Differences in Distribution of Esterase between Cell Fractions of Rat Liver Homogenates Prepared in Various Media

RELEVANCE TO THE LYSOSOMAL LOCATION OF THE ENZYME IN THE INTACT CELL

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The distribution of esterase in subcellular fractions of rat liver homogenates was compared with that of the lysosomal enzyme acid phosphatase and the microsomal enzyme glucose 6-phosphatase. Most of the esterase from sucrose homogenate sediments with glucose 6-phosphatase and about 8% is recovered in the supernatant. However, up to 53% of the esterase can be washed from microtome sections of unfixed liver, in which less cellular damage would be expected than that caused by homogenization. About 40% of both esterase and acid phosphatase are recovered in the soluble fraction after homogenization in aqueous glycerol or in a two-phase system (Arcton 113–0.25M-sucrose), although glucose 6-phosphatase is still recovered in the microsomal fraction of such homogenates. The esterase of the microsomal fraction prepared from a sucrose homogenate is much more readily released by treatment with 0.26% deoxycholate than are other constituents of this fraction. The release of esterase from the microsomal fraction by the detergent and its concomitant release with acid phosphatase after homogenization in glycerol or the two-phase system suggests that a greater proportion of esterase may be present in lysosomes of the intact cell than is indicated by the results of standard fractionation procedures.

The esterase of rat liver (carboxylic ester hydrolase EC 3.1.1.1) that hydrolyses indoxyl acetate and its derivatives is recovered mainly in the microsomal fraction of liver homogenates and the activity of the combined mitochondrial and lysosomal fractions represents only a small proportion of the total activity of a whole liver homogenate (Underhay, Holt, Beaufay & de Duve, 1956). This evidence conflicts with the localization of the enzyme seen in sections of fixed liver stained by using indoxyl acetates at pH 8.5 as substrates and subsequent oxidation of the released indoxyl to indigo by a redox buffer containing potassium ferricyanide and potassium ferrocyanide (Holt, 1958). Peribiliary granules are shown to be intensely stained but there is little cytoplasmic staining corresponding to the activity of the endoplasmic reticulum from which the microsomal fraction is derived. The granules also show acid phosphatase activity when stained by a cytochemical technique and have therefore been identified as lysosomes (Essner & Novikoff, 1961; Holt & Hicks, 1961).

It is difficult to reconcile the cytochemical results with the low esterase activity of fractions that contain lysosomes. The validity of both

experimental approaches therefore needs re-examination and in the present paper we describe and comment upon the results of a biochemical investigation into the stability of binding of liver esterase in tissue sections and fractions; we also investigate the effects of processes, such as freezing, used in preparing sections for cytochemical staining, upon this binding.

MATERIALS AND METHODS

Chemicals

Reagents of the highest available purity were used throughout. Indoxyl acetate of analytical purity was synthesized in our laboratory (Holt, 1958) or was obtained from TAA B Laboratories, Reading, Berks., U.K. The sodium salt of glucose 6-phosphate was obtained from Boehringer Corp. (London) Ltd., London W.5, U.K., and the disodium salt of 2-glycerophosphoric acid (Eastman) from Kodak Ltd., Kirkby, Liverpool, U.K. Triton X-100 was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., Triton WR-1339 from Winthrop Laboratories, Newcastle upon Tyne, U.K., and sodium deoxycholate from E. Merck A.-G., Darmstadt, Germany. Arcton 113 is a product of Imperial Chemical Industries Ltd., London W.C.1, U.K.

Animals

Male rats were bred at random from the Courtauld Institute strain of Wistar albino rats and were fed *ad libitum* on Rowett Research Institute diet no. 86. Rats weighing 190–210 g were starved overnight and killed by a blow on the head. Blood was immediately removed from the liver by perfusion with 0.85% (w/v) NaCl via the hepatic portal vein after section of the posterior vena cava, and the liver was then placed on ice.

For the preparation of isolated lysosomes (see below) rats were injected intravenously with a solution of Triton WR-1339 in 0.85% (w/v) NaCl (Wattiaux, Wibo & Baudhuin, 1963). Each rat was killed 4 days after receiving one 170 mg injection of Triton X-100. Rats were starved overnight before being killed.

Cell fractionation

Homogenization of liver. The liver was cut into small pieces with scissors and homogenized with 0.25 M-sucrose (1:3, w/v) in a 25 ml glass Potter–Elvehjem homogenizer fitted with a Teflon pestle (A. H. Thomas, Philadelphia, U.S.A.). The pestle, rotating at 1200 rev./min, was passed once up and down through the suspension during 60 s; this disrupted most of the cells but did not damage the lysosomal membrane, as judged by the low release of acid phosphatase into the supernatant (Table 1).

For experiments on the release of soluble esterase into the homogenizing medium (see below), some samples of liver were homogenized in 0.25 M-sucrose containing 0.1 M-, 0.5 M- and 1.0 M-NaCl, 0.5 M-KCl or 10% (w/v) pharmaceutical-grade polyvinylpyrrolidone.

Connective tissue and unbroken cells in the homogenate made it difficult to withdraw representative samples. Measurements were therefore made on the whole homogenate after sedimentation of the nuclear fraction as described below and adjustment of the volume of the remaining suspension (referred to as the cytoplasmic extract) to give a concentration equivalent to 100 mg of liver/ml. The sum of measurements made separately on the cytoplasmic extract and the nuclear fraction represents the whole homogenate and recovery values are based on this value.

Differential centrifugation. The homogenates were separated into five fractions: nuclear (N), mitochondrial (M), light mitochondrial (L), microsomal (P) and soluble (S), by the method of Appelmans, Wattiaux & de Duve (1955). The nuclear fraction was sedimented at 400 g for 10 min in a Servall RC-2 centrifuge (SS-34 rotor). The other fractions were prepared in a Spinco model L centrifuge (no. 40 rotor). All separations were performed at 0–4°C, and the supernatants were removed with a Pasteur pipette or by decanting. The pellets were resuspended in a suitable volume of 0.25 M-sucrose by using a TenBroeck hand-operated tissue grinder, capacity 2 ml (Kontes Glass Co., Vineland, N.J., U.S.A.). The resuspended pellets and final supernatants were kept at 0°C before the measurements described below were made.

Density-gradient centrifugation. A total mitochondrial fraction corresponding to the M and L fractions was separated from the cytoplasmic extract and resuspended in sucrose of density 1.21 at a concentration equivalent to

1 g of liver/ml. Portions (1 ml) of this suspension were placed in centrifuge tubes and 1 ml each of sucrose solutions of density 1.155, 1.14 and 1.06 were layered above the suspension. After centrifugation at 120 000 g for 2 h in the Spinco model L centrifuge (SW39L rotor) (Trouet, 1964), the clear top layer, the lysosomes accumulating at both interfaces of the solution of density 1.14 and the loose pellet at the bottom of the tube were collected separately and adjusted to a known volume with 0.25 M-sucrose. The acid phosphatase, esterase and glucose 6-phosphatase activities of these fractions were determined.

Release of esterase from liver preparations

Except for the preparations of sections all operations were done at 4°C and all preparations were kept at this temperature before measurements were made.

Preparation of sections. The following groups of sections were prepared from a perfused liver: (a) 300 µm sections cut on a mechanical chopper (McIlwain & Buddle, 1953) at room temperature; (b) 300 µm sections of liver previously frozen rapidly at –20°C, thawed at room temperature and then cut on the McIlwain chopper; (c) 25 µm frozen sections cut in the cryostat at –20°C; (d) 10 µm frozen sections cut in the cryostat at –20°C.

Weighed amounts of sections from (a), (b), (c) and (d), each equivalent to about 1 g of liver, were gently agitated in 10 ml of 0.25 M-sucrose for 30 min. The sections were removed from the solution by centrifuging at 1000 g for 10 min and the supernatant was centrifuged at 105 000 g for 60 min to ensure complete removal of particulate material from the medium. The pellets recovered at the two speeds were combined and homogenized in 0.25 M-sucrose with the TenBroeck tissue grinder and the volume of the suspension was adjusted to 10 ml. The suspension was centrifuged at 35 000 g for 6.7 min, then at 105 000 g for 60 min; the final supernatant was decanted and the two pellets were combined and suspended in 0.25 M-sucrose. The suspended pellets and the supernatants were assayed for esterase activity.

Homogenization in glycerol solution. A 3 g portion of finely chopped liver was homogenized in 5 ml of 75% (v/v) glycerol in the Potter–Elvehjem homogenizer, and then 25 ml of 0.25 M-sucrose–8.5 mM-NaCl was added, bringing the final concentration of glycerol to 12.5% (v/v) (Carruthers, Woernley, Baumber & Lilga, 1960). The suspension was thoroughly stirred and centrifuged by the method of Appelmans *et al.* (1955); then the fractions were assayed for acid phosphatase, esterase and glucose 6-phosphatase activities. As a control 3 g of liver was homogenized in 30 ml of 0.25 M-sucrose–8.5 mM-NaCl and then centrifuged as described above.

Homogenization in a two-phase system. Finely chopped rat liver was passed through an ice-cold hand-operated tissue press (Climpex Ltd., London N.W.7, U.K.). Then 0.5 g of the resulting tissue brei was homogenized for 1 min in 15 ml of Arcton 113 (1,1,2-trichloro-1,2,2-trifluoroethane)–0.25 M-sucrose (1:2, v/v). The emulsion was centrifuged at 30 000 g for 10 min in the Servall centrifuge (rotor SS-34). The clear esterase-containing aqueous upper layer (see below), the intermediate tissue layer and the clear lower layer of Arcton were collected separately

with a Pasteur pipette. The Arcton layer was evaporated to dryness and the slight residue was tested for the presence of phospholipid phosphorus by the method of King & Wootten (1956). Any contaminating Arcton was removed from the tissue layer by evaporation at 0°C in a rotary-film evaporator (Camlab Ltd., Cambridge, U.K.). The tissue was gently dispersed in 0.25 M-sucrose with the TenBroeck tissue grinder and the volume made up to 12 ml. The aqueous upper layer and the tissue suspension were separately centrifuged at 35 000g for 6.7 min in the Spinco Model L centrifuge (no. 40 rotor), and the supernatants centrifuged at 105 000g for 60 min. The pellets that sedimented at the two speeds were combined and suspended in 0.25 M-sucrose. The enzyme activities of these suspensions and those of the final supernatants were determined. As a control experiment 0.5 g of the liver was homogenized in 15 ml of 0.25 M-sucrose and centrifuged in the same way as the aqueous layer and the tissue suspension.

Release of enzyme from the microsomal pellet

Washing with media of different ionic concentration. A microsomal pellet, prepared as described above from a sucrose homogenate, was resuspended in 2.0 ml of each medium (Table 6) by using the TenBroeck tissue grinder. The volume was made up to 11 ml with the medium and the suspension was centrifuged at 105 000g for 60 min. In one experiment a microsomal pellet was frozen at -20°C for 1 h, thawed and then washed as described above with 0.25 M-sucrose. Another pellet was resuspended in 3 ml of the sucrose solution and the suspension was frozen at -20°C for 1 h before being thawed and made up to 11 ml and centrifuged. In each case the supernatant was decanted and the centrifuge tube and pellet rinsed with 0.25 M-sucrose before resuspending the pellet in a known volume of 0.25 M-sucrose. The resuspended pellets and washing media were assayed for esterase activity.

Digestion with ribonuclease. Microsomal pellets derived from 1 g of liver were resuspended in 10 ml of 0.25 M-sucrose and incubated at 37°C for 30 min with 1–5 mg of ribonuclease (Worthington), obtained from Cambrian Chemicals Ltd., Croydon, Surrey, U.K. The suspension was centrifuged at 105 000g for 60 min and esterase activity in the resulting pellet and supernatant was measured.

Ultrasonication of microsomal pellets. Microsomal pellets from 3 g of liver were resuspended in 3 ml of 0.25 M-sucrose, to give a protein concentration of approx. 30 mg/ml. This suspension was cooled in an ice bath and sonicated at 50 W at 16–24 kHz for 3 min by using a sonicator type E7680 (Mullard Ltd., London W.C.1, U.K.) with a titanium probe of dimensions 3 cm × 6 cm at the wide end and 1.9 cm × 6 cm at the narrow operating end. Sonicated suspensions were made up to 12 ml with 0.25 M-sucrose, 11 ml of which was then centrifuged at 105 000g for 60 min. Esterase and glucose 6-phosphatase activities and the cytochrome b_5 and protein contents of the pellets, supernatants and the sonicated suspension before fractionation were determined. In some cases the resulting supernatant was further centrifuged at 105 000g for 240 min and esterase activity and protein content were determined in this second pellet and supernatant.

Treatment of the pellet with deoxycholate. Microsomal

pellets were resuspended in 0.25 M-sucrose to a protein concentration of 4–5 mg/ml. Then 1 ml of 2.6% (w/v) sodium deoxycholate was added to 9 ml of the suspension to bring the final concentration of deoxycholate to 0.26%. The suspension was immediately centrifuged at 105 000g for 120 min, to give a clear supernatant, and a cloudy membranous layer lying over the hard-packed ribonucleoprotein pellet (Ernster & Jones, 1962). The pellet was resuspended in 0.25 M-sucrose and the esterase and glucose 6-phosphatase activities and the cytochrome b_5 and protein contents were measured in the three fractions. In some experiments the clear supernatant from the deoxycholate-treated pellet was centrifuged for a further 3–7 h at 105 000g either before or after diluting it five- and ten-fold with 0.25 M-sucrose. Measurements to determine whether any further sedimentation of esterase occurred were made on the supernatants because the pellets were too small to be handled quantitatively.

Absorption of soluble esterase by particulate components of the homogenate

Whole liver and sections homogenized in an aqueous extract of esterase. Esterase from 1 g of rat liver was extracted by cutting 10 μ m cryostat sections and washing them with 10 ml of 0.25 M-sucrose (see above). The sections were removed by centrifuging the mixture at 1000g for 10 min, to give a solution containing approximately half of the total esterase activity of the tissue. Two preparations were made in this way. One lot of washed sections was homogenized with the TenBroeck tissue grinder in one of the esterase extracts and 1 g of the same liver was homogenized in the second esterase extract. The second lot of washed sections and another 1 g of the liver were separately homogenized in 10 ml of 0.25 M-sucrose. Each of the four suspensions was separated into the five standard fractions and esterase activity of these was measured.

Whole liver homogenized in water–Arcton extracted esterase. Soluble esterase was prepared by homogenizing 1 g of liver in the two-phase Arcton–sucrose system (see above). Another 1 g portion of the same liver was homogenized in this extract and a third 1 g portion was homogenized in 0.25 M-sucrose. The two suspensions were separated into the five standard fractions and measurements for esterase activity were made.

Microsomal pellets homogenized in esterase extracts. Microsomal pellets, in each case derived from 1 g of liver, were separately resuspended in the TenBroeck tissue grinder in esterase extracts prepared from 1 g of liver by the cryostat technique and by the Arcton method, and also in 10 ml of 0.25 M-sucrose alone. The suspensions were centrifuged at 105 000g for 60 min, then the supernatants were decanted and the newly formed pellets resuspended in 0.25 M-sucrose and measured for esterase activity.

Assays

Esterase (EC 3.1.1.1). Esterase activities were determined potentiometrically by using a Radiometer pH-stat (TTT1a) and a Titrigraph (SBR 2a) (Radiometer, Copenhagen, Denmark). The assays were performed in a total volume of 3.0 ml at pH 7.5 and 37°C in a stream of CO₂-free air. The mixture contained 0.1 ml of an ethanolic solution of indoxyl acetate giving a final substrate concentration of

1 mM. After it had been established that the rate of autohydrolysis of the substrate was negligible over a period of 4–8 min, the esterase preparation was added and the enzymic reaction measured for at least 15 min by automatic titration with 0.01 M-NaOH. A portion of the cytoplasmic extract equivalent to 0.3 mg wet wt. of liver or of the microsomal pellet equivalent to 0.6 mg of liver gave a linear record at this substrate concentration over this period of time.

The esterase activity was calculated as μmol of H^+ released/min by 1 g of liver or by cell fractions derived from 1 g of liver.

Acid phosphatase (EC 3.1.3.2). The activity was assayed by the method of Berthet & de Duve (1952). For measurement of the total (free+bound) activity the enzyme preparation was incubated at pH 5.0 at 37°C in a solution containing 0.05 M-sodium 2-glycerophosphate, 0.05 M-sodium acetate buffer and 0.1% (v/v) Triton X-100. The reaction was stopped after 60 min by the addition of cold 8% (w/v) trichloroacetic acid. P_i in the protein-free filtrate was determined by the method of Fiske & Subbarow (1925). For determination of the free enzyme activity the incubation solution did not contain Triton X-100, and was made iso-osmotic with sucrose. The reaction was stopped after 10 min.

The enzyme activity was calculated as μmol of P_i released/min by 1 g of liver or by cell fractions from 1 g of liver.

Glucose 6-phosphatase (EC 3.1.3.9). Enzyme activity was determined by incubating the enzyme preparation at 37°C with 0.04 M-glucose 6-phosphate (sodium salt) in the presence of 7 mM-histidine and 1 mM-EDTA in 0.05 M-sodium cacodylate buffer, pH 6.5 (de Duve, Pressmann, Gianetto, Wattiaux & Appelmans, 1955). The P_i was determined by the method of King, Abdul-Fadl & Walker (1951) after precipitation of the protein with cold 30% (w/v) trichloroacetic acid.

The enzyme activity was calculated as μmol of P_i released/min by 1 g of liver or by cell fractions from 1 g of liver.

Cytochrome b_5 . The concentration of cytochrome b_5 was measured spectrophotometrically by the method of Ernster, Siekevitz & Palade (1962), the difference spectrum (reduced minus oxidized) between the 427 nm

maximum and 410 nm minimum being taken as an arbitrary measure of the cytochrome b_5 concentration.

Protein. Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin (30% solution, Armour Pharmaceutical Co. Ltd., Eastbourne, U.K.) as a standard, and expressed as mg of protein in 1 g of liver or in the fractions from 1 g of liver. Sodium deoxycholate at a concentration of 0.1% (w/v) was added to all samples to dissolve the protein. The standard protein solution was prepared in the same medium as that used for the tissue preparations to eliminate the variable effects of different sucrose concentrations on the colour development (Hinton, Burge & Hartman, 1969).

RESULTS

Distribution of enzyme activity in fractions of rat liver homogenate

The distribution of esterase, acid phosphatase and glucose 6-phosphatase activities between the five standard fractions obtained by differential centrifugation is shown in Table 1. The mitochondrial and light-mitochondrial fractions together contain 66.5% of the total activity in the homogenate of the lysosomal hydrolase acid phosphatase; 14.7% of this enzyme is recovered in the microsomal fraction and 5.7% in the soluble fraction. Of the total esterase activity 58.5% is recovered in the microsomal fraction, 8.3% in the soluble fraction and 19.6% in the two mitochondrial fractions. Of the microsomal enzyme glucose 6-phosphatase, 19.4% is found in these two fractions, indicating substantial microsomal contamination.

Various treatments of the light-mitochondrial fraction before enzyme determinations shows that the bound acid phosphatase activity of the lysosomes is released by incubation in a hypo-osmotic solution at pH 5.0, by maceration in a top-drive

Table 1. *Intracellular distribution of enzymes in rat liver*

Homogenates of liver in 0.25 M-sucrose were fractionated by differential centrifugation and the enzyme activities were determined by the methods described in the text. Absolute values are given in units/g of liver for enzymes and in mg of protein/g of liver. The numbers of experiments are given in parentheses and results are expressed as the mean $\pm$ s.d. CE, cytoplasmic extract; N, nuclear fraction; M, heavy-mitochondrial fraction; L, light-mitochondrial fraction; P, microsomal fraction; S, final supernatant.

Enzyme	Absolute value (CE+N)	Percentage values (CE+N=100%)					
		N		M	L	P	S
Protein (6)	205.0±16.6	22.2± 2.1	17.3±3.8	6.1±1.8	20.1± 3.4	33.6±2.3	99.8± 3.3
Acid phosphatase (4)	8.4± 0.9	13.8± 4.2	22.0±2.9	44.5±7.2	14.7± 6.9	5.6±3.8	102.0±10.6
Esterase (6)	173.0±19.4	11.3± 3.4	10.0±4.1	9.6±3.3	58.5± 7.6	8.3±1.4	97.5± 4.7
Glucose 6- phosphatase (4)	31.5± 2.1	23.8±10.0	10.0±5.0	9.4±5.1	53.7±16.1	2.2±2.1	96.9± 4.4

Table 2. *Effects of hypo-osmotic media, maceration and detergent on enzyme activities of light-mitochondrial and microsomal fractions*

The fractions were obtained by differential centrifugation of liver homogenate in 0.25 M-sucrose and were resuspended to a concentration of 100 mg/ml and then treated as shown. For free acid phosphatase activity the incubation was for 10 min at pH 5.0 and 37°C in 0.25 M-sucrose with 0.05 M-2-glycerophosphate; for total activity 0.1% of Triton X-100 was added and the incubation was for 60 min. Esterase activity was measured with 1 mM-indoxyl acetate at pH 7.5 and 37°C.

Procedure before enzyme determination	Light-mitochondrial fraction			
	Acid phosphatase activity (μmol of P_i released/ min per g)		Esterase activity (μmol of H^+ released/ min per g)	Microsomal fraction Esterase activity (μmol of H^+ released/ min per g)
	Free	Total		
Incubated for 60 min in 0.25 M-sucrose at pH 7.5 and 37°C	0.33	4.20	26.2	125
Incubated for 60 min in water at pH 5.0 and 37°C	4.25	4.18	25.0	120
Macerated in top-drive macerator for 3 min in water at 0°C	4.14	4.25	25.0	122
Incubated for 60 min in 0.1% Triton X-100 at 37°C	4.34	—	25.5	125

Table 3. *Enzyme activities in isolated lysosomes*

The total mitochondrial fraction, M + L, was prepared by differential centrifugation from a homogenate of the liver of a rat treated with Triton WR-1339. It was then separated into three fractions on a discontinuous sucrose gradient as described in the text. The results, expressed as percentages of the activities in cytoplasmic extract, refer to the means $\pm$ S.D. of four experiments, except for glucose 6-phosphatase activity, which was measured in one experiment only. Specific activities were measured as units/mg of protein and are given in parentheses.

Enzyme	Percentage values of cytoplasmic extract					
	Cyto-plasmic extract	Total mitochondrial fraction M + L	Fractions collected from sucrose gradient			Recovery (M + L = 100%)
			Top fraction	Lysosomes	Bottom fraction	
Protein	100	23.5 $\pm$ 4.8	0.5	2.2 $\pm$ 1.0	21.7 $\pm$ 4.2	104.0 $\pm$ 9.4
Acid phosphatase	100 (0.06)	52.4 $\pm$ 8.1 (0.17)	2.5 $\pm$ 2.2 (1.5)	44.3 $\pm$ 8.6 (1.6)	8.1 $\pm$ 3.1 (0.03)	105.0 $\pm$ 9.6
Esterase	100 (1.3)	11.3 $\pm$ 2.5 (0.73)	0	1.5 $\pm$ 0.1 (0.94)	9.8 $\pm$ 3.3 (0.75)	98.9 $\pm$ 6.4
Glucose 6-phosphatase	100 (0.20)	13.3 (0.15)	0	1.0 (0.13)	11.6 (0.13)	95.0

macerator (Measuring and Scientific Equipment Ltd., London S.W.1, U.K.), and by the detergent Triton X-100, but the esterase activity of this fraction remains unchanged, as does the esterase activity of the microsomal fraction after the same treatments (Table 2).

On isolation of lysosomes of liver from rats treated with Triton WR-1339, which decreases their density so that they may be separated from other organelles, 44.3% of the total acid phosphatase activity of the cytoplasmic extract and only 1.5% of the esterase and 0.65% of the glucose 6-phosphatase activities were recovered in the lysosomes (Table 3).

The specific activity of acid phosphatase of the isolated lysosomes was increased nearly 30-fold over that of the cytoplasmic extract but in the case of both esterase and glucose 6-phosphatase the specific activities were lowered, the values being approximately one-half of those of the cytoplasmic extract.

Release of esterase from liver preparations

Diffusion of esterase from frozen and unfrozen rat liver sections. The results, expressed as the percentage of the activity of samples of whole liver

homogenized in 0.25M-sucrose, are shown in Table 4. After centrifugation of this homogenate at 35000g for 6.7min to remove the particulate material, 8.3% of the esterase activity was recovered in the soluble fraction. Freezing of other samples of the liver at -20°C before homogenizing resulted in 30% of the activity of the homogenate being present in the soluble fraction.

About 10% of the total esterase activity diffused from 300µm sections of unfrozen liver cut on the McIlwain chopper when they were washed with 0.25M-sucrose. This amount is similar to that released by homogenizing unsectioned liver. On homogenization of the washed chopped sections there was no further loss of enzyme into the homogenizing medium. From the 300µm sections of prefrozen liver cut on the McIlwain chopper there was a loss of 28% of the activity into the washing medium and subsequent homogenization of these sections released a further 17% into the medium. There was greater diffusion from the more thinly cut sections but less subsequent release from these when they were subjected to homogenization.

Release of esterase into homogenizing media. The presence in the sucrose homogenizing medium of 0.1-1.0M-sodium chloride or 0.5M-potassium chloride or 10% (w/v) polyvinylpyrrolidone had no effect on the proportion of esterase recovered in the soluble fraction of liver homogenates, which was less than 10% of the total activity in all cases.

The results of fractionating liver homogenates prepared in 75% glycerol and in sucrose solution containing sodium chloride (see above) are presented in Fig. 1. This shows that 38% of the total esterase is recovered in the soluble fraction of the glycerol homogenates at the expense of the particulate fractions, compared with 11% in the case of the salt-sucrose preparation. The acid phosphatase

activity in both microsomal and soluble fractions was considerably higher in the glycerol than in the sucrose homogenates. In the case of glucose 6-phosphatase, this value increased to 10% of the activity in the soluble fraction of the glycerol homogenate, although the bulk of the enzyme remained in the microsomal fraction.

Esterase, acid phosphatase and glucose 6-phosphatase activities in pellets and supernatants obtained by homogenization of liver with Arcton 113 and in control preparations are given in Table 5, and show that the esterase and acid phosphatase activities of the soluble phase from the experiments with Arcton are considerably higher than those of the control supernatants. Arcton did not extract phospholipid from the tissue and caused no significant release of glucose 6-phosphatase activity into the supernatant.

Release of esterase from the microsomal pellet. None of the various media used for washing the pellet was effective in releasing the enzyme (Table 6); on freezing of the pellet before it was washed in sucrose solution 12.6% of its activity was released into the washing medium, but freezing of a suspension of the pellet did not cause release. Likewise, ribonuclease digestion of the pellet did not release esterase.

After sonication of microsomal pellets about half of the protein, half of the esterase and glucose 6-phosphatase activities and half of the cytochrome *b₅* content of the original microsomal preparation were recovered in the pellet obtained after centrifugation at 105000g for 60min (Table 7). When the supernatant was centrifuged for a further 4h an additional 13.4% of the protein and 8.3% of the esterase activity were sedimented in a second pellet.

On treatment of the microsomal pellet with 0.26% deoxycholate in 0.25M-sucrose, 86% of the

Table 4. *Diffusion of soluble esterase from rat liver*

The liver was homogenized as described in the text and esterase activities were measured with 1 mM-indoxyl acetate at pH 7.5 and 37°C. The washing medium was 0.25M-sucrose solution. Washes were centrifuged at 1000g for 10min and then at 105000g for 60min to sediment particulate material and the activity of the final supernatant was measured. Homogenates were centrifuged at 35000g for 6.7min and then at 105000g for 60min and the esterase activity of the pooled pellets and of the final supernatant was measured.

Tissue preparation	Esterase activity (%) compared with that of the whole homogenate					
	Washing medium	Homogenate of washed liver sections		Homogenate of whole liver		Recovery (%)
		Pellet	Supernatant	Pellet	Supernatant	
Unfrozen liver	—	—	—	92.0	8.3	100.3
Frozen liver	—	—	—	70.0	30.0	100
300µm sections of unfrozen liver	10.0	78.9	0	—	—	88.9
300µm sections of frozen liver	28.0	57.1	17.0	—	—	102.1
25µm sections of frozen liver	46.5	54.0	4.0	—	—	104.5
10µm sections of frozen liver	53.5	28.5	0	—	—	92.0

Fig. 1. Distribution of enzymes in liver homogenized in ionic sucrose solution and in glycerol solution. A 3 g portion of liver was homogenized either in 30 ml of 0.25 M-sucrose containing 8.5 mM-NaCl (□) or in 5 ml of 75% (v/v) glycerol solution after which 25 ml of the ionic sucrose solution was added (■). The ordinates show the protein content and enzyme activities of fractions obtained by differential centrifugation, expressed as percentages of the values for the whole homogenates. The fractions are shown on the abscissae in the order in which they are isolated; N, nuclear; M, mitochondrial; L, light-mitochondrial; P, microsomal; S, supernatant. (a) Protein; (b) esterase; (c) acid phosphatase; (d) glucose 6-phosphatase.

esterase content of the pellet was recovered in the supernatant after centrifugation at 105000g for 120 min (Table 8). Further centrifugation for up to 7 h sedimented only an additional 2% of the enzyme. As with the sonicated microsomal pellets, the cytochrome b_5 content of the three fractions is proportional to their protein content. The distribution of glucose 6-phosphatase activity was measured in this experiment but its recovery was

poor, probably because of extraction by deoxycholate of phospholipids (Dallner & Ernster, 1968) such as phosphatidylethanolamine or phosphatidylserine, known to be required for full enzymic activity (Duttera, Byrne & Ganoza, 1968).

On dilution of the deoxycholate supernatant fivefold with 0.25 M-sucrose and centrifugation at 105000g for 3–7 h, there was a progressive sedimentation of up to 20% of the esterase activity.

Table 5. *Distribution of enzyme activity between particulate and soluble fractions of liver homogenized in a two-phase system*

The liver was homogenized in either the two-phase Arcton 113–0.25 M-sucrose system or in sucrose alone and the homogenates were centrifuged as described in the text. Activities are expressed as percentages of the values for the whole homogenate in sucrose. The numbers of experiments are given in parentheses and results are expressed as the mean $\pm$ s.d. Particulate activity is the total sedimentable activity and soluble activity is that in the final supernatant.

Enzyme	Fraction	Activity (%) compared with that of whole homogenate in sucrose	
		Two-phase homogenate	0.25 M-Sucrose homogenate
Esterase (7)	Particulate	55.9 $\pm$ 10.9	89.3 $\pm$ 11.3
	Soluble	42.0 $\pm$ 8.1	8.2 $\pm$ 1.4
Acid phosphatase (4)	Particulate	61.9 $\pm$ 14.8	86.4 $\pm$ 0.1
	Soluble	37.5 $\pm$ 14.3	7.0 $\pm$ 0.7
Glucose 6-phosphatase (3)	Particulate	94.5 $\pm$ 2.1	97.0 $\pm$ 4.0
	Soluble	2.0 $\pm$ 0.1	2.6 $\pm$ 1.6

Table 6. *Effect of washing of the microsomal fraction in various media*

Microsomal fractions prepared from a homogenate of liver in 0.25 M-sucrose were resuspended in the various media, recentrifuged at 105 000g for 60 min, and then the pellets were resuspended in 0.25 M-sucrose and their esterase activity and that of the washes were measured with 1 mM-indoxyl acetate at pH 7.5 and 37°C.

Washing medium	Esterase activity (%) compared with that of unwashed microsomal fraction		Recovery (%)
	Washed pellet	Supernatant	
0.25 M-Sucrose	95.0	1.0	96.0
0.25 M-Sucrose*	81.0	12.6	93.6
0.25 M-Sucrose†	103.0	0	103.0
0.25 M-Sucrose–0.1 M-NaCl	93.0	0	93.0
0.25 M-Sucrose–0.5 M-NaCl	93.0	2.0	95.0
0.25 M-Sucrose–1.0 M-NaCl	94.0	0	94.0
0.25 M-Sucrose–0.5 M-KCl	93.0	1.0	94.0
0.1 M-Tris–HCl, pH 8.0	99.0	0	99.0
0.25 M-Sucrose–8.5 mM-NaCl–12.5% (v/v) glycerol	102.0	0	102.0
0.25 M-Sucrose–Arcton 113 (2:1, v/v)	94.0	0	94.0

* Microsomal fraction frozen at -20°C for 1 h before resuspension in 0.25 M-sucrose solution.

† Microsomal fraction resuspended in 0.25 M-sucrose then frozen at -20°C for 1 h before centrifugation.

With tenfold dilution a further 28% of the esterase was sedimented during centrifugation for 7 h.

stat sections of liver or by the Arcton–sucrose method (Table 9).

Adsorption of esterase by the particulate components of the homogenate

There was no adsorption of esterase by any of the particulate fractions of 10 μm cryostat sections of liver that had been homogenized in their own esterase extract, or by the fractions prepared from whole liver homogenized in a similar esterase extract or in one prepared by the Arcton–sucrose technique (Table 9).

The activity of microsomal pellets remained unchanged when they were sedimented after being resuspended in esterase extracts made from cryo-

DISCUSSION

Fractionation of a homogenate of rat liver in sucrose by differential centrifugation confirms that, whereas most of the esterase activity is recovered in the microsomal fraction, the light-mitochondrial fraction, which contains the lysosomes, also possesses about 9% of both the total esterase and glucose 6-phosphatase activities of the tissue. Further fractionation of homogenates of livers of rats pretreated with Triton WR-1339 gives a preparation of lysosomes that contains only about 1% of the total esterase activity of the homogenate

Table 7. *Recovery of enzymes in subfractions after sonication of microsomal fraction*

Resuspended microsomal fractions were sonicated for 3 min and then centrifuged at 105 000g for 60 min to give pellet (1) and supernatant (1). Supernatant (1) was further centrifuged for 4 h to give pellet (2) and supernatant (2). Results are expressed as the mean $\pm$ s.d. for four experiments. Specific activities, measured as units/mg of protein, and the specific content of cytochrome b_5 , measured as units/100 mg of protein, are given in parentheses.

Material	Percentage values (microsomal fraction before sonication = 100%)				
	Whole microsomal fraction after sonication	Microsomal subfractions			
		Pellet (1)	Supernatant (1)	Pellet (2)	Supernatant (2)
Esterase	98.3 $\pm$ 7.7 (21.2)	43.2 $\pm$ 8.4 (16.8)	54.6 $\pm$ 8.6 (20.6)	8.3	43.7
Cytochrome b_5	97.5 $\pm$ 3.7 (5.4)	47.3 $\pm$ 4.5 (4.9)	48.0 $\pm$ 3.7 (3.4)	—	—
Glucose 6-phosphatase	92.3 $\pm$ 9.6 (0.27)	42.3 $\pm$ 7.7 (0.22)	23.7 $\pm$ 2.0 (0.13)	—	—
Protein	102.8 $\pm$ 2.5	52.5 $\pm$ 6.8	40.7 $\pm$ 7.4	13.4	26.0

Table 8. *Recovery of enzymes in subfractions of a deoxycholate-treated microsomal fraction*

Resuspended microsomal fractions were treated with 0.26% deoxycholate and centrifuged at 105 000g for 120 min. The clear supernatant and the intermediate membranous layer were removed separately and the ribonucleoprotein pellet was resuspended in sucrose. Results are expressed as the mean $\pm$ s.d. The numbers of experiments are given in brackets. The specific activities of esterase, measured as units/mg of protein, and the specific content of cytochrome b_5 , measured as units/100 mg of protein, are given in parentheses.

Material	Percentage values (microsomal fraction before addition of deoxycholate = 100%)			
	Whole microsomal fraction after addition of deoxycholate	Microsomal subfractions		
		Supernatant	Membranous layer	Ribonucleoprotein pellet
Esterase [6]	92.5 $\pm$ 4.9 (20.0)	86.0 $\pm$ 8.5 (33.8)	9.5 $\pm$ 3.5 (10.6)	5.9 $\pm$ 3.6 (6.25)
Cytochrome b_5 [3]	100.0 $\pm$ 9.9 (5.9)	45.9 $\pm$ 10.0 (4.7)	25.9 $\pm$ 7.8 (10.1)	13.5 $\pm$ 9.9 (3.6)
Glucose 6-phosphatase [2]	98.5	9.6	2.75	5.65
Protein [6]	103.0 $\pm$ 8.9	54.8 $\pm$ 3.2	23.5 $\pm$ 2.5	23.4 $\pm$ 2.1

Table 9. *Adsorption of esterase by particulate components of liver homogenate*

Whole liver, 10 μ m sections of frozen liver (previously washed in sucrose solution) and microsomal fractions prepared from 0.25 M-sucrose homogenates of liver were homogenized in 0.25 M-sucrose or in an extract of esterase and then centrifuged. Esterase activities were measured with 1 mM-indoxyl acetate at pH 7.5 and at 37°C. Extract A refers to esterase washed from 10 μ m sections of frozen liver by gentle agitation in 0.25 M-sucrose; extract B refers to esterase recovered in the aqueous phase after homogenization of liver in the Arcton-sucrose medium.

Fractions	Esterase activity (%) compared with that of whole liver homogenate in sucrose solution				
	Liver homogenized in sucrose solution	Liver homogenized in extract A	Liver homogenized in extract B	Washed sections homogenized in sucrose solution	Washed sections homogenized in extract A
Nuclear + mitochondrial + light-mitochondrial	28	25	35	19	19
Microsomal	63.5	61	58	23	25

and also about 1% of the total glucose 6-phosphatase activity. These results suggest that esterase in lysosomal fractions is due to microsomal contamination. The experiments concerned with the

stability of binding of esterase in liver preparations show that binding is weakened by freezing and sectioning and that the enzyme diffuses more and more readily from sections as their thickness is

reduced. This effect is only partly due to freezing, since the release of esterase from homogenates of prefrozen liver is considerably less than that released from 25 and 10 μ m sections, and much more than that released by freezing a microsomal pellet. It might therefore be expected that homogenization, which completely disrupts cells, would result in an even greater release of the enzyme. However, the proportion of esterase recovered in the soluble fraction after centrifugation of sucrose homogenates is less than that released from liver sections exposed to 0.25M-sucrose. It thus appears that the enzyme associated with the microsomal fraction may have originated from some other cellular site, to become adsorbed by the components of this fraction.

However, neither homogenization of liver in ionic sucrose solutions, which decreases adsorption of protein by membranes (Rosenthal, Gottlieb, Gorry & Vars, 1956; Paigen & Wenner, 1962), nor homogenization in the presence of polyvinylpyrrolidone, which maintains the integrity of lysosomes (Novikoff, 1956) altered the proportion of esterase in the soluble fraction. On the other hand, there was a considerable increase in the proportion of esterase in the soluble fraction of rat liver after homogenization in aqueous glycerol, as with mouse liver (Carruthers *et al.* 1960), or with the two-phase Arcton-sucrose system. The source of the esterase released in these two media could not be determined directly. However, the distribution of glucose 6-phosphatase and acid phosphatase among the five standard fractions provided some evidence about this. The values for the glycerol homogenate are shown in Fig. 1 and, although the corresponding values for the fractions of the Arcton-sucrose homogenate are not shown separately in Table 5, most of the glucose 6-phosphatase sedimented in the usual way in the microsomal fraction in each case. However, a considerable proportion of acid phosphatase was recovered in the soluble fraction in both cases. For the Arcton-sucrose homogenate, the large amount of acid phosphatase activity in the soluble fraction indicates release of the enzyme from lysosomes, probably because of physical damage during homogenization rather than solvent action, since it was found that Arcton does not extract phospholipids from liver. Similar lysosomal damage presumably occurs in the glycerol homogenate, probably because of the high shearing force of the viscous medium (Carruthers *et al.* 1960).

The concomitant release of acid phosphatase and esterase when liver is homogenized in either the glycerol or Arcton-sucrose medium suggests that the lysosomes of intact liver may contain, as is also suggested by the cytochemical evidence, a substantial proportion of esterase activity. This

activity is released under normal homogenization conditions and adsorbed by fragments of the endoplasmic reticulum in a way similar to that reported for other enzymes (Kuff, 1954; Rosenthal *et al.*, 1956; Paigen & Wenner, 1962; Fonnum, 1968; Koeppen, Barron & Bernsohn, 1969). The alternative possibility that the soluble esterase produced during homogenization in glycerol or in Arcton-sucrose is due to direct release from microsomes is unlikely, since such treatments do not affect the distribution of glucose 6-phosphatase and do not release esterase from microsomal pellets.

If a substantial proportion of the esterase of a normal microsomal fraction is derived from lysosomes it might be expected to have properties different from those of constitutive proteins of this fraction. Such a difference was seen in the response of microsomal pellets to deoxycholate treatment. Nearly all the esterase, yet only about half the cytochrome *b₅* and half the total protein, was released from the pellets; other microsomal enzymes are released to the extent of less than half by the detergent (Ernster *et al.* 1962; Ernster & Jones, 1962). The ready release of esterase by deoxycholate suggests that the enzyme is bound to microsomes through lipid components of their membranes, particularly since it can be sedimented after dilution of the detergent, presumably because of reaggregation of micellar elements previously kept in solution at the higher concentration (Ernster *et al.* 1962). It was, in fact, the apparent association of liver esterase with lipid of microsomal membranes that led to the experiments with the Arcton-sucrose homogenizing medium. Previous work had shown that when vaccinia-virus-infected cells were homogenized in this medium, the phospholipid-containing cell debris passed into the Arcton layer, but the virus, polysaccharides and DNA passed into the aqueous layer (Holt & Epstein, 1958). This suggested that Arcton might compete with esterase released during homogenization for lipid-containing binding sites on cell membranes, and that the enzyme would pass into the aqueous phase, as was found (see above).

In contrast with the action of deoxycholate, ultrasonic vibration released approximately the same proportion of esterase, glucose 6-phosphatase, cytochrome *b₅* and total protein from the microsomal pellet. The sedimentation of another esterase-active pellet after further centrifugation indicates that sonication does not release the enzyme, but makes the membrane fragments smaller (Dallner & Ernster, 1968).

So far, attempts to induce membranes to adsorb esterase from enzyme extracts prepared as above have been no more successful than those of Underhay *et al.* (1956), who used a different esterase extract (Burch, 1954). This may be because of

structural changes in the extracted esterase and does not necessarily mean that microsomal membranes are unable to adsorb enzyme released at the time of homogenizing liver in the usual sucrose medium. Such release is, of course, not typical of lysosomal enzymes, nor is the lack of latency of esterase reported here and by Shibko & Tappel (1964) for a highly purified preparation of liver lysosomes. However, both of these phenomena could be attributed to the location of esterase on the cytoplasmic side of the lysosomal membrane (Shibko & Tappel, 1964) as opposed to the location of other lysosomal enzymes in the matrix.

In summary, the present evidence shows that rat liver esterase behaves like a microsomal enzyme under certain conditions and as a lysosomal enzyme under others. The most reasonable explanation for these findings is that it has a dual localization, with a greater proportion of the enzyme in lysosomes than is suggested by the results of standard fractionation procedures.

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