruloplasmin present in the density-gradient fractions isolated immediately after the animal had received the labelled compound could not be released by the repeated freezing procedure, indicating that this material was not located within particles of lysosomal nature.

The possibility that ^{125}I-labelled material present in particles of high density and low density had been subjected to degradation was investigated by gel chromatography on a column of Sepharose 6B of particles recovered from appropriate fractions and lysed by sonication. The results (Fig. 4) showed that the most pronounced degradation of the ^{125}I-labelled asialoglycoprotein had occurred in the high-density lysosomes, although the low-density lysosomes also contained degraded ^{125}I-labelled asialocaeruloplasmin. Gel chromatography of ^{3}H-labelled material in lysosomes of high and low density isolated after

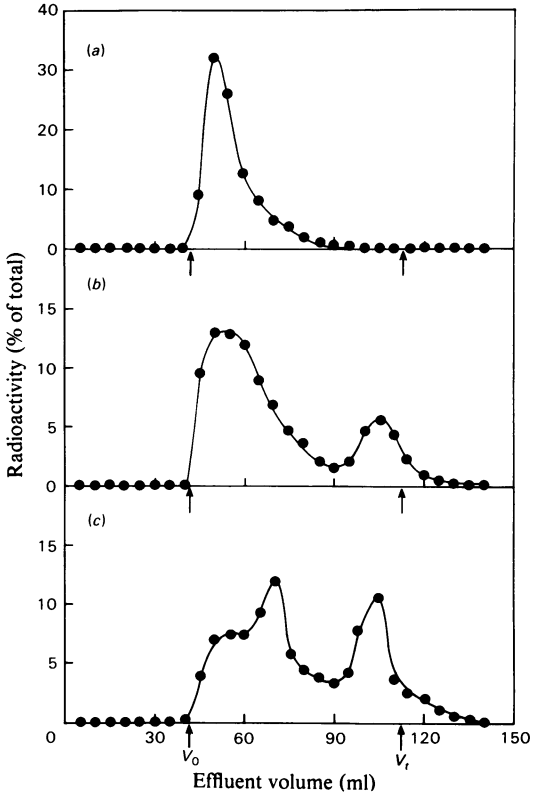


Fig. 5. *Gel chromatography of [^{3}H]asialocaeruloplasmin and ^{3}H-labelled material recovered from lysosomes of different density*

[^{3}H]Asialocaeruloplasmin (*a*; corresponding to 4.3×10^{5} c.p.m.) and ^{3}H-labelled material recovered after zonal centrifugation in fractions of density 1.06 g/ml (*b*; corresponding to 3.3×10^{3} c.p.m.) and 1.09 g/ml (*c*; corresponding to 7.8×10^{3} c.p.m.) respectively were chromatographed on a column of Sepharose 6B as described in the legend to Fig. 4.

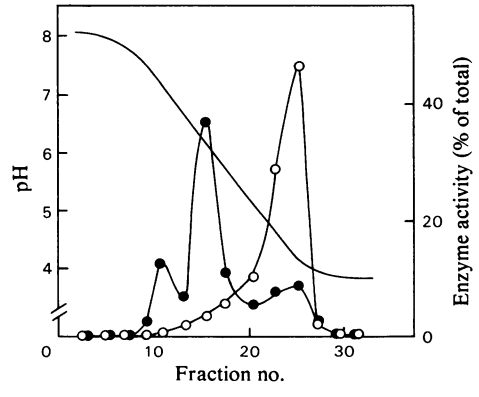


Fig. 6. *Free isoelectric focusing of lysosomal enzymes recovered from particles of different buoyant density*

Distribution of arylsulphatase after isoelectric focusing (carried out as described under 'Methods') of fractions recovered in the zonal density gradient at densities 1.06 (○) and 1.09 (●) g/ml respectively (see the legend to Fig. 4). ——, pH.

injection of [^{3}H]asialocaeruloplasmin gave similar results (Fig. 5).

The experiments described above indicate that the lysosomes in which asialocaeruloplasmin is originally captured are of comparably low density and that these particles continuously acquire a higher density with time. Evidence in support of this hypothesis was provided by analysing the isoelectric points of lysosomal enzymes.

Goldstone & Koenig (1974) have demonstrated a shift in the isoelectric points of lysosomal enzymes towards higher pH values during the aging of the lysosome. The isoelectric points of a lysosomal enzyme, arylsulphatase, in lysosomal subpopulations were therefore determined by isoelectric focusing of proteins recovered after lysis of the particles (see under 'Methods'; Fig. 6). Most of the arylsulphatase activity in the light fractions was associated with proteins having isoelectric points at pH 4–5. Virtually no proteins in fractions banding above pH 5.5 showed any arylsulphatase activity (Fig. 6). On the other hand, in the dense particles most of the arylsulphatase activity was associated with proteins recovered after isoelectric focusing in the range pH 5.5–7.8 (Fig. 6).

Discussion

According to the protocol (Scheme 1) for isolation of rat liver lysosomes the tissue is homogenized under fairly gentle conditions to avoid rupture of the lysosomal particles. The low content of lysosomal enzymes in fraction IV (Table 1) indicates that most lysosomes were indeed intact. Lysosomal enzymes released during the preparation can be adsorbed on other subcellular particles and interfere with the interpretation of lysosomal heterogeneity (Baccino *et al.*, 1971). A drawback of a gentle preparation procedure is, however, a lower recovery of lysosomes in fraction III. Many cells remain more or less intact and the lysosomes become associated with larger structures, which accumulate mainly in fraction I. There is no reason, however, to suspect that the lysosomes in fraction III should not be representative for the whole lysosomal population.

The present paper demonstrates, in agreement with previous studies (for review see Davies, 1976), a heterogeneity in the buoyant density of rat liver lysosomes. The densities found for the lysosomal particles in the iso-osmotic Percoll density gradients are considerably lower than those reported previously (Beaufay *et al.*, 1964). However, these latter authors used sucrose as gradient medium, which yielded a hyperosmotic solution. Lysosomal particles appear to be osmotically fragile (Reijngoud & Tager, 1977), and the observed discrepancy in density of lysosomes can be explained by a loss of water from particles to compensate for a high external osmotic pressure, thus yielding lysosomes of a higher density in hyperosmotic media. In support of this explanation our results are consistent with previously reported buoyant densities of rat liver lysosomes in iso-osmotic Ficoll density gradients (Futai *et al.*, 1972).

Part of the heterogeneities of lysosomal particles obtained from rat liver can be explained by the presence of more than one cell type in the tissue (e.g. Kupffer cells and hepatocytes). However, in the present study asialocaeruloplasmin, which is catabolized only in hepatocytes, was observed in lysosomal-like particles differing with regard to buoyant density. The lysosomal nature of particles containing ^{125}I-labelled asialocaeruloplasmin is suggested by (*a*) release of ^{125}I radioactivity and lysosomal enzyme activity in a parallel manner during repeated freezing and thawing of the particles and (*b*) the presence of degraded ^{125}I-labelled asialocaeruloplasmin in these particles. Further evidence for a lysosomal degradation of asialoglycoproteins in rat hepatocytes has been reported (Gregoriadis *et al.*, 1970; LaBadie *et al.*, 1975). Thus a heterogeneity in the buoyant density of lysosomal particles from rat hepatocytes can be deduced.

It should be noted that the organelles containing ^{125}I-labelled asialocaeruloplasmin recovered in fraction III immediately after administration of the asialoglycoprotein do not coincide with the lysosomal marker-enzyme activity in the next density-gradient centrifugation. The nature of these low-density particles is not known, although one can speculate that the labelled asialoglycoprotein is associated with plasma-membrane fragments containing the appropriate receptor or present in phagosomes.

A relationship between lysosomal subpopulations is suggested by the apparent transport of ^{125}I-labelled asialocaeruloplasmin from low-density to high-density lysosomal particles. The catabolism of asialocaeruloplasmin thus appears to involve a step where degradative active lysosomes progressively acquire higher buoyant densities during the course of asialocaeruloplasmin degradation.

In support of this model radioactively labelled material recovered 30min after administration of asialocaeruloplasmin was less degraded in lysosomes of low density than in the high-density particles. Further, arylsulphatase originating from the high-density particles appears to have a higher isoelectric point than the corresponding enzyme associated with the low-density particles, supporting the hypothesis of a positive relationship between 'age' and density of the lysosomes (Goldstone & Koenig, 1974).

The possibility that the demonstrated relationship between age and density of rat liver parenchymal lysosomes is valid also for other cell types should be considered, but obviously further experimental

work is needed before any conclusion on this matter can be made.

We are indebted to Professor T. C. Laurent for helpful discussions during the course of this work and to Professor S. Hjertén and Mrs. I. Blomquist for performing the isoelectric focusing. This work was supported by the Swedish Medical Research Council (03X-4, 05197), Gustaf V:s 80-årsfond and Svenska livförsäkringsbolagens nämnd får medicinsk forskning.

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