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Studies on Lipolytic Activities of Rat Liver Lysosomes

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Summary: The occurrence of phospholipase A, lysolecithinase, cholesterol esterase and lipase in rat liver lysosomes, mitochondria and microsomes has been investigated. Purified lysosomes contain an acid phospholipase A, cholesterol esterase and lipase; the lysolecithinase activity is minute. Mitochondria also contain phospholipase A, cholesterol esterase and in addition lysolecithinase, whereas the lipase activity is low in this fraction. The microsomal fraction exhibits only high lysolecithinase and cholesterol esterase activity and almost no phospholipase A and lipase activity. Lysosomes are free of acyl-CoA-transferases.

Zusammenfassung: Das Vorkommen von Phospholipase A, Lyso-Lecithinase, Cholesterinesterhydrolase und Lipase in Lysosomen, Mitochondrien und Microsomen der Rattenleber wurde untersucht. Gereinigte Lysosomen enthalten eine saure Phospholipase A, Cholesterinesterhydrolase und Lipase; die Lysolecithinaseaktivität ist gering. Mitochondrien enthalten Phospholipase A, Cholesterinesterhydrolase und dazu Lysolecithinase, dagegen ist die Lipaseaktivität in dieser Zellfraktion sehr gering. Die microsomale Fraktion weist eine hohe Aktivität von Lysolecithinase und Cholesterinesterhydrolase, eine sehr geringe von Phospholipase A und Lipase auf. Lysosomen sind frei von Acyl-CoA-Transferasen.

Most of the enzymes involved in the biosynthesis of complex lipid molecules are bound to membranes of the endoplasmic reticulum. Cholinephosphotransferase[1], cholinephosphate cytidylyltransferase[1], glycerolphosphate acyltransferase[1,2] and the polyenoic acid synthesizing system[3] have been described as microsomal enzymes. Only limited information about the localisation of lipolytic enzymes in the cell is available. These enzymes are of particular importance with regard to the continuous degradation and re-organisation processes occurring in metabolically active membranes. Furthermore the knowledge of their localisation in certain heterophagic cell types is highly desirable. The concept of a compartmentalisation of the intracellular digestion put forward by DEDUVE and his collaborators[4] is of particular relevance to this problem. Proteases, nucleases, polysaccharidases and glycosidases have been shown to be present in lysosomes. Myelin figures and membrane fragments within lysosomes have been detected by electronmicroscopic tech-

Enzymes:

Glycerolphosphate acyltransferase = Acyl-CoA:L-glycerol-3-phosphate *O*-acyltransferase (EC 2.3.1.15);
Acyl-CoA: lysophosphatidic acid *O*-acyltransferase (EC 2.3.1.?);
Acyl-CoA: lysolecithin *O*-acyltransferase (EC 2.3.1.?);
Phospholipase A = phosphatide-acyl-hydrolase (EC 3.1.1.4);
Phospholipase B = Lysophospolipase = lysolecithin acylhydrolase (EC 3.1.1.5);
Lipase = glycerol-ester hydrolase (EC 3.1.1.3);
Cholesterol esterase = sterol-ester hydrolase (EC 3.1.1.13);
Acyl-CoA hydrolase = acyl-CoA-hydrolase (EC 3.1.2.?).

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niques. Due to these findings the presence of lipolytic enzymes in lysosomes has been questioned.

In this respect, no systematic investigations on lipolytic enzymes of purified lysosomes have been carried out. A few reports indicate the lysosomal origin of some lipolytic enzymes on the basis of the low pH optimum of these enzymes. SEDGWICK et al.[5] measured the highest phosphatidate phosphohydrolase activity in a lysosomal fraction which confirms WILGRAM's et al.[1] finding. An acid lipase and phospholipase activity from rabbit polymorphonuclear leucocytes has been decribed by ELSBACH and RIZACK[6]. The occurrence of phospholipase A activity in rat liver mitochondria has been reported by SCHERPHOF and VAN DEENEN[7] and ROSSI et al.[8]. COHN et al.[9] report on a lipase from leucocytes which according to its pH optimum presumably is of lysosomal origin. HELLER and SHAPIRO[10] describe a sphingomyelinase present in the mitochondrial lysosomal fraction of rat liver. This enzyme splits sphingomyelin into ceramide and phosphorylcholine. With regard to a number of hereditary lipoidoses a determination of the localisation of all lipolytic enzymes is desirable. Thus a deficiency of sphingomyelinase activity in NIEMANN-PICK disease has been described by BRADY et al.[11,12]. Its absence in spleen from patients with the classical and visceral type of NIEMANN-PICK disease has recently been confirmed by SCHNEIDER and KENNEDY[13].

This paper describes investigations concerned with the localisation of the following lipolytic enzymes in the rat liver cell: 1. phospholipase A, 2. lysophospholipase (= lysolecithinase), 3. lipase, 4. cholesterol esterase. The isolation of hydrolysis products derived from specifically labeled substrates was applied as the method of choice for the enzymatic tests. The phospholipase A activity was followed by the release of [1-^{14}C]linoleic acid from 1-stearoyl-2-[1-^{14}C]linoloyl-glycerophosphoryl choline, that of lysolecithinase by the release of [9,10-^{3}H]stearic acid from 1-[9,10-^{3}H]stearoyl-glycerophosphoryl choline. Cholesteryl-[1-^{14}C]linoleate and 1,2-dipalmitoyl-3-[1-^{14}C]octadeca-8,11-dienoylglycerol were used as substrates for the cholesterol esterase and lipase, respectively.

Density gradient centrifugation permits the preparation of enzymatically and electronmicroscopically pure fractions. We prepared pure lysosomes according to the procedures of WATTIAUX et al.[14]. Nonhemolysing Triton WR-1339 was injected into rats and the lysosomes loaded with the detergent isolated in the mitochondrial fractions. Both types of particles were separated by density gradient centrifugation. The light lysosomes were clearly separated from the heavier mitochondria. The electronmicroscopic picture of the lysosome fraction is shown in fig. 1. Acid phosphatase was taken as the enzyme marker of the lysosomal fraction.

In order to compare the different lipolytic activities of lysosomes with those of other particles, mitochondria and microsomes were also purified. The purity of the latter two fractions was also controlled by electronmicroscopy. They proved to be homogenous.

Fig. 1—3 show electronmicrographs of 1. lysosomes, 2. mitochondria and 3. the microsomal fraction. The lysosomes are filled with the detergent surrounded by a limiting membrane, shown in fig. 4. After dialysis of the lysosomes against a hypotonic solution (0.05M phosphate buffer, pH 6.5) the membrane of the lysosome ruptures and forms small Triton-free vesicles, which can be isolated at 100,000 × g. This procedure then permitted the enzymic activities in the membrane and the lysosomal content to be measured (fig. 5). Ultrasonic treatment of the small vesicular fraction leads to the disappearance of the vesicular structure. A dense lamellar aggregation of the membranes is formed with a distance between the electron dense lines of 70—75 Å (fig. 6).

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Fig. 1. Electronmicrograph of lysosomes (20,000 × magnification).

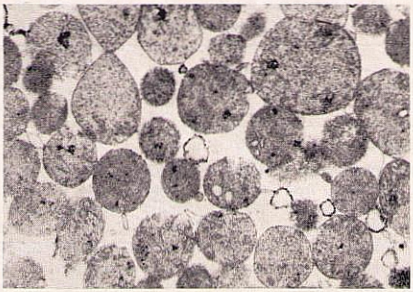

Fig. 2. Electronmicrograph of mitochondria (12,000 × magnification).

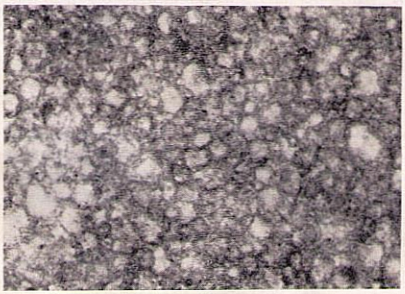

Fig. 3. Electronmicrograph of microsomes (40,000 × magnification).

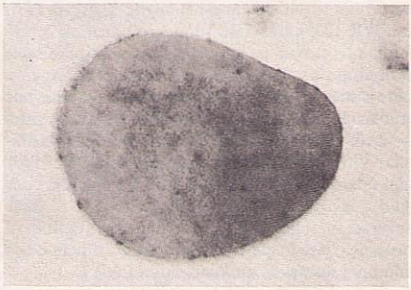

Fig. 4. Electronmicrograph of a single lysosome ("tritosome") (60,000 × magnification).

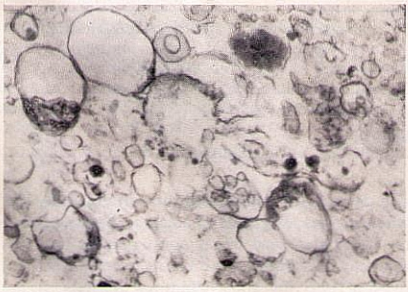

Fig. 5. Electronmicrograph of lysosomal membranes after dialysis against 0.01M phosphate buffer, pH 6.5 (15,000 × magnification).

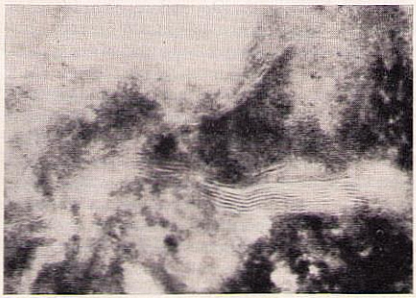

Fig. 6. Electronmicrograph of lysosomal membranes after ultrasonication and high speed (100,000 × *g*) centrifugation (48,000 × magnification).

Table 1. Distribution of phospholipase A, lysophospholipase (= lysolecithinase), cholesterol esterase and lipase in rat liver. A und B are mean values of series of two analyses with enzyme preparations from two different animals.

cell fraction	mμ moles substrate hydrolized per mg protein in 1 h						
	phosphatidyl choline		lysolecithin		triglyceride		cholesteryl-linoleate
	A	B	A	B	A	B	
lysosomes	4.20	3.45	1.87	3.40	5.70	8.70	151
mitochondria	2.15	2.83	15.1	10.0	1.74	2.4	125
microsomes	0	0.25	17.1	15,4	0.84	1.4	139
homogenate	1.00		16.6		8.00		57

Results

Centrifugation techniques permit the preparation of very homogenous sub-cellular particle fractions for enzymic studies. The method of WATTIAUX[14], according to which a non-hemolytic detergent (Triton WR-1339) after intravenous application is preferentially stored in lysosomes, represents a reliable procedure for the isolation of these particles in a pure state, which are then available for enzymic studies. By the use of specifically labeled substrates we have investigated the distribution of phospholipase A, lysolecithinase, cholesterol ester hydrolase and lipase in the rat liver cell and for the first time gained insight into the presence or absence of these enzymes in pure lysosomes. The distribution of these enzymes in rat liver lysosomes, mitochondria and microsomes is summarized in table 1.

Whereas the distribution of the enzymic activity remains constant, the rate of hydrolysis changes from one experiment to the other. The reason for this might be differences in the solubilisation of the water insoluble lipids. Optimal concentrations of the detergents had to be determined for each substrate. The pH optimum particularly of the lysosomal enzymes has been investigated between pH 5 and 8. The lysosomal phospholipase A, lipase and cholesterol esterase exhibited a pH optimum around 6.5 similar to those of the well-known lysosomal phosphatase, glycosidase, nucleotidase and protease. Cholesterol esterase had the highest specific activity in all cell fractions despite its substrate was least soluble. Phospholipase A, lipase and cholesterol esterase of lysosomes exhibited the highest specific activity in the cell fractions. Since the mitochondrial fraction still possessed acid phosphatase activity, we cannot exclude that the phospholipase A and lipase activities are due to lysosomes whose specific weight had not changed sufficiently by the uptake of Triton. It is obvious from table 1 that lysolecithinase, an enzyme which splits the 1-acyl group from the water soluble 1-acyl-glycero-3-phosphoryl choline is predominantly located in the microsomal and mitochondrial fraction. The electron microscopic examination of the lysosome fraction showed it to be homogenous. We therefore examined whether the enzymes are bound to the lysosomal membrane which appears as a single membrane, or freely soluble in the interior of the lysosome. During dialysis of the particles against 0.01M phosphate buffer, pH 7.0, the membrane ruptures and forms small vesicles, then the Triton is released in the supernatant solution. The membrane fraction and the supernatant solution were separated by centrifugation at 100000 × *g* and the two fractions were tested for various enzymatic activities. The activities are summarized in table 2.

Table 2. Hydrolysis of phosphatidyl choline, triglyceride and cholesteryl-linoleate by the lysosomal membrane and supernatant fraction.

fraction	% hydrolysis of substrates after 1 h incubation		
	phosphatidyl choline	triglyceride	cholesteryl-linoleate
membrane	9	11	50
supernatant	7	56	92

The data rule out the possibility that these hydrolytic enzymes are localised solely in the interior of the lysosomes. Part of membrane-bound enzymes also may have been solubilized by the detergent and released into the supernatant on dialysis of the lysosomes against the hypotonic buffer. These experiments however do not permit an estimation of the degree of solubilisation.

SEMADENI[15] observed the incorporation of [^{3}H]acetate into the lipids of isolated spherosomes of corn seedlings which are supposed to be equivalent to lysosomes. He concluded from this result that the lysosomes possess a great ability for lipid synthesis. The cell fractions were characterized by the occurrence of acid hydrolases. Since contaminations by microsomal membranes were not excluded these results must be interpreted cautiously. Using the sonicated fraction as enzyme source we studied the activity of acyl-CoA-transferases to lysophosphatidic acid and lysolecithin. We were unable to find any transferase-activity or acyl-CoA hydrolase activity.

Experimental

Preparation of labeled substrates

1. Cholesteryl-[^{14}C]oleate and cholesteryl-[1-^{14}C]linoleate: were prepared by trans-esterification of *cholesteryl acetate* and *methyl-[1-^{14}C]oleate* or *-linoleate* according to the method of MAHADEVAN[16] on a 1 mmolar scale. The two labeled cholesterol esters were purified by preparative thin layer chromatography (0.5 mm thickness, Kieselgel H; solvent system: hexane/ether/acetic acid 90:10:2). The yield was almost quantitative.

Spec. activity: 250,000 dpm/μmole.

2. 1,2-Dipalmitoyl-3-[1-^{14}C]linoloylglycerol: The acylation of optically active *1,2-dipalmitoylglycerol* with *[1-^{14}C]linoloylchloride* was performed on a 1 mmolar scale in dry chloroform and pyridine by keeping the reaction mixture at room temperature for 72 hours. The triglyceride was purified by silicic acid chromatography according to HIRSCH and AHRENS[17].

Spec. activity 250,000 dpm/μmole.

3. 1-[^{3}H]Stearoyl-2-[1-^{14}C]linoloyl-3-glycerophosphoryl choline: This substrate was prepared by the procedure of BAER and BUCHNEA[18]. The $CdCl_2$-complex of [^{3}H]*lysolecithin* was acylated with *[1-^{14}C]linoloylchloride*. The lecithin was purified by silicic acid chromatography according to HANAHAN et al.[19]

Spec. activity ^{14}C: 250,000 dpm/μmole; ^{3}H: $1.8 \cdot 10^6$ dpm/μmole.

4. 1-[^{3}H]Stearoyl-3-glycerophosphoryl choline was obtained by catalytic reduction of *1-oleoyl-* or *1-linoloyl-3-glycerophosphoryl choline* in a $^3H_2$-atmosphere.

Spec. activity $1.8 \cdot 10^6$ dpm/μmole. All substrates were shown to be pure in thin layer chromatographic analysis.

Cell fractionation

340 mg (2 m*l* of a 17% Triton WR-1339 solution in 0.9% NaCl) were injected into the tail vein of adult Wistar-rats (appr. 6 month old). The animals were sacrificed after 4 days, the liver was homogenized in 9 volumes of 0.25M sucrose solution and the mitochondrial and microsomal fractions isolated in the usual manner after discarding the 600 × *g* sediment. Mitochondria and microsomes were washed twice with 0.25M sucrose. The mitochondrial fraction was suspended in 2 m*l* sucrose solution and separated by centrifugation over a continuous sucrose gradient (0.5—2.0M) into lysosomes and mitochondria, both cleanly separated. The two fractions were collected by means of a Pasteur pipette. Comparison with *o*-dichlorobenzene (density = 1.306) and heptane (density = 0.684) mixtures indicated a density of the lysosomes of 1.100 and that of the mitochondria of 1.251. Aliquots of these fractions, fixed with OsO_4 and embedded in Epon[20], were controlled by electron microscopy. The distribution of acid phosphatase activity in the gradient was determined according to LINHARDT and WALTER[21].

Membranes of lysosomes were prepared by dialysis of the particles against 0.01M phosphate buffer, pH 6.5, for 12 hours. The washed sediment was suspended in 0.01M phosphate buffer and used for enzyme tests. The membrane fractions and substrate solutions were sonicated at 0°C and 20 Kc/sec., 0.6 A for 2 min. with a Schoeller-instrument (Frankfurt). Protein was determined by the biuret-method.

Radioactivity was measured in a Tricarb liquid scintillation spectrometer, model 3214, Packard, La Grange, USA. A 12 hour-hydrolysis of phospholipid containing samples with 1 m*l* of hyamin base* in 10 m*l* scintillator** in the vial yielded a constant counting efficiency.

Incubations

The lipolytic activities of the different cell fractions were determined with the four substrates in the following way:

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* 1% dimethyl-(2-[2-(*p*-diisobutyl-methyl-phenoxy)-ethoxy]-ethyl)-benzyl-ammoniumhydroxide.

** 5 g 2,5-diphenyloxazol (PPO) and 0.3 g 1,4-bis-[4-methyl-5-phenyloxazolyl-(2)]-benzene (dimethyl-POPOP) in 1 *l* of toluene.

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1. Phospholipase A: 0.5 μmoles of doubly labeled lecithin, 0.3 m*l* of a 0.5% sodium deoxycholate solution (pH 7.4), and 3 m*l* of tris-buffer (0.01M; 0.01M $CaCl_2$; pH 6.8) were sonicated whilst cooling in ice for 2 min. (20 Kc, 0.5 W). The cell fraction was added and the incubation mixture sonicated again for 2 × 1 min. at 0°C. The incubation proceeded at 37°C for 2 hours. The total lipids were extracted 4 times with 3 m*l* chloroform-methanol (2:1), the combined extracts concentrated to dryness, the residue dissolved in 1 m*l* of chloroform and the mixture separated by silicic acid chromatography (2 g silicic acid) into fatty acids, lecithin and lysolecithin as described before[22].

2. Lysophospholipase (= Lysolecithinase): 1.0 μmole of [^{3}H]lysolecithin was sonicated for 2 min. in 3 m*l* of tris-buffer (0.1M; pH 7.0) and incubated in the manner described for phospholipase A. The separation of the hydrolyzed fatty acids and unchanged substrate was carried out by thin layer chromatography (Kieselgel H; solvent system: chloroform/methanol/water 65:25:4). The fatty acid band was transferred quantitatively into the counting vial for radioactivity measurement. This method gave the same results as the more tedious column chromatography.

3. Cholesterol esterase: A mixture of 2.0 μmoles of cholesteryl-[1-^{14}C]linoleate, 2 mg of sodium taurocholate, 20 mg of albumin and 3.0 m*l* of phosphate buffer (0.1M; pH 6.6) was sonicated at 0°C for 1 min. and again for 2 min. after the addition of the cell fraction. Four incubations were carried out in each experiment, these were stopped after 10, 25, 50 and 300 min. The lipid extraction was carried out as described above. Labeled fatty acids, cholesterol and the labeled substrate were separated by thin layer chromatography (solvent system: petroleum ether (30—60°C)/ether/acetic acid 90:10:2), the bands quantitatively transferred into counting vials and the radioactivity measured.

4. Lipase: 0.50 μmoles of 1,2-dipalmitoyl-3-[1-^{14}C]linoloylglycerol was dispersed in 3 m*l* of tris-buffer (0.1M, pH 7.0) containing 5 mg of sodium deoxycholate by ultrasonication for 3 min. After the addition of the enzyme fraction the incubation mixture was again sonicated for 1 min. at 0°C and incubated for 2 hours at 37°. The total lipid mixture was separated by thin layer chromatography (Kieselgel G, solvent system: dichloroethane/methanol 98:2) into triglycerides, diglycerides and fatty acids.

Acyl-CoA hydrolase, acyl-CoA-lysophosphatidic acid- and *acyl-CoA-lysolecithin O-acyl transferase* activities were measured as described before[23].

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