

# Hydrolysis of retinyl esters by non-specific carboxylesterases from rat liver endoplasmic reticulum

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The four most important non-specific carboxylesterases from rat liver were assayed for their ability to hydrolyse retinyl esters. Only the esterases with pI 6.2 and 6.4 (= esterase ES-4) are able to hydrolyse retinyl palmitate. Their specific activities strongly depend on the emulsifier used (maximum rate: 440 nmol of retinol liberated/h per mg of esterase). Beside retinyl palmitate, these esterases cleave palmitoyl-CoA and monoacylglycerols with much higher rates, as well as certain drugs (e.g. aspirin and propanidid). However, no transacylation between palmitoyl-CoA and retinol occurs. Retinyl acetate also is a substrate for the above esterases and for another one with pI 5.6 (= esterase ES-3). Again the emulsifier influences the hydrolysis by these esterases (maximum rates: 475 nmol/h per mg for ES-4 and 200 nmol/h per mg for ES-3). Differential centrifugation of rat liver homogenate reveals that retinyl palmitate hydrolase activity is highly enriched in the plasma membranes, but only moderately so in the endoplasmic reticulum, where the investigated esterases are located. Since the latter activity can be largely inhibited with the selective esterase inhibitor bis-(4-nitrophenyl) phosphate, it is concluded that the esterases with pI 6.2 and 6.4 (ES-4) represent the main retinyl palmitate hydrolase of rat liver endoplasmic reticulum. In view of this cellular localization, the enzyme could possibly be involved in the mobilization of retinol from the vitamin A esters stored in the liver. However, preliminary experiments *in vivo* have failed to demonstrate such a biological function.

## INTRODUCTION

The biological functions of so-called 'non-specific carboxylesterases' (EC 3.1.1.1) are, apart from their involvement in drug metabolism, unknown so far [1,2]. Several attempts have been undertaken to ascertain such functions from their possible activity on physiological ester-type lipids. In 1969, Bertram & Krisch [3] demonstrated that vitamin A acetate is a substrate for purified pig, ox and human liver esterases. As a result, the Nomenclature Committee of the International Union of Biochemistry deleted the entry EC 3.1.1.12 (vitamin A esterase) [4]. However, a separate retinyl palmitate hydrolase (EC 3.1.1.21) has been defined, and enzymes with this activity have been partially purified from rat liver particulate fractions [5–8] and a cytosolic lipoprotein complex [9,10]. Recent experiments suggest that the particulate retinyl palmitate hydrolase may be different from the co-purifying lipase activities against cholesterol oleate and trioleoylglycerol [8,11], but an additional action on these lipids is not excluded. Meanwhile it has been shown in our laboratory that the non-specific microsomal carboxylesterases of pig [12] and rat [13] liver consist of several chemically and genetically different enzymes which act selectively on certain amphiphilic and moderately hydrophobic lipids [14–16]. It was therefore thought worthwhile to determine whether one of these esterases selectively hydrolyses retinyl esters or whether this is a function of all esterases of this type. Beside this, the question of whether these esterases only [3] act on

retinyl acetate or also on the palmitate needs to be reinvestigated. The latter substrate could indicate a physiological function, since this ester is the main storage form of vitamin A in the liver. The study was undertaken with the carboxylesterases from rat liver microsomes, which are the best defined and are obtainable in a highly purified form [14,17].

## MATERIALS AND METHODS

### Materials

Retinol and esters, emulsifiers, (Nph)<sub>2</sub>P and palmitoyl-CoA were obtained from Sigma, Munich, Germany. Silica-gel G<sub>60</sub> thin-layer plates were from Merck, Darmstadt, Germany. The enzymic assay kit for fatty acids was purchased from Wako Chemicals, Düsseldorf, Germany. [1-<sup>14</sup>C]Palmitoyl-CoA (52 Ci/mol), [*carbinol*-<sup>14</sup>C]retinol (48 Ci/mol) and [11,12(n)-<sup>3</sup>H]retinal (50 Ci/mol) were from Amersham-Buchler, Braunschweig, Germany. Propanidid {4-[2-(diethylamino)-2-oxoethoxy]-3-methoxybenzeneacetic acid propyl ester} was a gift from Bayer, Leverkusen, Germany.

### Enzymes and cell fractions

Carboxylesterases were highly purified from rat liver microsomes (non-inbred male Wistar rats, strain Han: WIST) as described by Heymann & Mentlein [17]. Subcellular fractions from rat livers were obtained essentially as described by Fleischer & Kervina [18].

Abbreviations used: (Nph)<sub>2</sub>P, bis-(4-nitrophenyl) phosphate; BSA, bovine serum albumin; ER, endoplasmic reticulum.

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### Enzyme assays

**Retinyl ester hydrolase activity.** Vitamin A acetate or palmitate was emulsified with 0.4% Triton X-100 and 20 mM-sodium taurocholate in 0.2 M-phosphate buffer, pH 7.4, in an ice-cooled beaker glass by ultrasonication [2 min, 150 W; Branson (Danbury, CT, U.S.A.) B12 sonifier] to yield a 0.2 mM emulsion. Alternatively the emulsifiers mentioned in the text (at double concentrations) were used. Portions (500  $\mu$ l) of these emulsions were incubated in glass vials at 37 °C for 2 h under gentle shaking with 10  $\mu$ g of the esterases or 1 mg of cellular protein and water added to 1 ml. The mixtures were covered with argon and protected from light. The reactions were stopped by boiling (2 min, 95 °C). Parallel incubations with boiled enzymes or cell fractions respectively served as controls. All incubations were performed in duplicate or triplicate. Liberated retinol was determined by extraction and conversion into anhydroretinol as described by Chen & Heller [9], except that benzene was replaced by toluene. Alternatively, with retinyl palmitate as substrate, the liberated fatty acid was measured enzymically with a commercial kit [15].

**Esterase activities.** The hydrolysis of methyl butyrate [17] and the anaesthetic propanidid [19] was monitored titrimetrically at 30 °C (or 37 °C) and pH 8.0 as described in the cited references.

**Marker enzymes.** 5'-Nucleosidase and glucose-6-phosphatase were assayed as described by Aronson & Touster [20]. The released phosphate was determined by the method of Baginski *et al.* [21]. The enrichment of several other markers, not mentioned in the Results and Discussion section, was determined to monitor the purity of the cell fractions, namely DNA, cytochrome *c* oxidase, acid phosphatase, urate oxidase, RNA and lactate dehydrogenase. These results were similar to those obtained by Fleischer & Kervina [18], and established the enrichment of the subcellular fractions listed in Table 2 (below). Protein was determined with the biuret procedure [17].

**Inhibition experiments.** These were performed by preincubation of enzymes or cell fractions with 0.1 mM-(Nph)<sub>2</sub>P for 30 min at 25 °C in 50 mM-Tris/HCl buffer, pH 7.4. In the subsequent enzymic assay the inhibitor was diluted approx. 10-fold.

**Palmitoyl-CoA:retinol acyltransferase.** Retinol was emulsified by ultrasonication with 20 mM-sodium taurocholate or 40  $\mu$ M defatted BSA in 0.2 M-phosphate buffer, pH 7.4, to give a 2 mM emulsion. Portions (100  $\mu$ l) of these emulsions, or of 4  $\mu$ M-retinol-saturated rat liver cellular retinol-binding protein prepared as described by Ong & Chytil [22], were incubated in glass vials, covered with argon and under protection from light, with 40  $\mu$ l of 200  $\mu$ M-[1-<sup>14</sup>C]palmitoyl-CoA (diluted with unlabelled substrate to a specific radioactivity of 1 Ci/mol) for 2 h at 37 °C with 5  $\mu$ g of each esterase or 200  $\mu$ g of microsomal protein in 60  $\mu$ l of water. The reactions were stopped by rapid freezing in liquid nitrogen. Then the samples were freeze-dried and the residues dissolved in 20  $\mu$ l of chloroform/methanol (2:1, v/v). Samples were applied on silica-gel thin-layer plates, which were developed with cyclohexane/diethyl ether (4:1, v/v). Autoradiographs were obtained as described by Mentlein

*et al.* [15]. The positions of retinol and retinyl palmitate were marked by co-chromatography of unlabelled reference material and detected by their fluorescence under u.v. light (366 nm). Blanks without enzyme served as controls.

**Influence of (Nph)<sub>2</sub>P on retinol metabolism *in vivo*.** Wistar rats (4 weeks old, male) fed with a standard diet (5.160 mg of vitamin A/kg; Altromin C 1000; Altromin, Lage, Germany) were put on a retinol-free diet (Altromin C 1016) 1 week before the experiment. Each rat received 40  $\mu$ Ci of [<sup>3</sup>H]retinol (50 Ci/mol) in 40  $\mu$ l of ethanol injected intraperitoneally (i.p.). After 3 days, one group received three additional doses of 500  $\mu$ l of 10 mM-(Nph)<sub>2</sub>P in 0.9% NaCl, pH 7.4, injected i.p. in 1-day intervals. At 6 days after the first injection, all rats were killed by decapitation and the tissues excised or serum collected after clotting. Tissue samples (1–2 g) or 0.2 ml of serum were digested and their radioactivity determined as described by Heymann *et al.* [23]. A similar experiment was performed by injecting 1.6  $\mu$ Ci of [carbinol-<sup>14</sup>C]retinol (48 Ci/mol) in 200  $\mu$ l of ethanol. In this case the liver lipids were extracted as described by Bhat & Lacroix [24], and labelled retinol and retinyl esters were detected by t.l.c. and autoradiography as described above.

## RESULTS AND DISCUSSION

### Retinyl ester hydrolase activity of purified liver carboxylesterases

The four most important non-specific carboxylesterases isolated from rat liver microsomes were assayed for their ability to hydrolyse retinyl esters. All purified isoenzymes cleaved retinyl acetate to a small extent, but the esterases with pI values of 5.6, 6.2 and 6.4 were most active (Table 1). In contrast, retinyl palmitate was an exclusive substrate for the esterases with pI values of 6.2 and 6.4 (Table 1). Such selective specificities of the esterase isoenzymes have already been observed with other lipid [15] and xenobiotic [19] substrates. The four esterases represent three chemically [13] and genetically [25] different proteins. The esterase with pI 5.6 is termed 'ES-3' in the genetic nomenclature [25] and selectively hydrolyses acetanilide or phenacetin [19]. The enzyme with a pI of 6.0 is also termed 'ES-10' [25] and acts on a variety of ester-type drugs (e.g. procaine, clofibrate) [19]. The esterases with pI values of 6.2 and 6.4 are microheterogeneous forms of a single protein named 'ES-4' in the genetic nomenclature [16,25]. Both forms selectively cleave the xenobiotics propanidid and aspirin [19], as well as CoA esters and monoacylglycerols of long-chain fatty acids [15,16]. The specific activities of the two forms of ES-4 are similar. The same was observed with retinyl esters (Table 1). However, as compared with their specific activities towards amphiphilic lipids or xenobiotics (in the range of  $\mu$ mol/min per mg of enzyme), the esterases acted on retinyl esters with low rates. The emulsifier for the vitamin A ester strongly influenced the specific activities, but not the specificities, of the esterase isoenzymes (Table 1). Esterase activities with water-soluble substrates, e.g. towards 4-nitrophenyl acetate, are not influenced by the emulsifiers in the concentrations applied [15].

In order to evaluate the reliability of the determinations, the hydrolysis of retinyl palmitate was monitored by two

**Table 1. Hydrolysis of retinyl esters at pH 7.4 and 37 °C by esterases purified from rat liver microsomes**

Results are means for two or three determinations.

Retinyl ester (0.1 mM)	Emulsifier	Esterase pI . . .	Retinol liberated after 2 h by 10 µg of esterase (nmol)			
			5.6	6.0	6.2	6.4
Palmitate	0.1 mM Defatted BSA		0.2	0.3	3.5	3.2
	10 mM-sodium taurocholate		0	0.2	2.6	2.5
	0.2% Triton X-100		0	0	7.3	6.8
	0.2% Triton X-100 plus 10 mM-sodium taurocholate		0	0	8.8	5.9
Acetate	0.1 mM Defatted BSA		3.6	0.8	2.8	3.6
	10 mM-sodium taurocholate		6.7	0.6	9.5	9.5

**Table 2. Specific enzyme activities and their enrichment in cell fractions obtained by differential centrifugation**

Enzyme . . .	Specific enzyme activities (SA) and enrichment (E)									
	5'-Nucleosidase		Glucose-6-phosphatase		Methyl butyrate esterase		Propanidid esterase		Retinyl palmitate hydrolase	
	SA (nmol/ min per mg)	E	SA (nmol/ min per mg)	E	SA (nmol/ min per mg)	E	SA (nmol/ min per mg)	E	SA (nmol/ h per mg)	E
Homogenate	26	1	114	1	1790	1	124	1	5.2	1
Nuclei			36	0.3	1170	0.6	34	0.3	12.4	2.4
Mitochondria			20	0.2	250	0.1	21	0.2	23.9	4.6
Lysosomes	18	0.7	43	0.4	790	0.4	24	0.2	11.0	2.1
Peroxisomes			23	0.2	320	0.2	7	0.1	2.2	0.4
Plasma membranes	983	36.9	48	0.4	1280	0.7	103	0.8	360.0	69.0
Rough ER			223	2.0	7130	4.0	189	1.5	8.2	1.6
Smooth ER			225	2.2	9610	5.4	257	2.1	22.0	4.2
Cytosol			18	0.2	150	0.1	10	0.1	0.6	0.1

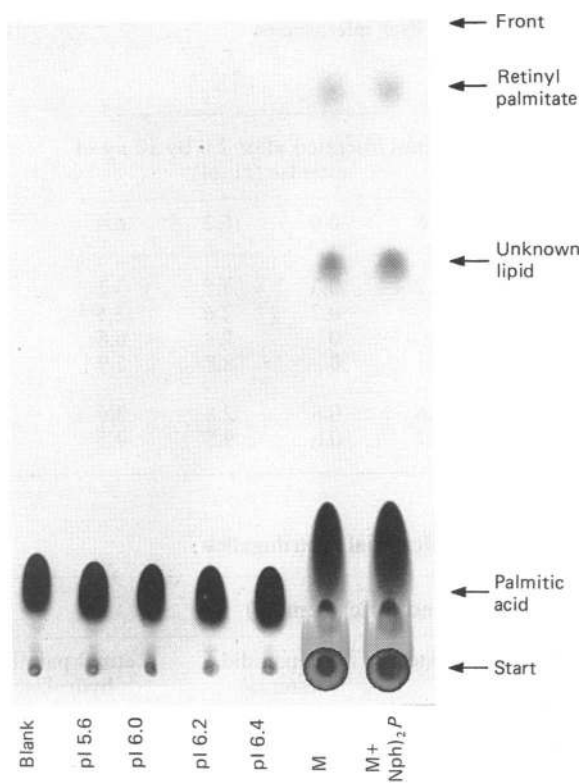
independent procedures: (1) the liberated retinol was converted into and estimated photometrically as anhydroretinol (all values in Tables 1 and 2), and (2) the liberated fatty acid determined by an enzymic procedure. From a single incubation mixture of 10 µg of pI-6.2 esterase with a 0.1 mM-retinyl palmitate emulsion in 0.2% Triton X-100 and 50 mM-phosphate buffer, pH 7.4, we determined after 2 h at 37 °C the liberated retinol (as anhydroretinol) to be 7.3 nmol and the liberated palmitic acid enzymically to be 8.1 nmol. Though the second procedure is simpler and therefore seems more reliable, it could only be used with purified enzymes. The enzymic hydrolysis of retinyl esters (acetate and palmitate) was also verified qualitatively by t.l.c. and fluorescence detection of retinol.

A recommended criterion [2,14-16] to check whether a hydrolase activity is an intrinsic property of the liver carboxylesterases investigated here or whether it is caused by an impurity is the irreversible inhibition of this type of esterase by (Nph)<sub>2</sub>P. Preincubation with 0.1 mM-(Nph)<sub>2</sub>P resulted in a decrease to 28% and 31% of the values reported in Table 1 for the hydrolysis of retinyl palmitate by esterases of pI 6.2 and 6.4 respectively, and to 10% for the hydrolysis of retinyl acetate by the pI 5.6 esterase (all with 0.1 mM defatted BSA as emulsifier). These inhibition values parallel those

observed with other esterase substrates [15,16] and therefore prove that these non-specific carboxylesterases hydrolyse retinyl esters.

The specific activities of the rat liver esterases for the hydrolysis of retinyl acetate are about 10-fold lower than those reported by Bertram & Krisch [3] for pig liver esterase. It is not clear whether their pig liver esterase preparation really lacked retinyl palmitate hydrolase activity or whether their measurement was not sensitive enough. The maximal specific activities of rat liver pI-6.2 esterase for the hydrolysis of retinyl palmitate are approx. 13-fold lower than those reported by Blaner *et al.* [11] for their hydrolase prepared from rat liver particulate fraction, but considerably higher than those reported for the rat cytosolic lipoprotein complex [9,10]. This indicates that an additional membrane-bound retinal palmitate hydrolase exists in rat liver (see below).

Since the esterases with pI values of 6.2 and 6.4 (= ES-4) hydrolyse palmitoyl-CoA with much higher rates [16] than retinyl palmitate, we investigated a possible transfer reaction of the activated fatty acid on free retinol. Using a sensitive radioactive assay, and taurocholate, defatted BSA or retinol-binding protein as emulsifiers, we could not detect palmitoyl-CoA:retinol transacylase activity with any of the esterases described in the present study (Fig. 1). In contrast, a microsomal



**Fig. 1. Transacylation between [ $^{14}\text{C}$ ]palmitoyl-CoA and retinol bound to cellular retinol-binding protein**

[ $^{14}\text{C}$ ]Palmitoyl-CoA ( $40\ \mu\text{M}$ ) and retinol-saturated rat liver cellular retinol-binding protein ( $2\ \mu\text{M}$ ) were incubated with water (Blank), purified esterases (indicated by their pI values), microsomes (microsomal fraction; M) or microsomes pretreated with  $(\text{Nph})_2\text{P}$  [M +  $(\text{Nph})_2\text{P}$ ] at pH 7.4 and  $37\ ^\circ\text{C}$  for 2 h. Lipids were extracted and separated by t.l.c. on silica gel. The Figure shows the autoradiogram. The esterases have no transacylase activity, and that of microsomes was not influenced by the esterase inhibitor. Only some traces of [ $^{14}\text{C}$ ]palmitoyl-CoA are extracted; they are found at the origin.

fraction catalysed the transfer under the conditions employed (Fig. 1). This proves that the rat liver palmitoyl-CoA:retinol acyltransferase [26] is not identical with one of the esterases. However, ES-4 is able to catalyse the transesterification between palmitoyl-CoA and ethanol (R. Mentlein, unpublished work).

#### Contribution to the total retinyl palmitate hydrolase activity of rat liver

A comparison of the catalytic properties already suggested that the esterases with pI values of 6.2 and 6.4 (= ES-4) may be not identical with the other retinyl palmitate hydrolases partly purified from rat liver so far [5–10]. Therefore we investigated the contribution of the esterases to the total retinyl palmitate hydrolase activity of rat liver. Accurate differential centrifugation demonstrated that the non-specific carboxylesterases are localized in the rough and smooth ER (Table 2). Esterase activity for methyl butyrate (a substrate selective for the esterases with pI values of 5.6 and 6.0) and propanidid (substrate-selective for the esterases with pI values of 6.2 and 6.4) enriched in the same manner as

the marker enzyme glucose-6-phosphatase in the ER. The highest specific retinyl palmitate hydrolase activity (at neutral pH) was detected in the plasma-membrane fraction characterized by the enrichment of the marker enzyme 5'-nucleosidase. This expands some earlier results. Mahadevan *et al.* [5] as well as Harrison *et al.* [6] found the highest specific retinyl palmitate hydrolase activities in the nuclear and mitochondrial lysosome-rich fractions. During simple differential centrifugation of liver homogenates in these, microsomal and supernatant fractions, enzymes of the plasma membranes may show such bimodal distribution [20]. In contrast, the retinyl acetate hydrolase activity was localized by both groups clearly in the microsomal fraction. Since the carboxylesterases cleave this substrate and are situated at the ER, probably they are mainly responsible for this activity.

However, the contribution of the non-specific carboxylesterases with pI values of 6.2 and 6.4 to the total liver retinyl palmitate hydrolase activity should be relatively low, as concluded from the data in Table 2. This can be quantified more accurately by inhibition studies with the selective esterase inhibitor  $(\text{Nph})_2\text{P}$ . Pretreatment of homogenate or plasma membranes with  $1\ \text{mM}$ - $(\text{Nph})_2\text{P}$  did not result in any measurable inhibition of retinyl palmitate hydrolysis. However, with two different smooth-ER fractions we measured 29 and 47% of the retinyl palmitate hydrolase activity of controls after pretreatment with  $(\text{Nph})_2\text{P}$ . The palmitoyl-CoA hydrolase activity in the microsomal fraction which is caused by esterases with pI values of 6.2 and 6.4 is decreased to 5% by  $(\text{Nph})_2\text{P}$  treatment under comparable conditions [16]. The somewhat lower inhibition of retinyl palmitate hydrolysis may indicate a contamination by plasma membranes or indicate the existence of further retinyl palmitate hydrolases in the ER. In conclusion, the contribution of non-specific carboxylesterases to total liver retinyl palmitate hydrolase activity is relatively low, but they do account for most of the activity of rat liver ER.

#### Physiological significance

More than 80% of the total vitamin A in the body of mammals is stored in the liver as retinyl palmitate. Since only free retinol is transported in the blood, a hydrolase might regulate mobilization [27]. Because the ER of hepatocytes is also involved in the processing of secretory proteins, including serum retinol-binding protein, the ER carboxylesterases/retinyl palmitate hydrolase might have a function in retinol delivery. To test this hypothesis *in vivo*, rats fed a vitamin A-low diet were injected with  $40\ \mu\text{Ci}$  of [ $^3\text{H}$ ]retinol. Under these conditions the labelled retinol is incorporated into retinyl esters in the liver during 24 h [24] and should then be moved to extrahepatic tissues (because of the vitamin A deficiency). One group of the animals received additional doses of the esterase inhibitor  $(\text{Nph})_2\text{P}$ . It has been shown in earlier studies that this inhibitor decreases carboxylesterase activities *in vivo* to about 5–30% of the control values [23,28,29]. After 6 days the accumulation of radioactivity (vitamin A and metabolites) in liver, serum and skin was investigated. For the two control animals we measured in liver 21 510 and 21 320 c.p.m./g of wet tissue, in serum 5440 and 7730 c.p.m./ml and in skin 5450 and 1400 c.p.m./g. The values for the two  $(\text{Nph})_2\text{P}$ -treated animals were not significantly different: liver, 24 700 and 29 870 c.p.m./g; serum, 7930 and

4390 c.p.m./ml; skin, 3550 and 2660 c.p.m./g. In contrast, the specific propanidid esterase activity in liver homogenates was decreased to 39% or 45% of the mean of the controls at the end of the experiment. As seen from autoradiograms of thin-layer chromatograms (not shown), (Nph)<sub>2</sub>P did not influence the ratio of retinyl palmitate to retinol in the liver lipids. Thus inhibition of liver ER carboxylesterases does not drastically influence vitamin A mobilization from the liver. However, more detailed experiments, such as a time course of the movement of radioactively labelled retinol, are necessary to evaluate such a function.

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