NEUTRAL LIPOLYTIC ACTIVITIES IN RAT LIVER

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Niet dat wij uit onszelf bekwaam zijn iets als ons werk in rekening te brengen, maar onze bekwaamheid is Gods werk, ...

(2 Cor. 3:5)

Aan mijn ouders

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CoA	coenzyme A
DEAE	diethylaminoethyl
dpm	desintegrations per minute
HDL	high density lipoproteins
IDL	intermediate density lipoproteins
K_m	Michaelis constant
LDL	low density lipoproteins
phospholipase A_1	phosphatide-1-acyl hydrolase (EC 3.1.1.32)
PC	*sn*-1,2-dipalmitoyl 3-phosphoryl-choline,phosphatidylcholine
TG	trioleoylglycerol
Tris	tris (hydroxymethyl) amino-methane
VLDL	very low density lipoproteins
V_{max}	maximal velocity of an enzyme reaction

CHAPTER I

GENERAL INTRODUCTION

1.1. Occurrence of lipoproteins

The transport of hydrophobic lipids in the blood is accomplished via lipid-protein complexes. Such complexes are usually called (serum or plasma) lipoproteins. They can be distinguished by their different physicochemical properties in: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL), each with different functions.

The commonly held concept, supported by physical and chemical data, is that plasma lipoproteins (compare TABLE I and ref. 1) are spherical particles with a core of triacylglycerols and/or cholesterolesters stabilized by a monolayer composed of phospholipids, cholesterol and specific proteins, so-called apolipoproteins (the "pseudomicellar" model) (2,3).

TABLE I

COMPOSITION AND PROPERTIES OF HUMAN PLASMA LIPOPROTEINS

Lipoproteins	Density range g/ml	S_f-rate	Composition (% dry weight)			
			Triacyl-glycerols	Total cholesterol	Phospho-lipids	Protein
Chylomicrons	<0.95	>400	78	12	7	3
VLDL	0.95 -1.006	$20<S_f<400$	57	20	13	10
IDL	1.006-1.019	$12<S_f<20$	39	32	17	12
LDL	1.019-1.063	$0<S_f<12$	12	49	18	21
HDL	1.063-1.210		2	24	26	48

Differences in density and size of these lipoproteins depend on the overall ratio of core to surface and on the relative proportions of their constituents (4). Shen *et al.* (5) found

a good correlation between size and chemical composition.

The apolipoproteins are divided in 5 main classes (A-E), which may be subdivided by their different electrophoretical and immunological properties (6). The apolipoproteins differ in structure, function and in distribution over the lipoprotein spectrum (1,7). Some, like apolipoprotein A-II and apolipoprotein B, appear to determine the structure of the lipoproteins, while others, like apolipoprotein A-I and the C apolipoproteins serve as modulators for lipoprotein metabolism.

1.2. Metabolism of lipoproteins

The different lipoproteins have different functions and are metabolized in a different way. Chylomicrons, which transport the dietary lipids, are synthesized in the small intestinal epithelium and are secreted by the lymphatic route into the circulation (8). This tissue also synthesizes VLDL and HDL (9). The liver synthesizes VLDL and HDL (1,9,10). LDL is a product formed during the degradation of chylomicrons and VLDL.

The triacylglycerols and a small part of the phospholipids of chylomicrons and VLDL are hydrolyzed by lipoprotein lipase (EC 3.1.1.3), which is located on the surface of vascular endothelium of extrahepatic organs, such as heart (11-13), adipose tissue (12,14,15), lung (16), mammary gland (17), intestine (18) and skeletal muscle (19,20). This activity is under hormonal control and is influenced by the diet. In the fed state the lipoprotein lipase activity of adipose tissue is increased (21-24), while the activity in heart is decreased (25). By intravenous injection of heparin a lipolytic factor is released into the bloodstream. This factor was first described by Hahn (26) and was capable to clear the plasma of hyperlipidemic dogs. It has been shown (27-29) that a number of lipolytic activities are found in postheparin plasma. Besides lipoprotein lipase from extra-

hepatic tissues a lipase released from the liver is released by heparin. The properties of the lipases can be studied *in vitro* after purification of the enzymes, which may be released from different tissues by *in vitro* organ perfusion with heparin, from whole postheparin plasma or from delipidated preparations of various organs. Lipoprotein lipase can also be purified from bovine milk (30).

Besides the heparin-releasable lipases a number of organs contain intracellular, non-heparin-releasable, lipolytic activities. These enzymes are involved in intracellular lipolysis, e.g. the mobilization of stored fat.

1.3. Role of the liver

During the lipolytic action of lipoprotein lipase, chylomicrons and VLDL are converted into smaller particles, intermediate density lipoproteins (IDL) or "remnant" particles. These particles can be taken up by the liver (31,32) or can be further degraded to LDL. The heparin-releasable liver lipase activity (liver lipase) differs from extrahepatic heparin-releasable hydrolases in substrate specificity (28,33), in apolipoprotein requirement (34-36), sensitivity to inhibitors (18) and in immunological properties (37-40). Up to 80% of the triacylglycerols originally present in VLDL is hydrolyzed during the conversion to IDL as described by Mjøs *et al.* (31) and Eisenberg and Rachmilewitz (41) for VLDL degradation in supradiaphragmatic rats and for breakdown by rat postheparin plasma *in vitro*, respectively. The latter authors found a relative enrichment of apolipoprotein B, cholesterol and phospholipid and a decrease of triacylglycerol and apolipoprotein C during the catabolism of VLDL to IDL. The relative amounts of the constituents, except apolipoprotein E, in VLDL and in IDL were: triacylglycerols 61 and 44%, free cholesterol 6 and 8%, esterified cholesterol 4 and 10%, phospholipids 14 and 19%, apolipoprotein B 3 and 10% and

apolipoprotein C 8 and 2%, respectively (41).

These data and the data concerning diameter, S_f-rate and mean weight indicate that only one IDL particle is formed from one VLDL particle (41).

For studying the metabolism of IDL the perfusion of the isolated rat liver with these particles provides much information. IDL particles are *in vivo* rapidly removed in intact animals. Therefore they must be produced in functionally hepatectomized animals or *in vitro* by incubation with lipoprotein lipase. Some differences were found between the various methods followed concerning diameter, triacylglycerol content, ratio of free to esterified cholesterol (compare 31,32,41-43). It turns out that the IDL particle is not a sharply defined particle, but a triacylglycerol-depleted VLDL or chylomicron or a triacylglycerol-rich LDL (1). Chylomicrons and VLDL particles are not removed by perfused rat liver in contrast to chylomicron remnants (31, 32,42-45) and VLDL remnants.

The metabolism of IDL varies in different species. In normal humans it is mainly further degraded to form LDL. In rats and in some hyperlipidemic states in man much of the IDL is removed from the circulation by hydrolysis or uptake by liver cells. The mechanism of this removal is obscure.

The heparin-releasable liver lipase can be involved in this process. Understanding of the exact role of this enzyme in plasma lipid metabolism requires further investigation.

1.4. Aim of the study

The purpose of this investigation is to contribute to the understanding of the nature and function of the neutral heparin-releasable and the non-releasable liver lipases. From earlier investigations in our laboratory (46) and from other studies (see section 1.3) it was supposed that liver lipase was involved in the degradation of lipoproteins. To elucidate the function of the liver lipase activities, the

substrate specificity of the enzyme must be known. Therefore, we have chosen to measure the enzyme activities with artificial substrates such as trioleoylglycerol, dipalmitoylglycerol, monoacylglycerols, phospholipids or palmitoyl-CoA. These substrates are easily obtained in purified form and the composition of the substrates can be varied. Rat liver lipase was obtained after partial purification of postheparin plasma or of liver perfusate. The non-releasable lipase was purified from the microsomal fraction of rat liver.

The heterogeneity and substrate specificities of both enzymes and the immunological relationship between the two enzymes are described in chapter II. A description of the influences of apolipoproteins on liver lipase and lipoprotein lipase is given in chapter III, while chapter IV reports on changes of liver lipase activity observed in diabetic and hypothyroid rats, as well as the effect of a linoleic acid-rich diet. A general discussion of the results on the possible contribution of the two lipases in lipid metabolism is given in chapter V.

CHAPTER II

NEUTRAL ACYLGLYCEROL HYDROLASE ACTIVITIES OF RAT LIVER

2.1. Occurrence of different neutral acylglycerol hydrolase activities

Lipolytic activity in rat liver homogenates has been studied by several authors (11,47-54) and has been identified in cytosol (48,49), microsomes (49-51), lysosomes (49,52), mitochondria (53) and plasma membranes (49,52,54).

On account of the different pH optima two triacylglycerol hydrolase activities have been distinguished in total liver homogenates. One is the acid (lysosomal) lipase (49,52) and the other the neutral lipase(s) (48,52).

The lipase activity in the microsomal and plasma membrane fractions has been measured with different substrates (triacylglycerol, monoacylglycerol, phosphatidylcholine or phosphatidylethanolamine) (51,54,55). Colbeau *et al.* (51) have attempted to separate the different activities. From differences in elution pattern, heat inactivation and the influence of 1 M NaCl, 1 M KCl or detergents they concluded that in the microsomal fraction two enzymes are present. One enzyme with monooleoylglycerol hydrolase activity, the other with trioleoylglycerol and phosphatidylethanolamine hydrolase activity. Others found an enzyme releasable by heparin, supposed to be present in the plasma membrane fraction (49,54), and in acetone powder extracts (56). The heparin-releasable liver enzyme is probably the same as the one present in plasma obtained after intravenous heparin administration ("postheparin plasma") (49,54,55,57).

Heparin releases neutral lipolytic activities from liver as has been shown in different species: man (36,58,59), dog (60-63), pig (29) and rat (38,64).

We have investigated which part of the total liver activities can be released by heparin. It is shown in

appendix paper I that the tri- and monooleoylglycerol, phosphatidylcholine and palmitoyl-CoA hydrolase activities are released in different proportions. Within 6 min the trioleoylglycerol hydrolase activity was released for 85%, while the phospholipase A_1 (measured with phosphatidylcholine vesicles) and monooleoylglycerol hydrolase activities were released for only 15 and 17%, respectively. Perfusion during 20 min led to 96% release of the trioleoylglycerol hydrolase activity from the liver, while the bulk of the other lipolytic activities remained in the liver. The remaining trioleoylglycerol hydrolase activity was found to be inhibited by an antiserum against the heparin-releasable liver lipase, in contrast to the non-releasable monooleoylglycerol hydrolase activity, which was not inhibited. In the liver perfusate (which contained the heparin-releasable enzyme) all the activities were completely inhibited by antiserum against liver lipase. An antiserum raised against non-releasable (microsomal) lipase (see section 2.3, appendix paper II) did not influence the activities released by heparin but inhibited the non-heparin-releasable activities completely.

The different enzymes found by some investigators in total liver (51) or plasma membrane fractions (65-67) may therefore be ascribed both to releasable and non-releasable enzymes.

The microsomal fraction will contain both enzymes, since they contain fragments from both endoplasmic reticulum and plasma membrane.

In conclusion: the neutral trioleoylglycerol hydrolase activity is completely heparin-releasable and localized on the surface of those liver cells which surround the heparin-accessible space. This localization is also supported by the observed loss of the activity by perfusion of livers with collagenase (68) (see also section 2.5). The monoacylglycerol hydrolase activity is catalyzed by at least two different enzymes: one localized on the cell

surface (extracellularly) (see section 2.2) and one within the cells (intracellularly) (see section 2.3).

2.2. Investigations of the substrate specificity of (heparin-releasable) liver lipase

2.2.1. *Heterogeneity of postheparin plasma lipase activity*

As discussed in the preceeding chapter heparin-containing perfusates of rat liver contain lipolytic activity against mono- and triacylglycerols and also phospholipase A_1 and palmitoyl-CoA hydrolase activities. Phospholipase A_1 activity has also been found in postheparin plasma (39,69-71), as well as palmitoyl-CoA hydrolase activity (72). Partial purification of lipolytic activities from postheparin plasma from man or rat was obtained by affinity chromatography on heparin-Sepharose. It yielded two major activity peaks. One was eluted by 0.75 M NaCl, the other one by 1.25 M NaCl (29, 39,71,73). The first activity peak was subsequently identified as being derived from the liver, while the other one was found to represent lipoprotein lipase present in extrahepatic tissues (13,73). The "liver peak" contained besides trioleoylglycerol hydrolase activity also activity against palmitoyl-CoA (39,74), phospholipase A_1 (measured with phosphatidylethanolamine) (71) or monooleoylglycerol hydrolase activity (28,75; appendix paper I).

2.2.2. *Heterogeneity of neutral liver lipase*

It has been attempted to separate the lipolytic activities towards mono-, di- and triacylglycerol, phospholipid and palmitoyl-CoA in postheparin plasma. The purification procedures of these lipolytic activities from postheparin plasma have been based on differences in affinity for Intralipid (an emulsion of triacylglycerols stabilized with phospholipids) and differences in binding to carrier-

bound mucopolysaccharides, such as heparin and concanavalin A (71,76,77). All attempts to separate the different "liver" activities, however, failed. Rat postheparin plasma, used in these experiments, was delipidated to remove endogenous substrates. The single activity peak, eluted at a low NaCl concentration (the "liver peak") hydrolyzed mono-, trioleoylglycerol and palmitoyl-CoA. The activities gave the same elution pattern (Fig. 1). This was in contrast with (delipidated) human postheparin plasma as was found by Ehnholm *et al.* (36). They were able to obtain three subfractions of liver trioleoylglycerol hydrolase activity. However, all enzyme activities were inhibited by antiserum

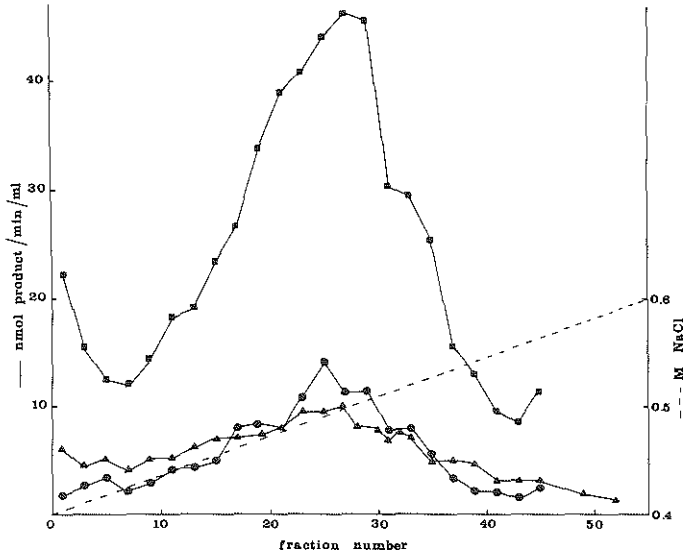

Fig. 1. Heparin-Sepharose chromatography of rat postheparin lipase(s). 112 ml postheparin plasma was mixed with 56 ml 10% Intralipid (Vitrum, Stockholm) and incubated during 15 min at 37°C. The fat cake was collected by centrifugation during 70 min at 100,000 g. The clear infranatant was again mixed with Intralipid and the incubation and centrifugation steps were repeated. The combined fat layers were delipidated subsequently by cold acetone, n-butanol and diethylether as described in appendix paper II for the enzyme purification. The activity in the dried powder was extracted with 5 mM Veronal buffer pH 7.4. The extract was applied to a heparin-Sepharose 4B column (1.5 x 10 cm) and the activity was eluted with a linear NaCl-gradient (600 ml 0.4-0.8 M NaCl). Fractions of 5 ml were collected and the hydrolase activity was determined with the substrates trioleoylglycerol (●-●), monooleoylglycerol (■-■) and palmitoyl-CoA (▲-▲) as described in appendix paper I.

against liver lipase (36). Moreover, as described in appendix paper I, purification of liver perfusates by heparin-Sepharose affinity chromatography did not alter the K_m and V_{max} parameters of the different activities, while the degree of purification for the three measured activities was found to be the same. When a suboptimal amount of antiserum, raised against liver lipase, was used, similar inhibition was obtained of the measured activities. Competitive inhibition was observed in the hydrolysis of both trioleoylglycerol and phosphatidylcholine substrates when a heparin-containing liver perfusate was used as enzyme source. In these experiments a constant trioleoylglycerol concentration (16.3 mM) and an increasing phosphatidylcholine concentration (0.13-1.3 mM) was used. At the higher phosphatidylcholine concentrations the trioleoylglycerol hydrolase activity was inhibited, while the phospholipase A_1 activity (measured with phosphatidylcholine) was stimulated (TABLE II).

TABLE II

INFLUENCE OF VARYING THE PHOSPHATIDYLCHOLINE CONCENTRATION ON THE RATES OF TRIOLEOYLGLYCEROL AND PHOSPHATIDYLCHOLINE HYDROLYSIS BY LIVER PERFUSATE

The lipid substrates were emulsified in 5% gum arabic by sonification during 6 min at 0°C (at 21 kHz, 7 Kcs). The incubation medium (0.25 ml) contained 0.1 ml substrate, 6.25 mg defatted bovine serum albumin, 40 mM Tris-HCl, pH 8.5, 0.25 mM $CaCl_2$, 0.1 M NaCl. The incubation was stopped by adding 1.45 ml chloroform/methanol/heptane = 1.45/1.25/1.0 (by volume). Fatty acids were extracted with 1.05 ml 0.5 M borate buffer pH 10.5, as described in appendix papers I and III. The reaction rate was constant during the 30 min incubation at 30°C.

Concentration of substrate (mM) of		nmol fatty acid release/min/ml from	
Trioleoylglycerol	Phosphatidylcholine	Trioleoylglycerol	Phosphatidylcholine
6.5	-	122	-
6.5	0.05	102	0.32
6.5	0.15	94	0.92
6.5	0.50	84	1.54

These results suggest a broad substrate specificity of liver lipase, rather than a mixture of different enzymes. The apparent molecular weight of 64,000 (71) seems to rule out a multi-enzyme complex.

2.2.3. *Further characterization of neutral liver lipase*

Liver lipase seems to be a single enzyme with a broad substrate specificity. For the study of the role of this enzyme in metabolism, the activities against different substrates, and the K_m and V_{max} values have been determined.

The activities of liver lipase were measured with trioleoyl and dipalmitoylglycerols emulsified in gum arabic. Long-chain monoacylglycerols were emulsified in defatted bovine serum albumin. The used long-chain monoacylglycerols were 1- or 2-monopalmitoylglycerol, a mixture of 1- and 2-monooleoylglycerol (88% and 12%, respectively), or a mixture of 1- and 2-monolinoleoylglycerol (87% and 13%, respectively). Phospholipase activities were measured with phosphatidylcholine vesicles. The hydrolysis of tri- and monooleoylglycerols and phosphatidylcholine had different kinetic parameters (compare appendix paper I). The V_{max} values were for monooleoylglycerol > trioleoylglycerol > phosphatidylcholine, while the apparent K_m values were for phosphatidylcholine < monooleoylglycerol < trioleoylglycerol. From the high V_{max} and low K_m values with monooleoylglycerol, it can be concluded that the enzyme preferentially hydrolyzes this substrate. Later it was found that liver lipase also catalyzes the transacylation of monoacylglycerol to form diacylglycerol and glycerol (appendix paper III). The same transacylation reaction was also described for a phospholipase A_1 activity in liver plasma membranes (55,78). Since the substrate, used for monoacylglycerol hydrolase measurements, was labeled in the glycerol moiety of the monoacylglycerol, glycerol was liberated in two reactions, both catalyzed by liver lipase: hydrolysis and transacylation.

The hydrolase activity was detected by measuring the formed glycerol, while for the transacylase activity the diacylglycerol formed was determined. When the amount of glycerol formed is corrected for the amount of diacylglycerol formed, the correct hydrolase activity can be calculated. This correction has not been made in the V_{max} and apparent K_m measurements. Therefore, the V_{max} value for true monooleoylglycerol hydrolase activity may be lower than shown in TABLE III of appendix paper I.

The activities of monoacylglycerol hydrolysis and transacylation are strongly dependent on the monoacylglycerol used and on the molar ratio of monoacylglycerol to albumin. Is this ratio lowered, then both activities decrease with different proportions (appendix paper III). This is probably caused by a preferential hydrolysis to glycerol and free fatty acid at the lower free monoacylglycerol concentrations, i.e. at the higher albumin concentration used. The V_{max} values of the rates of hydrolysis of monoacylglycerol have been found to depend on the kind of fatty acid esterified with glycerol. 1- or 2-Monopalmitoylglycerols were hydrolyzed at a slower rate than monooleoylglycerol and monolinoleoylglycerol. In the transacylation reaction this phenomenon was even more pronounced (appendix paper III). In summary, the ratio of hydrolysis to transacylation was 2.6 for 1- and 2-monopalmitoylglycerol, 0.8 for monooleoylglycerol and 0.6 for monolinoleoylglycerol.

2.2.4. *Positional specificity of fatty acid removal during hydrolysis and transacylation*

Liver lipase only removed the fatty acid on the *sn*-1 position of phosphatidylcholine (1.8 nmol/min.ml perfusate). It removed the fatty acid from the *sn*-1 position of phosphatidylethanolamine much faster than from the *sn*-2 position (20.1 *vs* 0.4 nmol/min.ml perfusate). 1- or 2-Monopalmitoylglycerol were equally active in the hydrolysis or trans-

acylation reactions (appendix paper III). The specificity of hydrolysis of dipalmitoylglycerol was also determined. It appeared that the fatty acids of dipalmitoylglycerol were hydrolyzed at different rates. The fatty acid on the *sn*-2 position was 2.7 times faster hydrolyzed than the one on the *sn*-1 position.

Incubation of liver lipase with various concentrations of antiserum against liver lipase resulted in similar percentual inhibition whether phosphatidylcholine, monooleoylglycerol or dipalmitoylglycerol was used as substrate (compare appendix paper III).

This broad substrate spectrum explains the ability of liver lipase to hydrolyze triacylglycerol completely to glycerol and fatty acid in contrast to lipoprotein lipase (compare ref. 39).

2.3. Investigations of non-releasable monoacylglycerol hydrolase activity

2.3.1. *Purification of non-releasable monoacylglycerol hydrolase activity*

In the preceeding chapter some of the properties of the heparin-releasable enzyme were discussed. In order to compare the properties of this enzyme activity with the non-releasable enzyme, the non-releasable monoacylglycerol hydrolase activity was partially purified from the microsomal fraction of rat liver, from which the heparin-releasable enzyme had been removed by prior heparin perfusion (appendix paper II). The microsomal fraction was delipidated with subsequently acetone, n-butanol and diethylether and the enzyme was solubilized in 50 mM ammonia buffer pH 8.5. The bulk of the enzyme activity was obtained by precipitation between 40 and 70% saturated ammonium sulphate. The precipitation was solubilized in 50 mM Tris-HCl buffer pH 8.0, dialyzed against this buffer and chromatographed on a

Sephacryl S-200 column. The most active fractions, eluting as a sharp peak, were combined and further purified on a DEAE-Sephadex A-50 column. The monooleoylglycerol hydrolase activity was purified up to 130 fold. On polyacrylamide gel electrophoresis rods the highest activity peak corresponded with the main protein bands (fig. 2). An antiserum against this enzyme was raised in a rabbit (compare section 2.4).

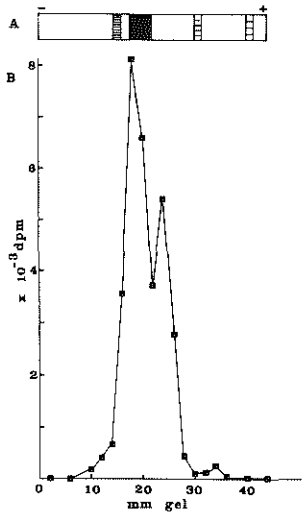

Fig. 2. Polyacrylamide gel electrophoresis of the most active fraction of microsomal lipase after chromatography on a Sephacryl S-200 column. A. Staining with Amido black. B. Monooleoylglycerol hydrolase activity. Polyacrylamide gel electrophoresis was performed in 5% polyacrylamide gels in 25 mM Tris-190 mM glycine at pH 8.5 with 4 mA per rod. On each gel 90 µg enzyme protein was applied. The enzyme activity was detected in 2 mm slices of the rods. After 1 h at 4°C in 0.5 M Tris-HCl (pH 9.0), to extract the enzyme, the monooleoylglycerol hydrolase activity was measured by adding 250 µl H_2O and 100 µl substrate (12.5 µmole). The substrate was prepared and the incubations were stopped (after 20 min at 37°C) as described in appendix paper I.

2.3.2. *Substrate specificity of the non-releasable monoacylglycerol hydrolase activity*

To investigate a possible relationship between microsomal monoacylglycerol hydrolase (microsomal lipase) and liver lipase, the substrate specificity of the first enzyme

was determined and will be described in this section. The rate at which the microsomal lipase catalyzes the hydrolysis of monoacylglycerols was dependent on the kind of fatty acid esterified in monoacylglycerol. Monolinoleoylglycerol was hydrolyzed much faster than monooleoylglycerol (appendix paper III). Monoacylglycerols with saturated, esterified, fatty acids, such as monopalmitoylglycerol, were hydrolyzed slower than monooleoylglycerol. The rate of hydrolysis was independent of the position of the fatty acid, for 1- and 2-monopalmitoylglycerol were hydrolyzed at the same rate (tested at a molar ratio of monoacylglycerol to albumin of 4.3 at 280 mU/mg enzyme protein). When monoacylglycerol was replaced by dipalmitoylglycerol the hydrolysis of the fatty acids on the *sn*-1 or *sn*-2 position of 1,2 dipalmitoyl-glycerol proceeded at different rates. The fatty acid on the *sn*-2 position of 1,2 dipalmitoylglycerol was 6.5 times faster hydrolyzed than the one on the *sn*-1 position (12.4 and 1.9 mU/mg enzyme protein, respectively). It appeared also that the hydrolysis of dipalmitoylglycerol was much slower than the hydrolysis of the monoacylglycerols used. Transacylation of monooleoylglycerol to form glycerol and diacylglycerol was undetectable with this enzyme. Trioleoyl-glycerols were not hydrolyzed at all. Therefore, it can be concluded that heparin-releasable liver lipase and the microsomal enzyme have different substrate specificities.

2.4. Immunological relationship between monoacylglycerol hydrolase activities of various tissues

Differences in substrate specificity between the liver lipase and the microsomal lipase (section 2.2 and 2.3) suggest that they are different enzymes. A relationship can, however, still exist between the two enzymes. Therefore, the immunological properties of the two enzymes were also investigated by using antisera against the liver lipase and the microsomal lipase (see section 2.3.1). Antiserum against

liver lipase inhibited the monooleoylglycerol hydrolase activity in a heparin-containing liver perfusate completely and in postheparin plasma partially (appendix paper II). However, the antiserum raised against the microsomal lipase did not inhibit the activity present in liver perfusate or in postheparin plasma.

Monoacylglycerol hydrolase activity has been described in several organs. Vaughan *et al.* (79) and Tornqvist *et al.* (80) determined these activities in adipose tissue, while Senior and Isselbacher (81) and De Jong *et al.* (82) found this activity in intestinal villus cells. In the previous chapter the partial purification of monoacylglycerol from liver has been described. By using the antiserum against this liver microsomal lipase the immunological relationship between the microsomal liver enzyme and the monoacylglycerol hydrolase activities of other organs was determined. Isolated microsomes of rat small intestinal villus cells and whole rat epididymal fat pads were delipidated. The bulk of the enzyme activity could be extracted from the delipidated powders and was incubated with antisera against liver lipase and microsomal lipase. The antiserum against liver lipase did not inhibit the enzyme activity of small intestinal villus cells, nor of epididymal fat pads, while about 50% inhibition was obtained with the antiserum against microsomal lipase. De Jong *et al.* (82) found three monoacylglycerol hydrolase activity peaks in microsomes of small intestinal villus cells after chromatography on a Sephadex G-200 column. Only one of these activity peaks was completely inhibited by the antiserum against liver microsomal enzyme. The other peaks were not inhibited.

Therefore, the antiserum against liver lipase inhibited only the monooleoylglycerol hydrolase activity present in liver perfusate and postheparin plasma, while the antiserum against microsomal lipase inhibited only the activity present in organs, such as liver, adipose tissue and small intestinal villus cells. The microsomal lipase represents

probably a (microsomal) monoacylglycerol hydrolase involved in the removal of endogenous monoacylglycerols.

2.5. Concluding remarks

The two neutral lipases in rat liver, i.e. the heparin-releasable liver lipase and the non-releasable microsomal lipase, are different in several aspects, as was described in the previous paragraphs.

The liver lipase hydrolyzed long-chain tri-, di- and monoacylglycerols, phosphadidylcholine and phosphatidyl-ethanolamine. The monoacylglycerols were found to be the preferential substrates. Monoacylglycerol could also be used in a transacylation reaction to form glycerol and di-acylglycerol. The ratio between hydrolysis and transacylation was dependent on the type of fatty acid esterified in the monoacylglycerol.

The microsomal lipase hydrolyzed long-chain monoacyl-glycerols and, at a much slower rate, dipalmitoylglycerols. Hydrolysis of trioleoylglycerol and transacylation between monoacylglycerols were found to be absent.

An antiserum raised against liver lipase inhibited only the activity released into the extracellular space by heparin perfusion, while the antiserum raised against the microsomal lipase cross-reacted with activity present in the intracellular space of liver, adipose tissue and small intestinal epithelium.

After isolation of parenchymal liver cells with collagenase, only the microsomal lipase could be detected, while in the total liver homogenate both enzymes were present (appendix paper III). The differences between the two enzymes in substrate specificity, localization and the absence of immunological cross-reactivity indicate that these enzymes are different. A summary of the properties of both enzymes is presented in TABLES III and IV.

TABLE III

COMPARISON OF HYDROLYTIC (AND TRANSACYLATION-) ACTIVITIES OF LIVER LIPASE AND MICROSOMAL LIPASE WITH DIFFERENT SUBSTRATES

Substrate	Liver lipase nmol/min.ml perfusate	Microsomal lipase nmol/min.mg protein
Trioleoylglycerol	50	0
1,2 Dipalmitoylglycerol (*sn*-1)	23	12.4
(*sn*-2)	62	1.9
1-Monopalmitoylglycerol	65 (26)*	n.d.
2-Monopalmitoylglycerol	67 (26)	n.d.
Monooleoylglycerol	169 (218)	373 (4)
Monolinoleoylglycerol	148 (244)	1295 (7)

n.d. = not determined

* Values between brackets are rates of transacylation for which the rates of hydrolysis have been corrected.

TABLE IV

OCCURRENCE OF (HEPARIN-RELEASABLE) LIVER LIPASE AND MICROSOMAL LIPASE IN THE RAT

	Liver lipase	Microsomal lipase
Total liver	+	+
Isolated liver parenchymal cells	-	+
Isolated liver microsomes	-	+
Heparin-containing liver perfusate	+	-
Postheparin plasma	+	-
Adipose tissue	-	+
Small intestinal epithelium	-	+

+ = present, - = absent.

The results mentioned in this chapter are described in more detail in appendix papers I, II and III. In these papers also the methods are given.

CHAPTER III

INFLUENCE OF APOLIPOPROTEINS ON LIVER LIPASE AND LIPOPROTEIN LIPASE

The two lipase activities in postheparin plasma, i.e. the (extrahepatic) lipoprotein lipase and the liver lipase, have the releasability by heparin in common. Both are probably involved in lipoprotein metabolism. In *in vitro* systems the two enzymes are differently influenced by C-apolipoproteins. The lipoprotein lipase is *in vitro* activated by apolipoprotein C-II (34,83,84). Liver lipase on the contrary is inhibited by apolipoprotein C-I, C-II and C-III (85).

We have found for lipoprotein lipase, partially purified from rat heart, that apolipoprotein C-II and apolipoprotein C-III influence the trioleoylglycerol hydrolase and phospholipase A_1 (measured with phosphatidylcholine) activities differently. The trioleoylglycerol hydrolase activity, stimulated by the addition of apolipoprotein C-II, could be inhibited by the simultaneous addition of apolipoprotein C-III. However, when apolipoprotein C-II-stimulated phospholipase A_1 activity was measured with this enzyme, apolipoprotein C-III further stimulated the activity (appendix paper V). On the other hand, both trioleoylglycerol and phospholipase A_1 activities of liver lipase were found to be inhibited by the addition of apolipoprotein C-II (TABLE V).

TABLE V

EFFECT OF APOLIPOPROTEIN C-II ON THE HYDROLYSIS OF TRIOLEOYLGLYCEROL AND PHOSPHATIDYLCHOLINE BY LIVER LIPASE

The hydrolase activities were measured with two substrates. For one substrate 6.25 µmol trioleoylglycerol (TG) and 0.32 µmol phosphatidylcholine (PC) were emulsified in 2.5 ml 5% gum arabic by sonification during 6 min at 21 kHz at 0°C. The incubation medium was as described in appendix paper V, but human serum was replaced by 20 µg apolipoprotein C-II. The reaction rate was linear during 40 min incubation. As enzyme source liver perfusate was used. For the other substrate 0.5 µmol phosphatidylcholine (PC) was sonified during 2 x 30 sec at 21 kHz at 0°C. The incubation was as described in appendix paper I. The apolipoprotein C-II concentration was 2 µg/ml incubation medium. The reaction was terminated after 15 min at 30°C by the addition of chloroform and methanol according to Bligh and Dyer (86). Free fatty acids were extracted as described in appendix paper III for the monoacylglycerol hydrolase activity and separated by thin layer chromatography. The R_f value was 0.41. As enzyme was used partially purified liver lipase from postheparin plasma, eluted from a heparin-Sepharose 4B column at 0.75 M NaCl (as described in ref. 39).

Substrates	Hydrolysis			
	Trioleoylglycerol		Phosphatidylcholine	
	-apo C-II	+apo C-II	-apo C-II	+apo C-II
	(nmol product formed/min.ml)			
TG + PC in gum arabic	8.05	3.33	0.41	0.11
PC vesicles			1.48	0.80

HEPARIN-RELEASABLE LIVER LIPASE IN DIABETIC AND HYPOTHYROID RATS. INFLUENCE OF A DIET RICH IN POLYUNSATURATED FATTY ACIDS

A lowered liver lipase activity has been reported in several diseases which are accompanied by hypertriglyceridemia, such as in diabetes, hypothyroidism (87), and some liver diseases (e.g. alcoholic, viral and acute hepatitis, biliary cirrhosis) (88-91). Lipoprotein lipase activity has been reported to be normal in some of these diseases (in some forms of chronic hepatitis, liver cirrhosis, maturity-onset diabetes), while it was lowered in acute hepatitis.

A lowered hepatic lipase has been described also in experimental models for diabetes (39,92,93, appendix paper V) and hypercortisolism in the rat (39). In hypothyroid rats (obtained by the administration of methimazole in the drinking water during 2 weeks) a reduction of 30% (2937 *vs* 2056 nmol fatty acid/min.ml) of the liver lipase activity (measured as monooleoylglycerol hydrolysis) was observed, while in diabetes (intravenous injection of 50 mg/kg body weight of streptozotocin) a reduction of 52% (2937 *vs* 1405 nmol fatty acid/min.ml) was observed (appendix paper IV). The liver lipase activity was measured in postheparin plasma both as hydrolysis of trioleoylglycerol in the presence of 1 M NaCl (to inhibit the lipoprotein lipase activity) and as the glycerol production from monooleoylglycerol, corrected for the hydrolase activity of preheparin plasma or postheparin plasma incubated with antiserum against liver lipase. The lowering of the liver lipase activity by experimental diabetes and hypothyroidism measured with monooleoylglycerol was more pronounced than measured with trioleoylglycerol.

The administration of drugs gives also rise to alteration of the liver lipase activity. For instance, oxandrolone (94) or gemfibrozil (95) given to patients to lower hypertriglyceridemia, led to an increase in the

activity of liver lipase.

The addition of certain steroid hormones may also result in alteration of liver lipase activity. Applebaum *et al.* (96) and Sauar and Stokke (97), for instance, found a decrease of the enzyme activity after estrogen administration to man.

Alterations in postheparin lipolytic activities cannot only be influenced by hormones and drugs, but also by dietary changes. In experimental diabetes and hypothyroid rats the lowered liver lipase could be restored by a diet rich in polyunsaturated fatty acids (containing 40 cal% fat, including 66 cal% linoleic acid and 4 cal% arachidonic acid) during one week. The liver lipase in postheparin plasma of diabetic and hypothyroid rats was restored to about 90% of the activity of control rats. The 100% value for trioleoylglycerol and monooleoylglycerol hydrolysis was 625 and 2719 nmol fatty acid/min.ml serum, respectively. In diabetic rats the liver lipase activity was restored by the diet, but this diet had no effect on the elevated serum glucose concentration. Hence, polyunsaturated fatty acids only correct certain diabetes symptoms, although in obese diabetic patients a linoleic acid-rich diet has been shown to lower both blood glucose and insulin, while the diet also decreased the hypertriglyceridemia (98). This suggests an increase of insulin efficiency by linoleic acid feeding. Diets rich in polyunsaturated fatty acids may also contribute to lowering of the blood triacylglycerols by inhibition of *de novo* fatty acid synthesis (99). Linoleic acid-rich diets may also stimulate prostaglandin synthesis. The increase of liver lipase activity in diabetic and hypothyroid rats by a linoleic acid-rich diet, however, was still observed after inhibition of prostaglandin synthesis by indomethacine. The influence of the linoleic acid-rich diet on the restoration of the liver lipase activity in both diseases, might be related to an increase of membrane fluidity, which can result in an improvement of the receptor for liver lipase

(compare ref. 100). Fluidity studies of liposomes as membrane models have elucidated that the fluidity of membranes depends on the molar ratio of cholesterol to phospholipids, the chain length and degree of unsaturation of the phospholipid acyl chains and the molar ratio of phosphatidylcholine to sphingomyeline (compare also ref. 101). Diets rich in polyunsaturated fatty acids decrease the cholesterol content in plasma and will increase the degree of unsaturation of the phospholipids. Both factors cause an increase of membrane fluidity. Borochov and Shinitzky (102) suggest that by increasing fluidity the surface receptors of red cell membranes sink deeper into the more fluid membrane bilayer. To what extent this also applies to liver lipase binding to plasma membranes or recognition of the lipoprotein particle by the liver cells is not yet clear.

CHAPTER V

ON THE ROLE OF THE NEUTRAL LIVER LIPASES IN LIPID METABOLISM

In this study the enzymatic properties of two neutral liver lipases (an "outer" and and "inner" enzyme) are described. The "outer" enzyme is removed from the liver by perfusion with heparin and is inactivated during cell isolation, using collagenase. Therefore, it is concluded that this (heparin-releasable) lipase is localized at the outside of liver cells. The heparin-releasable enzyme may act upon partial acylglycerols formed by the action of lipoprotein lipase on chylomicrons and VLDL, mainly 2-monoacylglycerol and 2,3 diacylglycerol (103). These products were found on the surface of the IDL or "remnant" particles, as was demonstrated electromicroscopically (104) and, in addition, these partial acylglycerols may be transferred to albumin. Mono- and diacylglycerols, *in vitro* bound to albumin, are good substrates for the liver lipase. The "inner" enzyme is not heparin-releasable and its activity is not affected during cell isolation. It can be purified from a microsomal fraction and is probably involved in the hydrolysis of endogenous long-chain mono- and diacylglycerols, which are formed within the cells during fat mobilization. This enzyme is not only present in hepatocytes, but also in other cell types, and involved in active fat metabolism (adipocytes and small intestinal epithelium cells).

The differences in substrate specificity and localization between the two monoacylglycerol hydrolase activities point to different enzymes. Moreover, the lack of immunological cross-reactivity between the two liver lipases, as described in this study, underlines the differences of enzyme structure, and also suggests that the microsomal lipase is not a precursor of the heparin-releasable liver lipase.

Although *in vitro* the liver lipase may hydrolyze long-

chain triacylglycerols, these substrates are probably of no importance for its *in vivo* action, since trioleoylglycerol hydrolysis is strongly inhibited when gum arabic is replaced by phospholipids in artificial mixtures. Triacylglycerol, the core material of lipoprotein particles, will be less exposed to the enzyme than more superficially localized phospholipids, which are only hydrolyzed at a low rate (but with a low apparent K_m) by the enzyme. This explains why it is unlikely that triacylglycerols from chylomicrons and VLDL are the substrate of liver lipase. In plasma lipoproteins, partial acylglycerols and phospholipids are located at the outside of the particles. The C-apolipoproteins inhibit, at least *in vitro*, the hydrolytic action of the enzyme. These apolipoproteins then may contribute to the resistance of lipid hydrolysis of chylomicrons and VLDL in the liver.

The localization of the liver lipase on the outside of liver cells and the degradation of IDL particles by the liver (31,42,43) suggest a relation between both. This is supported by the substrate specificity of liver lipase, the composition of the IDL particles and the inhibition of hydrolysis by C-apolipoproteins. In patients with a lowered liver lipase activity, as in hypothyroidism, an elevated level of IDL particles was observed (105).

El-Maghrabi *et al.* (67) showed in *in vitro* studies the hydrolysis of monoacylglycerols bound to IDL, LDL, HDL and albumin by parenchymal liver cells, isolated after collagenase treatment of liver, followed by centrifugation and washing of the cells. Parenchymal liver cells, isolated by us, were devoid of liver lipase. On the one hand this enzyme has been found to be susceptible to collagenase (compare also ref. 68) and on the other hand the enzyme is secreted by parenchymal cells and is virtually not bound by these cells (106). Therefore, it is not certain that the monoacylglycerol hydrolysis by isolated rat liver parenchymal cells observed by El-Maghrabi *et al.* (67) during isolation is due to liver lipase bound to the outside of parenchymal cells,

as suggested by these authors.

The observed transacylation catalyzed by liver lipase, between artificial monoacylglycerol substrates, was more strongly inhibited than hydrolysis of the substrate at higher albumin concentrations. This probably means that, although the enzyme may catalyze transacylation, *in vivo* monoacylglycerol hydrolysis of plasma components is the major function of the enzyme. Removal of long-chain monoacylglycerols can be an important detoxification mechanism since accumulation of these amphipatic molecules would cause damage to membranes.

Non-parenchymal liver cells possess binding sites for liver lipase, as was shown by binding by purified liver lipase to these cells (106). Parenchymal cells are capable of secreting liver lipase (107). It can be suggested that the bound enzyme contributes to the recognition of the IDL particles. The IDL particle is, at least in the rat, further catabolized in the liver. The restoration of serum liver lipase activity in diabetes and hypothyroid rats by a diet rich in polyunsaturated fatty acids is probably caused by a change in the fluidity of the cell membrane. Further work is required to elucidate the exact role of the enzyme in the metabolism of plasma lipid components.

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SUMMARY

The role of the liver in lipid hydrolysis at neutral pH is discussed in this work. Rat liver contains neutral and acid lipolytic activities. The neutral lipolytic activities can be detected with different substrates: tri-, di-, and monoacylglycerols, phosphatidylcholine, phosphatidylethanolamine and palmitoyl-CoA. *In vitro* perfusion of the liver with heparin for 6 min results in almost complete removal of the trioleoylglycerol hydrolase activity, while a major part of the monooleoylglycerol hydrolase activity was retained (section 2.1, appendix paper I). Therefore, in the liver heparin-releasable and non-releasable activities can be distinguished. The heparin-releasable enzyme causes most of the lipolytic activity of plasma after the intravenous injection of rats with heparin.

An antiserum raised against purified heparin-releasable lipase (liver lipase) inhibited completely the lipolytic activity present in a heparin-containing liver perfusate, whereas the non-releasable activities in the liver were not inhibited by this antiserum (section 2.4, appendix paper II).

The different lipolytic activities of liver lipase could not be separated by using several purification procedures. Determinations of V_{max} and K_m values for the hydrolysis of different glycerolesters revealed that monoacylglycerol is the preferred substrate for the liver lipase. Monoacylglycerols could participate in two reactions of the enzyme: hydrolysis to form glycerol and free fatty acids and transacylation to form glycerol and diacylglycerol. The ratio between hydrolysis and transacylation *in vitro* depends on the fatty acid esterified and on the molar ratio of monoacylglycerol to bovine serum albumin used. Monoacylglycerols with unsaturated fatty acids are preferentially transacylated, while monoacylglycerols with saturated fatty acid are largely hydrolyzed (section 2.2.3, appendix papers I and III).

The non-releasable lipase activity in rat liver was purified from the microsomal fraction and an antiserum was raised in a rabbit (section 2.3.1, appendix paper II). This microsomal lipase differed from the liver lipase immunologically, in substrate specificity and in localization. Microsomal lipase preferentially hydrolyzed (long-chain) 1- and 2-monoacylglycerols. Dipalmitoylglycerols were hydrolyzed at a much slower rate. Transacylation was nearly absent, while trioleoylglycerols were not attacked (section 2.3.2, appendix paper III). The microsomal lipase activity was not inhibited by the antiserum against heparin-releasable liver lipase. Liver lipase activity present in heparin-containing liver perfusates and postheparin plasma was not inhibited by antiserum against microsomal lipase.

Enzymes, immunologically related to the microsomal lipase, were found in adipose tissue and small intestinal epithelium. These activities were not inhibited by antiserum against liver lipase (section 2.4, appendix paper II).

It was concluded that liver lipase is involved in plasma lipid metabolism. The rapid release by heparin perfusion and the disappearance of the activity during liver cell isolation point in the direction of a localization on membranes, facing the vascular compartment.

The different enzyme activities of liver lipase are probably caused by a single enzyme, rather than by different enzymes, because of a) similar elution patterns of hydrolytic activities measured with various substrates during purification procedures, b) similar inhibition patterns measured with various substrates when different antiserum concentrations were used, c) competitive inhibition of trioleoylglycerol hydrolysis by phosphatidylcholine (section 2.2.2, appendix paper I).

The microsomal lipase is probably involved in intracellular lipid metabolism, in particular removal of partial acylglycerols, formed during fat mobilization.

Postheparin plasma contains in addition to liver lipase also lipoprotein lipase from extrahepatic organs. Addition of C apolipoproteins had different effects on these two enzymes. Apolipoprotein C-II activated the trioleoylglycerol hydrolase and phospholipase A_1 activity (measured with phosphatidylcholine) of partially purified heart lipoprotein lipase, while liver lipase was inhibited by apolipoprotein C-II. Apolipoprotein C-III inhibited the apolipoprotein C-II-stimulated trioleoylglycerol hydrolase activity of heart lipoprotein lipase, while the phospholipase A_1 activity of the enzyme was stimulated by apolipoprotein C-II *cum* apolipoprotein C-III (chapter III, appendix paper V).

The liver lipase activity measured in postheparin plasma of diabetic and hypothyroid rats was found to be lowered, and could be restored by a diet rich in polyunsaturated fatty acids. It is suggested that an increase of membrane fluidity by the diet restores the receptor binding and through this the enzyme activity (chapter IV, appendix paper IV).

The high *in vitro* activity of liver lipase towards monoacylglycerols suggests a function of the enzyme in the removal of partial acylglycerols in blood, present in intermediate density lipoproteins and/or bound to albumin.

Dit proefschrift handelt over twee lipolytische enzymen, die een neutraal pH optimum hebben en die in de lever voorkomen. Eén ervan is door perfusie van de lever met heparine uit dit orgaan te verwijderen. Het is verantwoordelijk voor de neutrale triacylglycerol hydrolase activiteit van dit orgaan en wordt "leverlipase" genoemd. Het andere enzym is gelocaliseerd in het endoplasmatische reticulum van levercellen en is door perfusie van de lever met heparine niet uit dit orgaan te verwijderen. Het is verantwoordelijk voor het grootste gedeelte van de monoacylglycerol hydrolase activiteit van dit orgaan en wordt "microsomale lipase" genoemd.

Leverlipase hydrolyseert mono-, di- en triacylglycerol verbindingen, alsmede fosfatidylcholine, fosfatidylethanolamine en palmitoyl-coenzym A. Uit de bepaling van de V_{max}- en K_m-waarden voor deze esters met hogere vetzuren bleek dat monoacylglycerolesters de preferentiële substraten voor dit enzym zijn (hoofdstuk II en artikel I). Monoacylglycerolesters zijn niet alleen bij hydrolytische, maar ook bij transacyleringsreacties betrokken. De hydrolyse van monoacylglycerol levert naast vrije vetzuren glycerol op, terwijl de transacylering leidt tot de vorming van diacylglycerol en glycerol. De verhouding van beide activiteiten hangt af van de aard van het gebruikte monoacylglycerol substraat. Hydrolyse treedt voornamelijk op met monopalmitoylglycerol en transacylering met monooleoyl- en monolinoleoylglycerol. De verhouding tussen hydrolyse en transacylering wordt mede beïnvloed door de hoeveelheid albumine die in de substraatemulsies wordt gebruikt (hoofdstuk II en artikel III). De verschillende hydrolytische activiteiten van leverlipase met de eerdergenoemde vetzuuresters zijn waarschijnlijk te wijten aan beperkte substraatspecificiteit van leverlipase en niet aan het bestaan van verschillende specifieke leverlipasen. Argumenten hiervoor zijn: a) afwezigheid van scheiding van

verschillende activiteiten tijdens zuivering door kolomchromatografie; b) gelijke remming van de verschillende activiteiten bij gebruik van suboptimale concentraties van een antilichaam, dat tegen gezuiverd ratteleverlipase in het konijn werd opgewekt; c) remming van de trioleoylglycerol hydrolase activiteit door toegevoegd fosfatidylcholine en *vice versa* (hoofdstuk II en artikelen I en III).

Het leverlipase is waarschijnlijk gelocaliseerd aan die zijde van (niet-parenchymale-) levercellen welke grenst aan de vasculaire ruimte. Het enzym is derhalve waarschijnlijk betrokken bij het plasma lipoproteine metabolisme, in het bijzonder de monoacylglycerol hydrolase activiteit (hoofdstukken II en V en artikel III).

Het microsomale lipase verschilt van het leverlipase a) in immunologisch opzicht b) in substraatspecificiteit en c) in localisatie. Dit enzym hydrolyseert voornamelijk 1- en 2-monoacylglycerol met lange vetzuurketens. Transacylering met deze substraten wordt vrijwel niet waargenomen, terwijl de trioleoylglycerol hydrolase activiteit afwezig is (hoofdstuk II en artikel III). Een antilichaam, dat tegen het gezuiverde ratte microsomale leverenzym werd opgewekt, reageert ook met het microsomale lipase van dunne darmepitheelcellen en met de monoacylglycerol hydrolase activiteit van vetweefsel. Dit antiserum remt niet de activiteit van heparine-bevattende leverperfusaten of van plasma dat verkregen wordt na intraveneuze injectie met heparine ("postheparine plasma"). Het microsomale lipase speelt waarschijnlijk een rol bij het intracellulaire vetmetabolisme, in het bijzonder de hydrolyse van intracellulaire partiële glyceriden, die gedurende de mobilisatie van opgeslagen weefselvetten worden gevormd (hoofdstukken II en V en artikel II).

Postheparine plasma bevat behalve leverlipase ook lipoproteine lipase, dat afkomstig is van extrahepatische organen. Toevoeging van C-apolipoproteinen beïnvloedt leverlipase en lipoproteine lipase verschillend. Apolipo-

proteine C-II activeert de trioleoylglycerol hydrolase en fosfolipase A_1 activiteiten van gedeeltelijk gezuiverd lipoproteine lipase uit hart, terwijl de leverlipase activiteit juist geremd wordt door apolipoproteine C-II (hoofdstuk III en artikel V).

De leverlipase activiteit gemeten in postheparine plasma van ratten, wordt door experimentele diabetes en hypothyreoïdie verlaagd. De enzymactiviteit kon worden verhoogd door deze ratten voor te behandelen met een dieet dat rijk is aan meervoudig onverzadigde vetzuren. Er wordt gesuggereerd dat een toename van de vloeibaarheid van de leverplasmamembranen door dit dieet de binding en/of de activiteit van leverlipase verhoogt (hoofdstuk IV; artikel IV).

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APPENDIX PAPERS

DIFFERENTIAL RELEASE OF HEPATIC LIPOLYTIC ACTIVITIES

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DIFFERENTIAL RELEASE OF HEPATIC LIPOLYTIC ACTIVITIES

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SUMMARY. Perfusion of rat liver with a heparin-containing medium results in a release of upto 95% of the total neutral (or alkaline) triacylglycerol hydrolase activity (EC 3.1.1.3). Of the monoacylglycerol hydrolase (EC 3.1.1.23), phospholipase A_1 (EC 3.1.1.32) and palmitoyl-CoA hydrolase (EC 3.1.2.2) activities only 17 to 4% were found to be heparin-releasable. Antiserum raised against heparin-releasable liver lipase inhibits triacylglycerol hydrolase, monoacylglycerol hydrolase, palmitoyl-CoA hydrolase and phospholipase A_1 activities in perfusates of isolated livers completely. Non-heparin-releasable monoacylglycerol hydrolase, palmitoyl-CoA hydrolase and phospholipase A_1 activities are not inhibited by the antiserum and are therefore catalyzed by different enzyme(s). The difference in substrate specificities of releasable and non-releasable liver enzymes is discussed in relation to their possible function in lipoprotein metabolism.

Various lipolytic activities are released from liver by heparin[1-9], of which triacylglycerol hydrolase at least may also be measured with palmitoyl-CoA as substrate[4,10]. Both heparin-releasable triacylglycerol and palmitoyl-CoA hydrolases were purified together, both were inhibited by the same antibody[4,10], were varied to the same extent by diet[10], the hormonal state[11] or drugs[12]. Therefore both activities may be catalyzed by the same enzyme. The possible existence of other heparin-releasable lipolytic enzymes in liver, however, may not be excluded[13]. It is the purpose of the present paper to identify heparin-releasable besides non-releasable lipolytic activities in liver and to discriminate the different proteins with the antibody prepared against heparin-releasable lipase [4,10], as well as by difference in substrate specificity.

METHODS AND MATERIALS. Fed, male Wistar rats of about 250 g were used. Livers were perfused at 30°C through the portal vein with a Krebs-Ringer bicarbonate buffer, containing 5 mM glucose at a rate of 20 ml/min. Release of lipase from the liver was obtained by per-

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fusion with the same medium, containing 1.2% bovine serum albumin (w/v; Sigma fraction V), as well as 5 I.U. heparin/ml, for a period of 6 min (TABLE I) or 20 min (TABLE II). In a number of experiments (not shown) it was found that perfusion beyond 6 min released less than 6% more of the total heparin-releasable lipase activity. Phospholipase A_1 activity was measured with [1-^{14}C]-L-α-dipalmitoylphosphorylcholine (Applied Science Laboratories, Inc., State College, Pennsylvania, USA) as the substrate. Each test contained 0.5 mM phospholipid (1 mCi/μmole), 0.1 M Tris-HCl pH 8.5, 5 mM $CaCl_2$, 5 to 20 μl sample and water to a final volume of 55 μl. After incubation for 10 min at 30°C the reaction was stopped with 1 ml of a mixture of heptane, isopropanol and 10 N H_2SO_4 (400:400:1 by volume). Separation of phases was obtained with 200 μl water. After vigorous stirring, the mixture was centrifuged, the upper layer isolated and treated with about 5 mg silicic acid (100 Mesh, Mallinckrodt Chem. Works), to remove phospholipids. After centrifugation radioactivity was determined by liquid scintillation counting. Monoacylglycerol hydrolase activity was estimated with 2-[^{3}H]-glycerolmonooleate. Each test contained 2.5 mM glycerolmonooleate, 5 mg bovine serum albumin, 0.1 M Tris-HCl (pH 8.5), 20 to 200 μl sample and water to a final volume of 0.5 ml. After 10 min at 37°C the reaction was stopped with 0.5 ml 10% trichloroacetic acid. After centrifugation all glycerides are found in the pellet, while glycerol is present in the supernatant[14]. 0.2 ml of the supernatant was counted in "Instagel". Triacylglycerol hydrolase activity was estimated with tri[1-^{14}C]-oleoylglycerol, emulsified with gum acacia as the substrate, essentially as described by Ehnholm et al.[12] with the exception that 1 M NaCl was omitted from the incubation medium. The lipid substrate was purified by thin layer chromatography before use. Palmitoyl-CoA hydrolase activity was measured as described before[15].

RESULTS AND DISCUSSION

It has been shown earlier by us[11] that post-heparin serum may be separated into two clearcut activity peaks on a Sepharose-heparin column. The first peak, eluted with 0.7 M NaCl, contained the bulk of the palmitoyl-CoA hydrolase activity and about half of the triglyceridase activity, while the second peak, eluted with 1.2 M NaCl, contained very little palmitoyl-CoA hydrolase activity as well as the other half of total triglyceridase activity. It was further observed[11] that triglyceride emulsions were hydrolyzed completely to fatty acids and glycerol (molar ratio of 3:1), while the second peak yielded relatively much partial glycerides (molar ratio of fatty acid to glycerol 8:1). This indicated to us, since a separate diglyceridase has never been found, that the first peak should contain considerable monoglyceridase activity. An antibody against the purified protein, giving one band on polyacrylamide gel electrophoresis, was raised in rabbits[4,10,11] and was used in the present study.

TABLE I

RELEASABILITY OF DIFFERENT LIPOLYTIC ACTIVITIES FROM RAT LIVER BY HEPARIN

Rat livers were perfused with a heparin-containing medium as described under METHODS. During 6 min the perfusate was collected. Then the liver was homogenized in 0.25 M sucrose. In the perfusate and in the homogenates (10% w/v) lipolytic activities were estimated as described under METHODS. Mean liver weights were 10 g. Results are expressed as the activities found in a whole liver after perfusion or in the total perfusates. The activities are given as μmoles free fatty acids produced/min ± S.D. When monooleate and palmitoyl-CoA were the substrates, glycerol and coenzyme A formed were measured respectively. Number of perfusions was 4.

Substrates	Activity in homogenates	Activity in perfusates	% of total activity released
Trioleate	0.5±0.02	2.6±0.2	85
Palmitoyl-CoA	74.2±10	3.0±0.2	4
L-α-dipalmitoyl glycero-phosphocholine	1.15±0.22	0.17±0.03	15
Monooleate	340±46	57±9	17

It can be seen from TABLE I that almost all of the neutral triglyceridase is released by perfusion of isolated livers with heparin, while the other hydrolytic activities were only partially released. This suggests heterogeneity of enzyme pattern.

Similar enzyme activity ratios were found in the homogenates after heparin perfusion, whether homogenates were tested directly (TABLE I) or after delipidation by making an acetone powder prior to extraction (not shown). TABLE II shows that 95% of the triglyceridase activity was removed by heparin and the the antiserum against releasable hepatic lipase inhibits both releasable and non-releasable triglyceridase activities completely. This suggests that the variable small amount of triglyceridase that is not released with heparin in 6 min may be the same enzyme but not (readily) accessible to the high molecular heparin. The other activities of which the bulk is not released by heparin (TABLE II) are only sensitive to the antibody insofar the activities may be released by heparin. Therefore it is most likely that heparin-releasable liver lipase(s) may act on a variety of substrates and is (are) immunologically

TABLE II

INFLUENCE OF ANTISERUM AGAINST RELEASABLE LIVER LIPASE ON THE HEPARIN-RELEASABLE AND NON-RELEASABLE ACTIVITIES

Rat livers were perfused with a heparin-containing medium as described under METHODS. During 20 min the perfusates were collected. Then the livers were homogenized in cold 0.25 M sucrose and 5 volumes of acetone (-20°C) added. After 10 min stirring at 0°C, followed by centrifugation, the pellet was reextracted with cold acetone, again centrifuged and n-butanol (-20°C) added to the pellet instead of acetone. The butanol was removed by washing twice with acetone and finally 2 washings were carried out with cold diethylether, after which the preparation was dried. Extraction of the acetone powder was carried out with 0.05 M NH_4OH/NH_4Cl buffer of pH 8.5 for 60 min. The extracts were incubated overnight with antiserum. After centrifugation during 4 min at 15,000 g the remaining activity was estimated with different substrates as described under METHODS.

Substrate	Non releasable mU/total liver		Releasable mU/total liver	
	Control	Antiserum	Control	Antiserum
Triglyceride	315	0	5710	0
Monoglyceride	89754	84390	29693	0
Palmitoyl-CoA	16725	14967	2183	0
Phospholipid	42	42	203	0

different from the non-releasable lipases. Antibody titration curves[4,10,11] are presented in Fig. 1. The lower curves represent the activities of liver perfusates. They are all readily inhibited by the antibody. The upper curves are obtained with post-heparin serum of hepatectomized rats[11]. These extrahepatic activities were not inhibited by the antibody (Fig. 1).

In TABLE III apparent K_m and V_{max} values calculated from Lineweaver Burk plots of experiments not presented, are shown. Since Sepharose-heparin chromatography purifies both triglyceridase and monoglyceridase activities 240-fold (TABLE III), it is tempting to conclude that both activities are catalyzed by one enzyme, with a higher affinity and a higher velocity with the monoglyceride substrate. Hence the enzyme could be denominated as monoglyceridase, were it not that considerable activity with tri- (and di-) glyceride is also present. Hence the name glycerolester lipase may be more

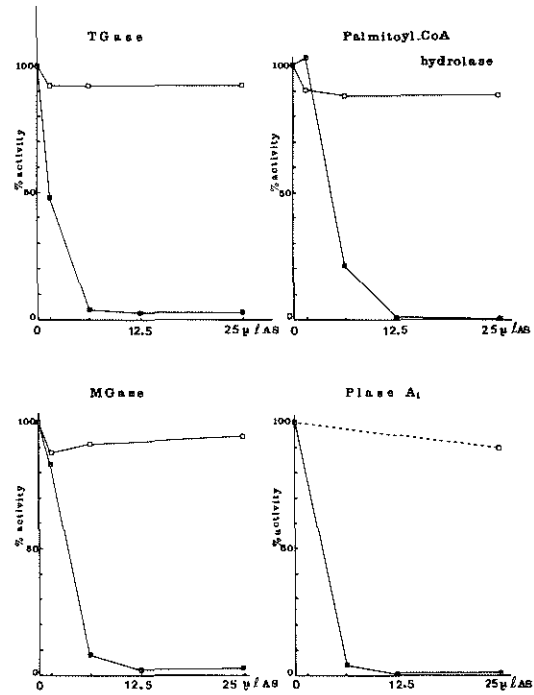

Fig. 1. Inhibition of triacylglycerol hydrolase (TGase), monoglyceridase (MGase), palmitoyl-CoA hydrolase and phospholipase A_1 (PLase A_1) activities in heparin containing perfusates of rat liver (closed symbols) or post-heparin serum of hepatectomized rats (open symbols) by antiserum (AS) raised against heparin-releasable rat liver lipase[4]. 500 µl liver perfusate or 50 µl post-heparin serum of hepatectomized rats was incubated overnight with various amounts of AS (as shown) and/or control rabbit serum to match the amount of protein used. Overnight incubation did not cause inactivation. After centrifugation, the remaining activities were determined as described under METHODS.

appropriate. Heparin-releasable phospholipase (A_1, since A_2 activity could not be detected - not shown) activity was purified almost 160-fold (TABLE III), so that the activity may partly be due to the same glycerolester lipase. A comparison of this enzyme with the heparin-releasable phospholipase A_1 activity of liver plasma membranes, described by Waite and Sisson[5], employing the antibody used in the present study, is being undertaken.

The high affinity and low velocity with phospholipid may be the reason why the heparin-releasable liver lipase activity is

TABLE III

K_m AND V_{max} VALUES OF HEPARIN-RELEASABLE LIVER LIPASE MEASURED WITH DIFFERENT SUBSTRATES

K_m and V_{max} values were calculated from linear Lineweaver Burk plots. The liver enzyme was purified on a heparin Sepharose column by elution with a NaCl gradient[11]. TG, MG and PL are the abbreviations for triglyceride, monoglyceride and phospholipid respectively.

Enzyme source	K_m (mM)			V_{max} (mU/mg protein)		
	TG	MG	PL	TG	MG	PL
Liver perfusate	6.2	0.5	0.1	10	83	0.7
Purified liver perfusate	6.2	0.6	0.2	2390	19833	116

higher when monooleate is sonicated with gum acacia (present study) than with phosphatidylcholine as emulsifyer[11].

In conclusion: The heparin-releasable neutral liver lipase activities measured (triglyceridase, monoglyceridase, palmitoyl-CoA hydrolase and phospholipase A_1) are probably catalyzed by one enzyme. It may attack both 1- and 2-monoglycerides[2] and may be involved in the breakdown of 2-monoglyceride-rich remnants of chylomicrons and very low density lipoproteins produced by (extra hepatic) lipoprotein lipase, which has exclusively α-lipolytic activity. Another activity may be triglyceride breakdown especially when blood levels are high, then having an overflow function in hypertriglyceridemia. The non-releasable enzyme(s) are at least partly located within the liver cells, beyond the plasma membrane barrier for heparin. The non-releasable neutral monoglyceridase activity has recently been purified from post mitochondrial supernatant 140-fold, using ammoniumsulphate fractionation and molecular sieving. It also has both 1- and 2-monoglyceridase activities and may therefore play a role when remnants of lipoprotein particles are further degraded within the liver cells[16] or if monoglycerides tend to accumulate otherwise. The properties of this enzyme, that has no activity towards di- and triglycerides, and therefore should

be denominated a monoglyceridase or glycerolester esterase, will be described elsewhere.

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ON THE ORGAN SPECIFICITY OF NEUTRAL GLYCEROLESTER HYDROLASE OF VARIOUS TISSUES; AN IMMUNOLOGICAL STUDY

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ON THE ORGAN SPECIFICITY OF NEUTRAL GLYCEROLESTER HYDROLASE OF VARIOUS TISSUES; AN IMMUNOLOGICAL STUDY

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SUMMARY. Rat liver microsomal glycerol monoester hydrolase (EC 3.1.1.23) has been purified 130 fold. The enzyme has a molecular weight of about 60,000. An antibody raised against this enzyme in rabbit did not inhibit heparin-releasable liver lipase, which hydrolyses long-chain 1- and 2-monoglycerides effectively. This confirms an earlier conclusion, based on results obtained with an antibody raised against the latter enzyme, that the non-releasable and heparin-releasable liver enzymes are different proteins. The antibody against the liver microsomal glycerol monoester hydrolase, however, inhibited also the monoglyceridase activities of acetone powder extracts of rat small intestinal epithelial microsomes and rat epididymal fat pads, suggesting structural similarities between the endoplasmic reticulum hydrolases of various tissues. These findings also apply to pig where an antibody against adipose tissue lipases inhibits the monoglyceridase activities of small intestinal and liver microsomal acetone powder extracts.

More than two decades ago (comp. ref. 1), it was found that in a number of species heparin injection causes the release of two distinct glycerolester hydrolase activities. These are probably identical with (extrahepatic*-) lipoprotein lipase (EC 3.1.1.3) and hepatic postheparin lipase. The two enzymes are immunoglocigally different[2,3], have different substrate specificity[4,5] and may be inhibited differentially[1,6,7]. Whereas lipoprotein lipase preferentially hydrolyzes long-chain triacylglycerol esters, when compared with monoacylglycerol esters, the liver enzyme preferentially hydrolyzes monoglycerides[4,5]. This is reflected by the molar ratio of the fatty acids and glycerol released during triglyceride hydrolysis which exceeds 8 when lipoprotein lipase is the catalyst (due to

*) During perfusion of isolated rat liver with heparin not only salt resistent liver lipase is released but also salt sensitive, and relative labile, lipoprotein lipase. Up to one fifth of the total trioleoylglycerol hydrolyzing activity of a freshly obtained perfusate is inhibited by 1 M NaCl (unpublished).

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accumulation of 2-monoglycerides) and is 3 with the liver enzyme. The function of the heparin-releasable liver enzyme therefore may not only be triglyceride hydrolysis, when plasma levels are high, but mainly monoglyceride hydrolysis for which the apparent K_m is much lower and the V_{max} much higher[5] than for triglyceride hydrolysis.

A glycerol monoester hydrolase non-releasable by heparin that has no activity with long-chain di- and triacylglycerol esters, in contrast to long-chain monoacylglycerol esters, has also been described. This activity is present in a number of tissues, such as small intestinal epithelium[9,10], liver[5,11,12] and adipose tissue[13,14]. It was the purpose of the presented work to obtain more information on this (these) non-releasable microsomal enzyme(s) and to eliminate the possibility that in liver this enzyme is a precursor of the heparin-releasable lipase. Therefore, our immunological studies[2,3,5] were extended.

METHODS

Purification of rat liver microsomal glycerol monoester hydrolase

In order to remove heparin-releasable liver lipase, rat liver was *in vitro* perfused[3] for 20 min with 5 I.U. heparin per ml Krebs-Ringer bicarbonate buffer prior to homogenization in 0.25 M cold sucrose, containing 10 mM Tris-HCl (pH 7.4) and 1 mM ethylenediamine tetraacetate. After removal of nuclei by centrifugation for 5 min at 800 x *g*, mitochondria were removed at 8700 x *g* (10 min) and microsomes sedimented at 100,000 x *g* (60 min). An acetone powder was prepared by homogenization of the microsomes with acetone at -10°C. This step was repeated and the acetone precipitate extracted with n-butanol at -10°C. Butanol was subsequently removed by acetone and diethylether extractions and the powder dried. Acetone powders were extracted by stirring with 50 mM ammonia buffer (pH 8.5) for 60 min at 0°C, followed by centrifugation and ammoniumsulphate fractionation. A fraction obtained between 40 and 70% saturation was then subjected to molecular sieving on a column (70x1.5 cm) with Sephacryl S-200 (Pharmacia), which was equilibrated and eluted with 50 mM Tris-HCl buffer of pH 8. The monooleoyl-hydrolyzing activity emerged in a sharp peak preceded by a small shoulder. The bulk of this material was found to have a molecular weight of about 60,000, since it was eluted just after bovine serum albumin, as used in a calibration study. Finally, the hydrolase was purified on DEAE Sephadex A-50 (Pharmacia) equilibrated with 50 mM Tris-HCl and eluted stepwise with 1.5 bed volumes of 50 mM, 100 mM and 200 mM Tris-HCl of pH 8. The enzyme activity emerged in the last fraction as a single peak. Polyacrylamide gel electrophoresis (5% gels in rods in 25 mM Tris - 190 mM glycine of pH 8.5) revealed two bands (Rf's 0.4 and 0.5). The main enzyme activity was measured in the first band. The purification is briefly summarized in Table I.

Preparation of antibody

0.3 mg protein of the purified rat liver microsomal enzyme

TABLE I

PURIFICATION OF NON-HEPARIN RELEASABLE GLYCEROL MONOESTER HYDROLASE

Details of the purification steps and enzyme activity measurements are mentioned under METHODS. Enzyme activity is expressed as μmol glycerol released from monooleoylglycerol per min (U).

Fraction	Tot.protein (mg)	Tot.activity (U)	Spec.activity (mU/mg prot.)	Purification factor
Whole homogenate	1429	156	109	1
Microsomes	241	135	559	5
40-70% (w/v) amm. sulphate cut of acetone powder extract	29	53	1821	17
Molecular sieving (Sephacryl S-200) (3 topfractions of 2 ml each)	3.8	18	4729	43
DEAE-Sephadex 0.2 M Tris-HCl eluate (3 topfractions of 2 ml each)	0.7	10	14187	130

was mixed with Freund's adjuvant (1:1) and injected into the foot pads of a rabbit. After 10 days a booster injection was given intramuscularly and this was repeated twice, after which blood was collected by heart puncture.

Enzyme activity measurements

In the monoglyceridase activity measurements reported monooleoyl 2-[^{3}H]-glycerol was used as the substrate[5]. In a few experiments with rat adipose tissue acetone powder extracts and extracts of microsomes from rat liver and rat small intestinal epithelial cells virtually identical activities were measured when monooleoylglycerol was replaced by 2-monopalmitoylglycerol (not shown). Each test contained 2.5 mM monoglyceride, 5 mg bovine serum albumin, 0.1 M Tris-HCl (pH 8.5) and 10-100 μl enzyme sample in a final volume of 0.5 ml. After 10 min at 37°C, the reaction was stopped with 0.5 ml 10% trichloroacetic acid which precipitates the unhydrolyzed long-chain monoglycerides[15]. 0.5 ml supernatant was counted in "Instagel". When lipoprotein lipase or liver postheparin

lipase activities were measured (see footnote and Fig. 2), this was done with tri[1-^{14}C]-oleoylglycerol, emulsified with gum acacia (5% w/v), as the substrate, essentially as described by Ehnholm et al.[16], in the presence of 0.1 or 1.0 M NaCl, to distinguish salt sensitive lipoprotein lipase from salt resistent liver lipase[6,7].

RESULTS AND DISCUSSION

The glycerol monoester hydrolase of rat small intestinal endoplasmic reticulum is not inhibited by the antibody prepared in our laboratory against heparin-releasable liver lipase[17]. This also holds for the non-releasable glycerol monoester hydrolase from rat liver[5]. Hence it is likely that non-releasable monoglyceridase activity is different from the heparin-releasable liver activity, which was the conclusion of our previous paper[5]. Yet it is possible that the protein moieties are related, but that immunological, substrate specificity and kinetic differences[5] are induced by glycosylation, phosphorylation or otherwise modification of the protein. Therefore, we decided to purify non-releasable rat liver microsomal glycerol monoester hydrolase, to raise an antibody against this protein and to extend our immunological studies. The enzyme purification is briefly depicted in Table I. Microsomes were prepared as the starting material, since neutral esterases are enriched in these particles. In fact, neutral esterase activity is a marker for microsomes[18]. An antibody raised against this non-releasable enzyme was found to react neither with heparin-releasable monooleoylglycerol hydrolase activity of a perfusate of a rat liver, obtained by *in vitro* perfusion of liver with 5 I.U. heparin per ml perfusion medium[3], nor with the activity contained in postheparin serum of an intact rat (Fig. 1). An antibody prepared against postheparin liver lipase[2] gave almost complete inhibition of postheparin liver perfusate and partial inhibition of total post-heparin rat plasma (Fig. 1, comp. also refs. 3, 5 and 8). The monooleoylglycerol hydrolase activities (or 2-monopalmitoylglycerol hydrolase activity, which gave similar results; not shown) of acetone powder extracts of microsomal preparations of rat liver, of isolated rat small intestinal villus cells[19] and of whole acetone powders of epididymal fat pads[13], were all found to be inhibited by antiliver microsomal glycerol monoester hydrolase (Fig. 1) and not by antiheparin releasable liver lipase (Fig. 1). Hence, it is likely that the monoacylglycerol hydrolase(s) that occur in the organs tested are structurally more related to each other than to heparin-

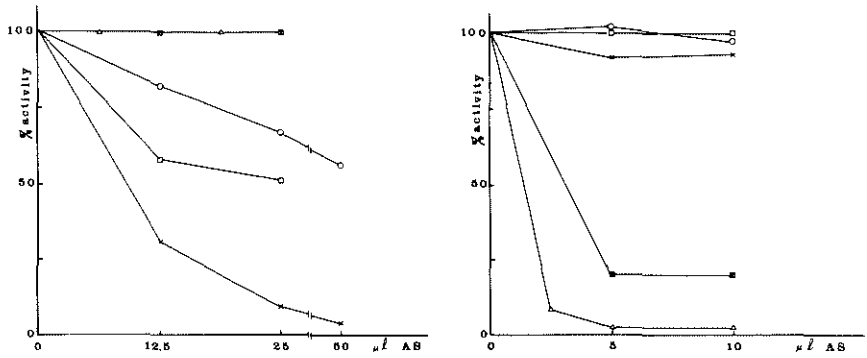

Fig. 1 Immunological relationship between non-releasable monooleoylglycerol hydrolases of different rat tissues. Left: titration with antiserum against purified non-releasable rat liver glycerol monoester hydrolase. Right: titration with antiserum against heparin-releasable liver lipase[2,3]. Equal activities (25 mU) of enzyme samples were preincubated for 3 h at 4°C with the indicated amounts of antiserum (AS). Control serum was used to match the different amounts of antiserum. After centrifugation for 4 min at 15,000 x g, assays were carried out as described under METHODS. Preincubation with control serum did not cause inactivation. Control activities, as indicated, were set 100%. Acetone powder extracts (comp. METHODS) of liver microsomes (1 U/ml) x-x; small intestinal villus cell microsomes[19] (1 U/ml) □-□ or epididymal fat pads[13] (1 U/ml) o-o. Liver perfusate (in vitro perfusion with 200 I.U. heparin in 40 ml Krebs-Ringer bicarbonate buffer, activity 230 mU/ml) Δ-Δ. Postheparin plasma (obtained after in vivo injection of 200 I.U./kg rat; activity 4000 mU/ml) ■-■.

releasable enzymes that are localized in heparin accessible spaces.

The present finding of similarity of glycerol monoester hydrolase(s) in various organs of rat also holds for pig. It can be seen from Fig. 2 that an antibody prepared by Nieuwenhuizen et al.[20] with activity against pig adipose tissue lipases A and B inhibits monooleoylglycerol hydrolase activities of acetone powder extracts of pig liver and small intestinal mucosa microsomes completely. It does not inhibit mono- or trioleoylglycerol hydrolysis in postheparin pig plasma in the absence or presence of 1 M NaCl (Fig. 2). An antibody prepared by Serrero et al. against a highly purified pig intestinal lipase[21] also showed cross reactivity with liver microsomal monoglyceridase activity (not shown).

It is clear from the figures presented that the antibody

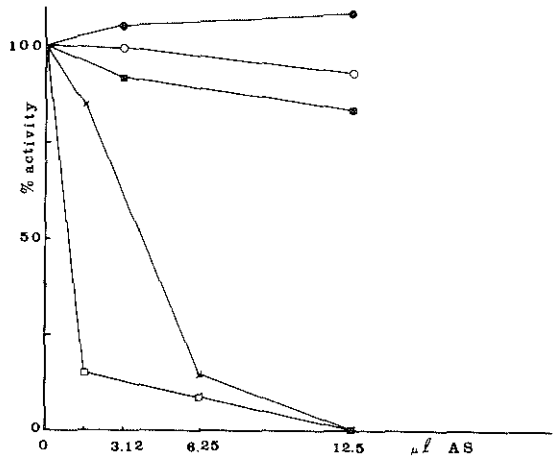

Fig. 2 Immunological relationship between glycerol monoester hydrolases of various pig tissues. The antibody used was prepared by Nieuwenhuizen et al.[20] against purified pig adipose tissue lipase. Activities were measured with monooleoylglycerol substrate (comp. METHODS and Fig. 1), except when lipoprotein lipase was tested. In that case the substrate was trioleoylglycerol (METHODS); 0.1 M NaCl (o-o) or 1 M NaCl (●-●) were present as indicated. Equal activities (10 mU) of enzyme samples were preincubated with the indicated amounts of antiserum (AS) and centrifuged (Fig. 1).

Monoglyceridase activities of liver microsomal acetone powder extract x-x, intestinal microsomal acetone powder extract from a small intestinal scraping (after repeated rinsing of the mucosal surface with cold saline) □-□ and postheparin plasma (collected 6 min after the injection of 100 I.U. heparin per kg pig) ●-■ are shown. 100% of the monoglyceridase activity corresponded to 400 mU/ml enzyme sample and 100% triglyceridase activity to 250 mU/ml enzyme sample.

effectivity varies between the various organs. Therefore it is concluded that the enzymatic activities in the tissue studied are catalyzed by related (iso)enzymes, that differ immunologically from heparin-releasable enzymes completely. There is species specificity since at least the antibody against the pig adipose tissue glycerol monoester hydrolase[20] was not found to inhibit the rat liver enzyme (not shown). The similar lipase-like immunoreactivity of pig adipocytes, myocardium and aorta, as found histochemically by Nieuwenhuizen et al.[20], also suggests the presence of structually related lipases (which according to the experiments presented in Fig. 2

hydrolyze long-chain monoacylglycerol esters) in various organs. It remains to be determined whether the similar non-releasable monoglyceridase activities of the various organs described are due to related isoenzymes or to one enzyme with different availability of antigenic determinants, when isolated from different tissues. Louvard et al.[22] have shown that different amounts of antibodies may be required to precipitate membrane-bound and free aminopeptidase, which is partly due to different availability of antigenic determinants.

Generally, a weak long-chain monoacylglycerol ester hydrolase activity is also found in preheparin serum. In pig, the serum activity is almost negligible and in rat the total activity amounts to about one U, while the long-chain monoglyceridase activity of rat liver amounts to 400 U/10 g liver, 15% of which is heparin-releasable[5]. The heparin-releasable activity may be more favourably localized than the non-releasable activity for the breakdown of plasma 2-monoglycerides that arise during lipoprotein lipase action upon very low density lipoproteins and chylomicrons. The non-releasable enzyme in liver endoplasmic reticulum may supplement plasma lipoprotein breakdown, while the similar enzymes of small intestinal epithelium and adipose tissue mainly have different functions: hydrolyzing monoglycerides from exogenous and endogenous triglyceride sources, which arise during pancreatic and hormone-sensitive lipase actions, respectively. The immunological similarity of these enzymes of liver, small intestine and adipose tissue on the one hand and the immunological difference of these enzymes from the heparin-releasable liver lipase strengthen the opinion[5] that the non-releasable liver enzyme is not a precursor of the heparin-releasable one.

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PAPER III

STUDY OF NEUTRAL LONG-CHAIN ACYLGLYCEROL HYDROLASE AND MONOACYLGLYCEROL TRANSFERASE ACTIVITIES OF HEPARIN-RELEASABLE AND NON-RELEASABLE LIPASES FROM RAT LIVER

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STUDY OF NEUTRAL LONG-CHAIN ACYLGLYCEROL HYDROLASE AND MONOACYLGLYCEROL TRANSFERASE ACTIVITIES OF HEPARIN-RELEASABLE AND NON-RELEASABLE LIPASES FROM RAT LIVER

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SUMMARY

1. The substrate specificities of heparin-releasable and non-releasable acylglycerol hydrolases from rat liver were compared.
2. Heparin-releasable liver lipase hydrolyzes the 2-acylgroup of dipalmitoylglycerol 2.7 times faster than the 1-acylgroup. For non-releasable microsomal lipase the rate of hydrolysis of the 2-acylgroup from dipalmitoylglycerol is 6.3 times higher than 1-acylgroup removal.
3. Both the heparin-releasable lipase and the non-releasable microsomal lipase are able to hydrolyze monoacylglycerols. The heparin-releasable lipase, in contrast to the non-releasable microsomal lipase, catalyzes transacylation, forming diacylglycerols and glycerol from monoacylglycerols. The ratio of the rates of hydrolysis to transacylation depends on the substrates used. Under V_{max} conditions this ratio for 1- and 2-monopalmitoylglycerol is 2.6, for monooleoylglycerol 0.8 and for monolinoleoylglycerol 0.6.
4. At lower substrate concentrations the ratio of hydrolysis to transacylation increases independent of the monoacylglycerol used. The hydrolytic activity of heparin-releasable lipase relative to the transacylation activity increases at higher concentrations of albumin, probably because albumin binds monoacylglycerols.

5. The heparin-releasable lipase and microsomal lipase, of which earlier studies revealed immunological differences, are probably located in different cell types. The activity of the heparin-releasable enzyme is lost during isolation of parenchymal liver cells using collagenase. The non-releasable activity is hardly affected.

INTRODUCTION

The liver contains two neutral long-chain acylglycerol hydrolases. One can be released by *in vitro* heparin-perfusion, suggesting an extracellular localization [1]. The other one is not heparin-releasable and has a microsomal localization [2]. The enzymes are immunologically different. Antisera have been prepared against both enzymes. The microsomal enzyme is probably present in a number of extrahepatic organs as well [2,3]. The heparin-releasable liver lipase activity (LLA) hydrolyzes triacylglycerol, 1- and 2-monoacylglycerol [1,4], diacylglycerol [4], the *sn*-1 position of phosphatidylcholine or phosphatidylethanolamine [1,5]. The extracellular localization of the enzyme suggests a role of the enzyme in extracellular lipid metabolism.

Waite *et al.* [6,7] described an enzyme from liver plasma membranes with properties which varied to some extent with the ones described by us for LLA [1,2] . Their enzyme preparation had both monoacylglycerol hydrolase and transferase activities but no triacylglycerol hydrolase activity, unless treatment with detergent had been taken place.

An absolute preferential hydrolysis of the *sn*-1 acyl-group in phosphatidylcholine or -ethanolamine and absence of specificity for the position of the acylgroup during monoacylglycerol hydrolysis was found. Therefore, the positional specificity of the hydrolysis of 1,2 diacylglycerol was studied. With this substrate the acylgroup at the *sn*-2 position was found to be hydrolyzed faster than the acylgroup at the *sn*-1 position.

LLA can also form diacylglycerol from monoacylglycerol. Therefore, monoacylglycerols are involved in two reactions: hydrolysis and transacylation. Practically no transacylation was found when the heparin-releasable lipase was substituted by the non-releasable (microsomal) lipase. The description of these findings on the two liver lipases is the purpose of the present paper, as well as the discussion of their different functions in relation to their different localization.

MATERIALS AND METHODS

sn-1-Palmitoyl 2-[9,10-^{3}H]palmitoyl 3-phosphorylcholine and *sn*-1-[1-^{14}C]palmitoyl 2-palmitoyl 3-phosphorylcholine were obtained from Applied Science Laboratories Inc., State College, Pennsylvania, U.S.A.). Heparin was obtained from Organon (Oss, The Netherlands) and phospholipase *C* from Bacillus cereus was from Boehringer (Mannheim, Germany). 1,2 Dipalmitoylglycerol was a product of Serdary (London, Ontario, Canada) and *sn*-1,2-dipalmitoyl 3-phosphorylcholine a product of Fluka (Brucks, Switzerland). All chemicals used were of analytical grade.

Substrates. *sn*-1-Palmitoyl 2-[9,10-^{3}H]palmitoylglycerol and *sn*-1-[1-^{14}C]palmitoyl 2-palmitoylglycerol were synthesized according to Hanahan and Vercamer [8] from *sn*-1-palmitoyl 2-[9,10-^{3}H]palmitoyl 3-phosphorylcholine and *sn*-1-[1-^{14}C]palmitoyl 2-palmitoyl 3-phosphorylcholine respectively by the action of phospholipase *C* (EC 3.1.4.3). After lipid extraction according to Bligh and Dyer [9], the products were separated on silicagel G thin layer chromatography plates (Merck, Darmstadt, Germany) and developed in hexane/diethylether/acetic acid (30/70/1 by volume). Labeled 1,2 dipalmitoylglycerol (R_f = 0.68) was eluted with diethylether and stored in hexane at -20^{o}C to avoid transesterification to 1,3 dipalmitoylglycerol. This product contained 94% 1,2 dipalmitoylglycerol and 6% 1,3 dipalmitoylglycerol and free

fatty acid. Labeled monopalmitoylglycerol and monooleoylglycerol were obtained as described by De Jong and Hülsmann [10]. Monolinoleoyl [2-^{3}H]glycerol was a gift of Unilever Research Laboratories (Vlaardingen, The Netherlands) and consisted of 87% 1-monolinoleoylglycerol and 13% 2-monolinoleoylglycerol. Monooleoyl [2-^{3}H]glycerol consisted of 88% 1-monooleoylglycerol and 12% 2-monooleoylglycerol.

Enzyme assays. For the estimation of the diacylglycerol hydrolase activity fatty acid release was measured in a medium (0.25 ml) containing 3.2 mM substrate (0.1 ml diacylglycerol emulsified in 2.5% gum arabic pH 7), 5 mg bovine serum albumin (defatted as described by Chen [11]), 40 mM Tris pH 8.5. After 10-20 min at 30°C the reaction was terminated by adding 3.45 ml chloroform/methanol/heptane (1.45/1.25/1.0 by volume). Fatty acids were extracted in 1.05 ml borate buffer pH 10.5 as described by Belfrage and Vaughan [12]. After centrifugation 1 ml of the upperphase was counted in Lumagel (Lumac, Basel, Switzerland). Monoacylglycerol hydrolase and transferase activities were measured with labeled monoacylglycerols, emulsified by sonification (5 x 1 min at 21 kHz at 0°C) in defatted bovine serum albumin in a molar ratio varying from 1.57 to 20 (in 0.125 mM Tris-HCl pH 8.0). After 5-10 min at 30°C the reaction was terminated by the addition of chloroform and methanol according to Bligh and Dyer [9]. [2-^{3}H]Glycerol was determined in 1 ml of the upperphase. 0.5 ml of the underphase was separated on silicagel G thin layer chromatography plates, developed in heptane/diethylether/acetic acid (60/40/1 by volume). The different lipids were stained with iodine vapor. They were scraped from the plates and after sonification in 5 ml water counted in 10 ml Lumagel. R_f values for 1,2 diacylglycerol and 1,3 diacylglycerol were 0.20 and 0.27, respectively. Radioactivity was counted in a Packard 2650 liquid scintillation counter. One mU corresponds with one nmol formed product/min.

Enzyme preparation. Fed male Wistar rats of about 250 g

were used. Livers were *in vitro* perfused through the portal vein with a Krebs-Ringer bicarbonate buffer containing 5 mM glucose at a rate of 20 ml/min at 30°C for 5 min to remove blood. Then liver lipase was obtained by perfusion with a similar medium containing in addition 1.2% (w/v) bovine serum albumin (Sigma, fraction V) and 5 I.U. heparin/ml for a period of 2 min. Liver perfusate was collected in ice and divided in 0.5 ml portions and stored at -20°C. Just prior to enzyme incubations the samples were thawed. Microsomal monoacylglycerol hydrolase was purified as described before [2].

Antisera. Antisera against heparin-releasable liver lipase and microsomal hydrolase were prepared as described by Jansen *et al.* [13] and Oerlemans *et al.* [2], respectively. The Ig G fractions of the antisera were obtained by precipitation with 50% saturated ammoniumsulphate at 0°C. The precipitate was dissolved and dialysed against 0.9% NaCl (w/v).

Cell isolation. Parenchymal liver cells were isolated [14] and acetone powders were prepared as described [15]. Afterwards the dried powder was suspended in acetone (-20°C). After centrifugation for 10 min at 12,000 g the pellet was treated with n-butanol (-20°C). This was removed on a Büchner funnel and the powder was treated subsequently with acetone and diethylether.

RESULTS

Hydrolysis of diacylglycerol by LLA, *in vitro* released from liver by heparin perfusion

For the *in vitro* hydrolysis of dipalmitoylglycerol by LLA we used gum arabic as emulsifier with a fixed amount of bovine serum albumin to stabilize the emulsion and to act as fatty acid acceptor as well. Liver lipase hydrolyzes both the fatty acids on the *sn*-1 and *sn*-2 position of 1,2 dipalmitoylglycerol. The hydrolysis was found to be linear

during 15 min. The rate of hydrolysis of the *sn*-2 position compared to that of the *sn*-1 position, measured under optimal conditions with respect to incubation time and protein concentration, is 2.68±0.07 (± SEM; n=6) times higher (Fig.1).

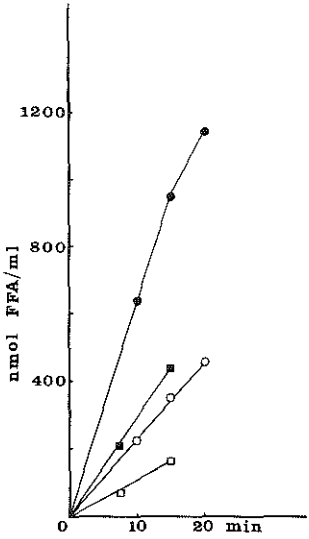

Fig. 1. Influence of antiserum against heparin-releasable liver lipase on the hydrolysis of dipalmitoylglycerol by liver perfusate. The substrate was labeled in the 1-position with [^{14}C]palmitic acid or in the 2-position with [^{3}H]palmitic acid. The incubation medium (0.25 ml) contained 3.2 mM diacylglycerol (0.1 ml of 8 mM diacylglycerol in 2.5% gum arabic pH 7), 5 mg bovine serum albumin and 0.01 µmol Tris pH 8.5. The liver perfusate was incubated during 3 h at 0°C with the Ig G fraction of antiserum (□,■) or with 0.9% NaCl (o,●). The activity was measured after removal of precipitate by centrifugation during 4 min at 12,000 g. The reaction was terminated and free fatty acids extracted as described under Materials and Methods.
Closed symbols (●,■) release of [^{3}H]palmitic acid, open symbols (o,□) release of [^{14}C]palmitic acid.

Different reaction velocities might be caused by different enzymes or by different kinetics for the different substrates for one enzyme or a combination of these. Previously [1] we have discussed the broad substrate specificity of this heparin-releasable liver lipase. Although the apparent K_m's

for the various substrates were different, the addition of an antiserum against the purified enzyme resulted in similar titration curves. A concentration of antiserum, which inhibited 55%, gave similar partial inhibition for the various substrates tested, including dipalmitoylglycerol, whether 1- or 2-acyl-release was measured (Fig. 1; compare ref. 1).

Hydrolase and transacylase activities of LLA. Dependency on unsaturation of fatty acid

The monoacylglycerol acyltransferase, described by Waite *et al.* [6,16], which is located at the plasma liver membranes has no activity against triacylglycerol unless purified and tested in the presence of Triton X-100 [7]. This enzyme hydrolyzes mono- and diacylglycerols, phosphatidylethanolamine as well as phosphatidylcholine. Waite *et al.* observed considerable transacylation activity with different substrates. Heparin-containing liver perfusates were also able to catalyze transacylation reactions when monoacylglycerol was used as the substrate (Fig. 2). Therefore, this substrate may be involved in two reactions: hydrolysis to yield glycerol and free fatty acid and transacylation to form diacylglycerol and glycerol. In our studies the substrate used was labeled in the glycerol moiety, so that for the determination of the hydrolytic activity the rate of glycerol formed has to be corrected for glycerol formed by transacylation. Under V_{max} conditions the ratio of hydrolysis to transacylation is dependent on the type of monoacylglycerol used. For 1- and 2-monopalmitoylglycerol this ratio is 2.6, for monooleoylglycerol 0.8 and for monolinoleoylglycerol 0.6 (TABLE I). The V_{max} values were estimated at a constant molar ratio of monoacylglycerol to albumin of 8.0. It can be seen from TABLE I that V_{max} values for the hydrolysis of the different substrates used increased when the fatty acid moiety was unsaturated. 1- and 2-Monopalmitoylglycerol was hydrolyzed more slowly

TABLE I

HYDROLYSIS AND TRANSACYLATION OF LIVER PERFUSATE MEASURED WITH DIFFERENT MONOACYLGLYCEROLS

The monoacylglycerols were emulsified in bovine serum albumin in a molar ratio of monoacylglycerol to albumin of 8. The substrate concentration was 2.5 mM. Enzyme activities were determined as described under Materials and Methods. The hydrolase activity is corrected for the glycerol production during the transacylation reaction.

Substrate	V_{max} (mU/ml)				Average ratio hydrolysis / transacylation
	hydrolysis		transacylation		
	expt.1	expt.2	expt.1	expt.2	
1-monopalmitoylglycerol	58	72	22	30	2.5
2-monopalmitoylglycerol	62	72	21	30	2.7
monooleoylglycerol*	170	167	225	211	0.8
monolinoleoylglycerol*	145	150	260	228	0.6

* mainly 1-monoacyl derivative (compare Materials and Methods).

than monooleoylglycerol or monolinoleoylglycerol. This was even more pronounced for the transacylation reactions studied.

When the amount of albumin in the incubation medium is kept constant at increasing substrate concentrations, a sigmoid curve instead of a hyperbolic saturation curve is obtained (Fig. 2). When at a constant substrate concentration (1 mM) the monooleoylglycerol to albumin ratio was changed from 20 to 1.57, the rates of hydrolysis and transacylation decreased 85% and 99%, respectively. A typical experiment is shown in TABLE II. When monolinoleoylglycerol was used as substrate to measure the hydrolysis and transacylation rates of liver lipase, a predominant hydrolysis was found at a low substrate concentration (0.3 mM). At higher substrate concentrations transacylation exceeded hydrolysis. In Fig. 3 it is shown that the ratio of

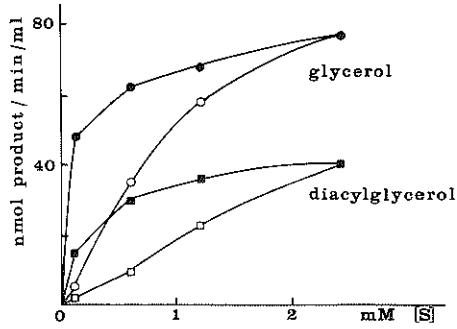

Fig. 2. Saturation curves for 1-monopalmitoylglycerol hydrolysis (o-o, ●-●) and transacylation (□-□, ■-■). Liver perfusate was incubated with different amounts of substrate complexed with a constant amount of albumin (0.15 mM, open symbols) or with a variable amount of albumin, so that the molar ratio of monoacylglycerol to bovine serum albumin was constant (16.2, closed symbols). The incubation time was 10 min. Incubation medium as described under Materials and Methods.

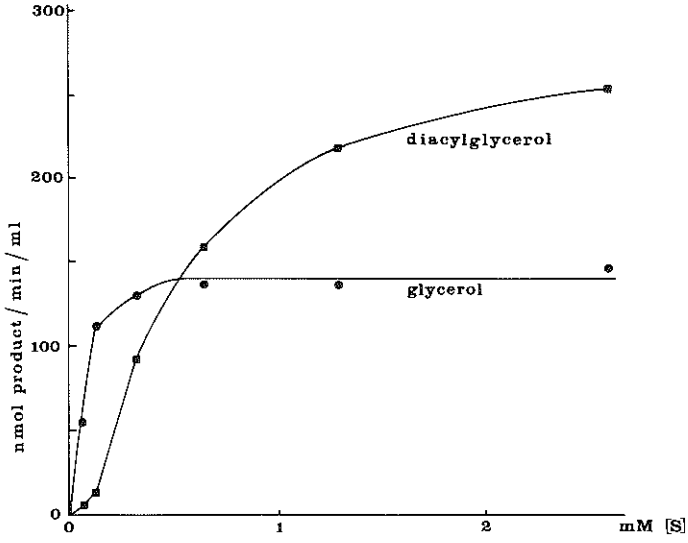

Fig. 3. Hydrolysis (●-●) and transacylation (■-■) of monolinoleoylglycerol by liver perfusate. The molar ratio monolinoleoylglycerol to albumin is 8. The incubations during 10 min were performed as described under Materials and Methods. Correction for glycerol production by transacylation is made for hydrolysis.

TABLE II

INFLUENCE OF ALBUMIN ON THE HYDROLASE AND TRANSACYLASE ACTIVITIES OF LIVER PERFUSATE

Liver perfusate was obtained from fed male Wistar rats by 2 min perfusion with Krebs-Ringer bicarbonate-albumin (1.2% w/v) and heparin (5 I.U./ml)-containing medium as described under Materials and Methods. Hydrolase and transacylase activities were detected with monooleoyl[2-^{3}H]glycerol, emulsified in albumin, as substrate. The substrate concentration was 1 mM. The reactions were performed as described under Materials and Methods. Corrections were made for the glycerol production due to transacylation.

Molar ratio monoacylglycerol / albumin	[albumin] mM	Activity of (mU/ml) hydrolysis	transacylation	Ratio hydrolysis / transacylation
20.0	0.05	112	172	0.65
16.6	0.063	99	161	0.61
10.0	0.10	81	137	0.59
5.0	0.20	67	70	0.96
2.0	0.50	19	12	1.58
1.57	0.638	18	1	18.00

hydrolysis to transacylation changed from 9.4 to 0.6. This is quite different in intestinal epithelium, where acylation (with acyl-CoA as the acyldonor instead of a glycerolester) is favoured relative to deacylation at low free monoacylglycerol concentrations [10].

Microsomal lipase; substrate specificity

The monoacylglycerol hydrolase activity of microsomal origin is immunologically different from LLA [2]. The former enzyme does not hydrolyze long-chain triacylglycerol at all. Dipalmitoylglycerol is hydrolyzed by this enzyme. The acylgroup on the *sn*-2 position is 6.3 times faster hydrolyzed than the one on the *sn*-1 position (data not shown).

This microsomal enzyme hydrolyzes monoacylglycerols more rapidly than diacylglycerols. Transacylation between monoacylglycerols, whether tested with monooleoylglycerol or monolinoleoylglycerol, under conditions mentioned for LLA in TABLE I, was almost undetectable (TABLE III). The differences in substrate specificity of LLA and the microsomal enzyme again point to different enzymes.

TABLE III

HYDROLYSIS AND TRANSACYLATION BY MICROSOMAL LIPASE MEASURED WITH DIFFERENT MONOACYLGLYCEROLS

The monoacylglycerols were emulsified in albumin in a molar ratio of 8. The substrate concentration was 2.5 mM. Enzyme activities were determined as described under Materials and Methods.

Substrate	V_{max} (mU/mg protein)				Ratio hydrolysis / transacylation	
	hydrolysis		transacylation			
	expt.1	expt.2	expt.1	expt.2	expt.1	expt.2
Monooleoylglycerol	393	353	4	4	104	82
Monolinoleoylglycerol	1338	1252	8	5	164	265

Localization of heparin-releasable and non-releasable lipases

After the separation of parenchymal and non-parenchymal liver cells by collagenase perfusion [14] triacylglycerol hydrolase activity could not be detected in either preparation [17-19]. This is due to the use of collagenase [18]. However, acetone powders of isolated parenchymal cells showed considerable monoacylglycerol hydrolase activities. Therefore monoacylglycerol hydrolase activity is not lost during the isolation of parenchymal cells, probably because the micro-

somal enzyme is shielded from added proteolytic enzymes by the plasma membrane or resistant against these enzymes all together. It was found that in acetone powders of the isolated parenchymal cells, treatment with an antibody against purified microsomal liver lipase [2] indeed resulted in strong inhibition of monopalmitoylglycerol hydrolysis. This sensitive part was denominated microsomal lipase (TABLE IV), while the decrease of enzyme activities by treatment with the antibody against purified heparin-releasable liver lipase [13] was denominated LLA. The data of TABLE IV indicate that the bulk of the non-releasable microsomal liver enzyme is localized in the parenchymal cells, while the other

TABLE IV

INFLUENCE OF ANTISERUM AGAINST HEPARIN-RELEASABLE AND MICROSOMAL LIPASE ON THE 1-MONOPALMITOYLGLYCEROL HYDROLASE ACTIVITIES OF ACETONE POWDERS OF LIVER HOMOGENATE AND ISOLATED PARENCHYMAL LIVER CELLS

Liver cells and acetone powders were prepared as described under Materials and Methods. Acetone powders were dissolved in 5 mM Veronal buffer pH 7.4 (5 mg powder/ml buffer). Undissolved particles were removed by centrifugation (10 min at 12,000 g). Incubation of antisera were performed during 3 h at $0^{o}C$. Enzyme activities were measured after centrifugation for 15 min at 12,000 g. The molar ratio of monopalmitoylglycerol to albumin was 7.0 and the substrate concentration 1.64 mM. The enzyme activities were determined as described in Materials and Methods. The reaction was, however, terminated by adding an equal volume 10% TCA, as described by Schotz and Garfinkel [20]. The LLA and microsomal lipase activity was determined by subtraction of the activity after incubation with antiserum against microsomal lipase and LLA respectively from the activity after incubation without antiserum (= total activity).

	Activity in whole liver (nmoles glycerol/min/ml extract)			Activity in parenchymal cells (nmoles glycerol/min/ml extract)		
	LLA	Microsomal lipase	Total	LLA	Microsomal lipase	Total
Expt. 1	13	144	197	2	142	169
Expt. 2	10	91	141	0	87	103

enzyme resides for the largest part outside the parenchymal cells, probably at the outer surface of the non-parenchymal cells [17,19] which constitute about 10% of the total liver protein [14]. In untreated liver a saturating amount of the antibody against LLA was found earlier [1] to inhibit only a small part of the total monoacylglycerol hydrolase activity which is confirmed here (TABLE IV).

DISCUSSION

Although in earlier studies [1] the main activity of the heparin-releasable liver lipase (LLA) has been attributed to monoacylglycerol hydrolase activity, it has become evident from the present data that this only holds for saturated monoacylglycerols or long-chain unsaturated monoacylglycerol-esters, when the free concentrations are low. At higher availability of unsaturated monoacylglycerols, the major function of LLA may be transacylation. This preferential preservation of unsaturated fatty acids in ester bonds relative to hydrolysis may have physiological significance. Saturated fatty acids on the contrary are predominantly liberated by LLA. Transacylation does not contribute to free fatty acid formation in the liver, but still may decrease the concentrations of monoacylglycerol in the surface film of lipoproteins, which may promote removal from the surface of other molecules, such as cholesterol and phospholipids. The ultimate fate of the diacylglycerol formed is not clear. We have not found significant triacylglycerol formation from monoacylglycerol by LLA.

The arguments that LLA has a broad substrate specificity [1,4,21] rather than that the enzyme preparation used is a mixture of different enzymes may be presented as follows. When using the antibody against LLA similar titration curves were obtained when hydrolysis was tested with different substrates [1]. Moreover, when incomplete inhibition was obtained by the addition of suboptimal antiserum concentra-

tions, similar degrees of inhibition were observed whether triacylglycerol, diacylglycerol, monoacylglycerol or phosphatidylcholine were used as substrates for the enzyme released from isolated livers by heparin perfusion.

Several investigators have tried to separate the different activities. Ehnholm *et al.* [21] purified an enzyme from human postheparin plasma which contained phospholipase A_1 and triacylglycerol hydrolase activities and ascribed it to one enzyme. Waite *et al.* [6,7,16] isolated an enzyme from liver plasma membranes, which had phospholipase A_1, monoacylglycerol hydrolase and acyltransferase activities but no triacylglycerol hydrolase activity, unless Triton X-100 was added in the assay medium. Earlier [1,4] we showed that the heparin-releasable liver lipase has palmitoyl-CoA hydrolase, triacylglycerol hydrolase, monoacylglycerol hydrolase and phospholipase A_1 activities. The present study adds diacylglycerol hydrolase and monoacylglycerol acyltransferase activities. Therefore, we think that LLA is an enzyme with broad substrate specificity, rather than a mixture of different active enzymes. If LLA is not removed prior to the isolation of liver microsomes [22] or plasma membranes [23] a mixture of LLA and the microsomal enzyme may be obtained.

The hydrolysis of various substrates by LLA makes it difficult to assign a distinct function to LLA, unless the exact localization of the enzyme is known.

Recent work in our laboratory [17,19] shows that LLA is produced in the parenchymal cells and from there transported to non-parenchymal elements, probably vascular endothelium, where on the outside of the cells (the enzyme is heparin-releasable) the function is exerted. LLA, in contrast to the microsomal enzyme, exerts its function on plasma components. The highest *in vitro* activity is obtained with monoacylglycerols, which are products of lipoprotein lipase action on chylomicrons and very low density lipoproteins. Therefore, LLA will probably hydrolyze monoacylglycerol, present in the surface of lipoproteins or bound to plasma components of

higher density, such as albumin. Recently El-Maghrabi *et al.* showed extracellular monoacylglycerol hydrolysis with isolated parenchymal liver cells [24]. The isolated parenchymal cells, as isolated by us, contained no more LLA. Therefore, it may be that microsomal lipase can exert its activity outside the cells or that in their cell preparations LLA is present. The microsomal enzyme, of which the bulk resides in the parenchymal cells, is present in microsomes of a variety of other cell types as well [2,3,10] and is probably involved in the removal of excess monoacylglycerols, if acylation to higher acylglycerols is relatively slow compared to the rate of monoacylglycerol formation. The finding that the bulk of that enzyme is present in the parenchymal cells, suggests a different localization and another function.

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PAPER IV

EFFECT OF HYPOTHYROIDISM, DIABETES AND POLYUNSATURATED FATTY ACIDS ON HEPARIN-RELEASABLE RAT LIVER LIPASE

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EFFECT OF HYPOTHYROIDISM, DIABETES AND POLYUNSATURATED FATTY ACIDS ON HEPARIN-RELEASABLE RAT LIVER LIPASE

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SUMMARY. In the rat both hypothyroidism and diabetes decrease heparin-releasable liver lipase activity. This defect may be reversed by feeding a diet rich in polyunsaturated fatty acids. It is suggested that a diet-induced increase of membrane fluidity restores liver lipase activity, which contributes to the hypolipidemic effect of polyunsaturated fatty acids.

A decrease in heparin-releasable liver lipase activity may be expected[1,2] to occur in a number of atherogenic conditions, in which the plasma concentrations of intermediate density lipoprotein particles (IDL) have been shown to be increased. Indeed, in diabetes and in hypothyroidism not only the IDL levels are increased[3-5], but liver lipase activities have been shown to be low. We have described the latter for streptozotocin diabetic rats[1], which was recently confirmed by Elkeles and Hambley[6], while Krauss et al.[7] described a 40% decrease in 4 hypothyroid women. The reason for the decreased activity is not known, although a decreased membrane fluidity[8] may be involved to some extent since diets rich in polyunsaturated fatty acids have repeatedly been shown to have a hypolipidemic effect. In the present paper it will be shown that also in the hypothyroid rat heparin-releasable liver lipase activity is low and that both in diabetes and hypothyroidism the normal levels of activity may be restored by a diet rich in polyunsaturated fatty acids.

METHODS AND MATERIALS. Fed, male Wistar rats of 220-270 g were used. Hypothyroid rats were obtained by including methimazole (1-methyl-2-mercaptoimidazole of Nogepha, Alkmaar, The Netherlands) in the drinking water (0.5 mg/ml). After 2 weeks the thyroxin concentration in the plasma had dropped from 36 ± 1.6 to 4.7 ± 0.7 nmol/l (n=8), while the total plasma cholesterol level had increased from 55 + 6 to 74 ± 7 mg/100 ml. Diabetes was induced by injecting 50 mg per kg body weight of streptozo-

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tocin (Calbiochem, dissolved in 10 mM citrate buffered saline of pH 4.5) into a tailvein. These rats were sacrificed 6-10 days later, when the blood glucose level had increased from 6.5 ± 0.5 (n=14) to 32.8 ± 1.2 mM (n=8). Glucose was determined as described by Werner et al.[11], thyroxin as described by Visser et al.[12] and cholesterol plus cholesterolester as described by Röschlau et al.[13]. Postheparin serum was obtained by injecting 200 I.U. heparin (Organon)/kg body weight intravenously. After 6 min, blood was withdrawn and liver lipase measured in the plasma with either tri-1-[^{14}C]-oleoylglycerol, in the presence of 1 M NaCl, or with 2-[^{3}H]-glycerolmonooleate as the substrate, as described previously[14]. Since the monoglyceridase assay measures both "preheparin" and extrahepatic postheparin plasma activities, the liver contribution could only be determined by comparing activities in plasma, preincubated with control rabbit serum, with plasma preincubated with an antiserum raised in rabbits against purified heparin-released liver lipase[1] as described previously[14]. The amount of antiserum used was sufficient to remove the liver activity completely[14] during a 3 h preincubation at 0°C (followed by centrifugation). The antiserum was kindly provided by Dr. H. Jansen. The figures given are mean values ± S.E.M.

RESULTS

Control sera contain a "NaCl-resistent" triglyceridase activity, which reflects the heparin-releasable liver lipase activity[7] (TABLE I). The enzyme may also be measured with a monoglyceride substrate, provided the preheparin and extrehepatic contributions are excluded by preincubation with antibody (see METHODS). Both activities are decreased significantly in hypothyroidism and in diabetes. The latter has been reported earlier[1]. When the standard chow (containing 18 cal% fat, including 6.4 cal% linoleic acid) was replaced by a diet rich in sunflowerseed oil (containing 40 cal% fat, including 66 cal% linoleic acid and 4 cal% arachidonic acid), liver lipase activities in both hypothyroid and diabetic rats increased to the normal range (TABLE I).

DISCUSSION

The reversal of the lowered liver lipase activity by a diet rich in polyunsaturated fatty acids, as observed in diabetes and hypothyroidism, suggests that in both diseases membrane fluidity may be decreased. Mercuri et al.[15] and Lerner et al.[16] observed a fatty acid pattern in the diabetic liver in which the arachidonic acid is low in comparison to the linoleic acid level. This suggested a desaturase deficiency, which indeed has been demonstrated[15]. A similar defect may be expected in hypothyroid

TABLE I

LIVER LIPASE ACTIVITY IN POSTHEPARIN RAT SERA UNDER DIFFERENT HORMONAL AND DIETARY CONDITIONS

$C_{18:2}$ rich diet was given for one week; for composition see text. The significance (Student t-test) of the differences in the triglyceridase activity on the one hand and the monoglyceridase activity on the other hand between the normal and hypothyroid states are $P<0.005$ and $P<0.001$ respectively; between the normal and diabetic states $P<0.05$ and $P<0.001$ respectively. The significance of the increase of activity by the $C_{18:2}$ rich diet in hypothyroidism was for the triglyceridase and monoglyceridase activities $P<0.005$ and $P<0.05$ respectively; for the increase of activities by diet in diabetes were the P values <0.025 and <0.001 respectively. The alterations of activities by the $C_{18:2}$ rich diet in control rats were not significant.

Condition	Hydrolysis of Trioleoylglycerol (nmoles fatty acid/min/ml serum	Hydrolysis of Monooleoylglycerol (nmoles fatty acid/min/ml serum
Control rats	517 ± 20 (n=7)	2937 ± 93 (n=6)
Control rats; $C_{18:2}$ rich diet	625 ± 33 (n=3)	2719 ± 219 (n=3)
Hypothyroid rats; control diet	411 ± 9 (n=5)	2056 ± 135 (n=6)
Hypothyroid rats; $C_{18:2}$ rich diet	564 ± 32 (n=4)	2500 ± 114 (n=4)
Diabetic rats; control diet	445 ± 21 (n=5)	1405 ± 41 (n=5)
Diabetic rats; $C_{18:2}$ rich diet	558 ± 34 (n=6)	2402 ± 173 (n=6)

liver membranes, where Chen and Hoch[17] also found decreased arachidonic acid and increased linoleic acid levels, resulting in an overall lowered desaturation index. Thyroxin-stimulated microsomal fatty acid desaturation has been reported recently[18].

Decreased membrane fluidity could influence membrane-bound enzymes[8] and binding proteins, which are laterally mobile[19]. Defective binding may cause cholesterol accumulation in the blood, a finding well known to occur in both diabetes and hypothyroidism. This in turn may further decrease membrane fluidity since cholesterol has been shown to increase the phospholipid packing [20-22], especially of the less unsaturated phospholipid species. According to Cooper[8], an increase in the cholesterol/phospholipid

ratio may be expected to lead to instable particles from which cholesterol may escape into cell membranes.

Liver lipase probably does not extend through the liver plasma membrane, because it is easily removed by heparin perfusion. Therefore it is possible that the lowered activity observed in diabetes and hypothyroidism results from defective binding. Normal binding requires intact surface glycosylation. In diabetes sialic acid synthesis is low[23]. The linoleic acid rich diet, however, did not increase the sialic acid content of liver microsomes and plasma proteins (not shown) in the diabetic rat. It is most likely therefore that the diet increased binding and possibly insertion of newly synthesized protein molecules by increasing the membrane fluidity.

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PAPER V

TRIGLYCERIDASE AND PHOSPHOLIPASE A_1 ACTIVITIES OF RAT-HEART LIPOPROTEIN LIPASE. INFLUENCE OF APOLIPOPROTEIN C-II AND C-III

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TRIGLYCERIDASE AND PHOSPHOLIPASE A_1 ACTIVITIES OF RAT-HEART LIPOPROTEIN LIPASE

INFLUENCE OF APOLIPOPROTEINS C-II and C-III

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Summary

The influence of purified human apolipoprotein C-II on phospholipase A_1 and triglyceridase activities of lipoprotein lipase were compared. Lipoprotein lipase was obtained from rat hearts by perfusion with a medium containing heparin and purified on a heparin-Sepharose 4-B column. Using phosphatidylethanolamine-coated triglyceride particles as substrate it was found that the phospholipase A_1 and triglyceridase activities of lipoprotein lipase similarly depend on the presence of apolipoprotein C-II. Apolipoprotein C-III cannot replace apolipoprotein C-II. However, addition of apolipoprotein C-III in the presence of C-II affects both lipase activities. While strong inhibition of triglyceridase activity was observed under these conditions, phospholipase A_1 activity was slightly stimulated. On the basis of these findings a model was constructed for the role of apolipoprotein C-II in lipoprotein lipase action.

Introduction

Lipoprotein lipase is considered to be a key enzyme in the hydrolysis of triglycerides from chylomicrons and very low density lipoproteins (VLDL). The functional enzyme is localized on the vascular endothelial surface and can be released into the circulation by intravenous injection of heparin. Artificial triglyceride emulsions are practically not hydrolyzed by lipoprotein lipase in vitro, unless serum is present. It is now well established that apolipoprotein C-II is the factor, present in serum, necessary for lipoprotein lipase action [1—6].

Substrate specificity studies have shown that lipoprotein lipase has positional specificity for primary ester bonds of tri- and diglycerides [7—8]. 1-(3)Monoglycerides are also hydrolyzed but in contrast to tri- and diglyceridase activity,

monoglyceride hydrolysis is not stimulated by the addition of serum or apolipoprotein C-II [9,10]. Early reports by Vogel and co-authors [11,12] have shown the presence of phospholipase A_1 activity in post-heparin serum which was ascribed to lipoprotein lipase. Although later studies have shown that most of this phospholipase A_1 can be ascribed to a hepatic lipase [13], recent work confirmed the original supposition that lipoprotein lipase is able to hydrolyze the primary acyl bond of phosphatidylethanolamine and phosphatidylcholine [14,15]. This activity of lipoprotein lipase may be of physiological importance in the attack of phosphatidylcholine present in the surface film of chylomicrons and VLDL, thus exposing the neutral-lipid core to the action of the enzyme [16]. Because phospholipids are localized in the surface of lipoproteins it is interesting to see if apolipoprotein C-II is an obligatory factor of the phospholipase A_1 activity of lipoprotein lipase. These studies will give information about the role of apolipoprotein C-II in lipoprotein lipase action. We have studied this using a lipoprotein lipase preparation obtained from rat heart and purified human apolipoprotein C-II.

Materials and Methods

Glycerol tri[1-^{14}C]oleate and [9,10-^{3}H]palmitate were products of the Radiochemical Centre (Amersham, U.K.). Ultrapure urea was a product of Schwarz-Mann (Orangeburg, U.S.A.) and was purified before use by passage through a mixed bed ion-exchanger. Phospholipase A_2 from Crotalis was purchased by Boehringer (Mannheim, G.F.R.). Heparin and protamine sulphate were products of Organon (Oss, The Netherlands).

Human apolipoproteins C-II, C-III (1) and C-III (2) were isolated from purified VLDL ($d < 1.006$) * by Sephadex G-200 chromatography followed by DEAE-cellulose chromatography, both in 6 M urea, essentially the same as described [17,18]. The purity of the peptides was checked on polyacrylamide gels in the presence of 8 M urea [19] and found to be greater than 95% as judged from the intensity of the protein stain (Fig. 1). Purified apolipoproteins were stored after lyophylization in a dessicator at 4°C over P_2O_5. Labeling of rat-liver microsomal phosphatidylethanolamine was performed by incubation of rat-liver microsomes with [9,10-^{3}H]palmitate, ATP, $MgCl_2$ and coenzyme A under conditions described by van den Bosch et al. [20]. The product was purified according to established methods. Phospholipase A_2 incubations [21] revealed that 98.6% of the acyl label was associated with position 1. Phosphatidylethanolamine was used in a specific activity of 1.8 Ci/mol. Triglyceridase activity was determined according to Ehnholm et al. [22] with some modifications. 20.3 μmol purified glycerol tri[1-^{14}C]oleate (specific activity approx. 0.4 Ci/mol) were mixed with 2.5 ml 2.5% gum acacia in 0.03% $NaHCO_3$

* Very low density lipoproteins were isolated from hyperlipaemic plasma obtained from the local blood-bank. Chylomicrons were removed by centrifugation in the Beckman L-2 ultracentrifuge using the 60 Ti rotor (25 min, 20000 rpm at 12°C) without adjustment of the plasma density. The top layer was removed and VLDL was flotated from the infranatant in a second spin (18 h, 50000 rev./min in the 60 Ti rotor). The flotating cake was removed from the top, suspended in 0.85% NaCl, 0.1% EDTA solution (pH 7.4) and centrifuged again (18 h, 40000 rev./min, SW 40 rotor). This procedure was repeated once more and the washed VLDL was used to isolate apolipoproteins C.

and emulsified by sonication. The assay volume (0.25 ml) contained 10 μmol Tris · HCl (pH 8.5), 33 μmol NaCl, 0.0625 μmol $CaCl_2$, 6.25 mg defatted bovine serum albumin, 0.27 μmol triolein and 0.02 ml dialyzed human serum. Tubes were preincubated for 5 min, enzyme was added and the reaction was terminated after 30 or 60 min incubation at 30°C. The radioactive free fatty acids formed were extracted according to Corey and Zilversmit [23] and counted in a liquid scintillation counter.

Phospholipase A_1 activity was determined using 1.25 μmol ^{3}H-labeled phosphatidylethanolamine sonicated in the presence of 20.3 μmol triolein in gum acacia solution, similarly as described above. We have chosen for this substrate because it is to be expected that phosphatidylethanolamine will arrange itself on the surface of the triglyceride particles, leading to a substrate more comparable with chylomicrons or VLDL than phosphatidylethanolamine-deoxycholate micels, often used in a phospholipase A_1 assay. Final concentrations of trioleate and ^{3}H-labeled phosphatidylethanolamine were 1.08 and 0.066 mM, respectively. Other assay constituents and conditions were similar as described above for the triglyceridase assay. The reaction was terminated by extraction of the lipid constituents according to Bligh and Dyer [24] and [9,10-^{3}H]palmitate was separated from the other lipid classes on silicagel G thin-layer chromatography plates, eluted and counted. Enzyme activities are expressed in nmol free fatty acids formed per min at 30°C.

Results

When rat hearts are perfused with a medium containing heparin, 40—70% of the total lipoprotein lipase is released into the medium [25,26]. This lipase is considered to be present on the endothelial surface of the heart capillaries and is most likely involved in chylomicron or VLDL clearance [25,27]. For this reason we preferred to use heparin perfusates as source of lipoprotein lipase instead of acetone powders of hearts as used by Twu et al. [28].

We found that in addition to triglyceridase activity, phospholipase A_1 activity can be detected in those perfusates (Table I). Under our assay conditions, phosphatidylethanolamine was hydrolyzed with a rate of 3—5% compared with trioleate hydrolysis. Phosphatidylcholine was also hydrolyzed. Its activity, however, was 10 times lower than phosphatidylethanolamine hydrolysis (unpublished data). When perfusates were applied to a heparin-Sepharose 4-B column, both lipase activities were quantitatively bound to the matrix. No activity was eluted by washing the column with 0.7 M NaCl, but 1.5 M NaCl eluted both lipase activities (Table I) which were extremely labile. In 2 h when kept on ice, activities drop by 50%. High unstability of purified rat heart lipoprotein lipase has also been reported by Twu et al. [28]. For this reason, experiments with this enzyme preparation were performed as soon as possible after the elution from the column. We think that both lipase activities can be ascribed to the heart lipoprotein lipase, analogous to the results found for milk lipoprotein lipase [15] and rat post-heparin lipoprotein lipase [14]. Our supposition is substantiated by the effects of inhibitors of lipoprotein lipase on triglyceridase and phospholipase A_1 action (Table II).

We used high activity fractions of the heparin-Sepharose 4-B column eluate

TABLE I

ISOLATION OF HEPARIN RELEASABLE TRIGLYCERIDASE AND PHOSPHOLIPASE A_1 FROM RAT HEART

Hearts from 5 Wistar rats (body weights 220—280 g), fasted overnight, were perfused retrogradely in a non-recirculating system according to Langendorff at 37°C. Blood was removed during 5 min preperfusion with Krebs-Ringer bicarbonate solution, saturated with 95% O_2, 5% CO_2 and containing 2 mg/ml glucose. Lipoprotein lipase was released during a 2-min perfusion with the same medium to which 3% (w/v) bovine serum albumin and 5 U/ml heparin were added (pH 7.4) and these perfusates were immediately applied to a column (15 × 1 cm) containing heparin-Sepharose 4-B, prepared according to Iverias [29] by H. Jansen. The operation temperature was 4°C. The column was washed with 20 ml 0.7 M NaCl in 5 mM Tris · HCl (pH 7.4), followed by 40 ml 1.5 M NaCl in the same buffer. Fraction 4 plus 5, containing the bulk of eluted lipase activity, contains 12 μg protein per ml.

Fraction		ml	Triglyceridase			Phospholipase A_1		
			mU/ml	mU	%	mU/ml	mU	%
Perfusates		160	0.98	156	100	0.052	8.35	100
Perfusate after passage column		160	0	0	0	0	0	0
0.7 M NaCl		20	0	0	0	0	0	0
1.5 M NaCl	1	5	0	0	0	0	0	0
	2	5	0	0	0	0.01	0.05	0.6
	3	5	0.37	1.80	1.2	0.007	0.04	0.5
	4	5	4.85	24.35	15.5	0.351	1.75	21
	5	5	4.52	22.6	14.4	0.306	1.53	18.3
	6	5	2.02	10.1	6.4	0.156	0.78	9.3
	7	5	0.80	4	2.6	0.082	0.41	4.9
	8	5	0.96	4.8	3.1	0.019	0.10	1.1
Recovery					43.2			55.7

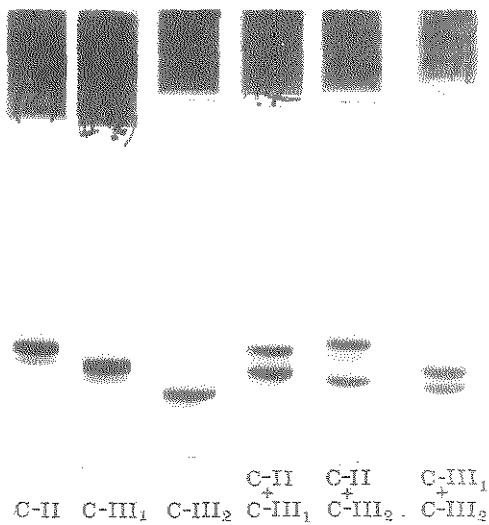

Fig. 1. Polyacrylamide gel electrophoresis of human apolipoprotein C-II, apolipoprotein C-III (1) and apolipoprotein C-III (2) and mixtures thereof. Gels were run according to Kane [19], omitting a preincubation with tetramethylurea.

TABLE II

INFLUENCE OF LIPOPROTEIN LIPASE INHIBITORS ON HEPARIN-RELEASABLE TRIGLYCERIDASE AND PHOSPHOLIPASE A_1 FROM RAT HEART

Fraction 4 plus 5 of the 1.5 M NaCl eluate were used as enzyme source. Incubations, shown in this Table, were started 2 h after elution of the enzyme from the heparin-Sepharose 4-B column. Where indicated NaCl or protamine sulphate was present in final concentrations of 1.125 M and 1 mg/ml, respectively. Activities are expressed as percentage of control values, which are 2.2 and 0.066 mU/ml for triglyceridase and phospholipase A_1, respectively. Electrostatic interaction of protamine with phosphatidylethanolamine may explain the more pronounced effects of protamine sulphate on phospholipase A_1 compared with triglyceridase activity.

Addition	Triglyceridase (%)	Phospholipase A_1 (%)
None	100	100
1 M NaCl	7	3
Protamine sulphate	47	0

TABLE III

EFFECTS OF HUMAN APOLIPOPROTEIN C-II, APOLIPOPROTEIN C-III(1), APOLIPOPROTEIN C-III (2) AND MIXTURES THEREOF ON HEPARIN-RELEASABLE TRIGLYCERIDASE AND PHOSPHOLIPASE A_1 FROM RAT HEART

For more details, see legend of Fig. 2. In this experiment fractions of the heparin-Sepharose 4-B chromatography were mixed instantly with glycerol (20%, v/v) to stabilize lipoprotein lipase (see ref. 28). 0.02 ml of the most active fraction eluted was used in all incubations. The data in this Table are expressed as percentage of the basal lipase activities, obtained in the absence of apolipoproteins C (1.06 and 0.12 mU/ml for triglyceridase and phospholipase A_1, respectively). All values were corrected for blanks, incubated analogue to the tests, in which enzyme was added after the incubation was terminated. Blanks were not influenced by the presence of apolipoproteins C. Basal lipase incubations were run in quadruplicate, all other tests in duplicate.

Additions *			Triglyceridase (%)	Phospholipase A_1 (%)
Apolipoprotein C-II	Apolipoprotein C-III (1)	Apolipoprotein C-III (2)		
—	—	—	100	100
2			1150	350
12			1840	600
	5.4		120	150
	16.2		80	210
	54		5	260
		5.4	40	150
		16.2	80	210
		54	15	330
2	5.4		895	460
12	5.4		1170	680
2	54		5	590
12	54		20	685
2		5.4	910	365
12		5.4	1090	610
2		54	30	550
12		54	70	780

* In μg/ml incubation medium.

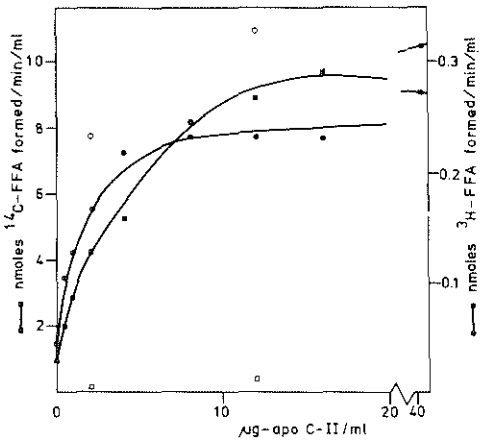

Fig. 2. Effect of human apolipoprotein C-II and apolipoprotein C-II plus apolipoprotein C-III (1) on heparin-releasable triglyceridase and phospholipase A_1 from rat heart. Top fractions in lipase activity of the 1.5 M NaCl eluate were used as enzyme source (see legends to Tables I and II). Triglyceridase and phospholipase A_1 activities were determined in the absence of serum but with human apolipoprotein C-II in concentrations indicated in the figure. ■———■, triglyceridase activity; ●———●, phospholipase A_1 activity; ◻, triglyceridase activity in the presence of apolipoprotein C-II (2 or 12 μg/ml) and apolipoprotein C-III (1) (54 μg/ml); ○, phospholipase activity under similar conditions as triglyceridase activity. FFA, free fatty acids.

with 1.5 M NaCl to study the effects of purified human apolipoproteins C on triglyceridase and phospholipase A_1 activities of lipoprotein lipase. Effects of increasing concentration of human apolipoprotein C-II on both lipase activities are shown in Fig. 2. In these incubations serum was omitted from the incubation mixture. Both lipase activities are stimulated rather parallel by very small amounts of apolipoprotein C-II. We also tested the influence of addition of apolipoprotein C-III (1) on incubations containing 2 concentrations of apolipoprotein C-II. While triglyceridase activity is strongly inhibited by apolipoprotein C-III (1) addition, phospholipase A_1 activity is slightly stimulated (Fig. 2). In experiments shown in Table III, the effects of apolipoprotein C-III (1) were studied in more detail. In the absence of apolipoprotein C-II, apolipoprotein C-III (1) tended to be inhibitory to the basal triglyceridase activity, while the basal phospholipase A_1 was slightly stimulated (up to 2.6 times, Table III). When apolipoprotein C-III (1) was added to incubations, containing apolipoprotein C-II, an inhibition of the (apolipoprotein C-II activated) triglyceridase activity was observed which was extremely evident at high concentrations of apolipoprotein C-III (1), both at high (12 μg/ml) and at low (2 μg/ml) concentrations of apolipoprotein C-II. In contrast, the (apolipoprotein C-II-activated) phospholipase A_1 was stimulated under these conditions as before (Fig. 2 and Table III). All these effects of apolipoprotein C-III (1) were reproduced with apolipoprotein C-III (2) (Table III).

Although apolipoprotein C-III (1) and apolipoprotein C-III (2) were stimulatory to basal phospholipase activity in our experiment, effects of apolipoprotein C-II were by far superior (2 μg apolipoprotein C-II is more stimulatory to the basal phospholipase activity than 54 μg apolipoprotein C-III (1) or apolipo-

protein C-III (2), see Table III) and a small contamination of apolipoprotein C-III peptides with apolipoprotein C-II, which could explain this phenomenon, is diffucult to exclude.

Discussion

According to the lipid-core model, chylomicrons and VLDL are composed of a central core of neutral lipids, mostly triglycerides, covered by a surface of apolipoproteins, phospholipids and free cholesterol. In this model an active site of lipoprotein lipase must penetrate in the surface film to interact with triglycerides. How this is accomplished is presently unknown. Recently Eisenberg and Schurr [16] speculated that phospholipase A_1 action of lipoprotein lipase may create space in the surface film, thus exposing triglycerides to the enzyme action. Alternatively, apolipoprotein C-II may have a function in the penetration process. High affinity of apolipoprotein C-II for lipoprotein lipase has been demonstrated [30,31]. Our results with the rat heart enzyme show that apolipoprotein C-II not only stimulates the hydrolysis of triglycerides but also of phospholipids. Therefore, the function of apolipoprotein C-II may be more general in that it functions as a binding site for lipoprotein lipase on the lipoprotein particle. Absence of effects of apolipoprotein C-II on hydrolysis of micellar or soluble substrates by lipoprotein lipase [9,10] excludes a true coenzyme function.

It has been shown previously that stimulation of triglyceridase activity of lipoprotein lipase by apolipoprotein C-II can be diminished by addition of apolipoprotein C-III and other apolipoproteins [3,5,6]. This inhibition by apolipoprotein C-III was confirmed in the present study. However, phospholipase A_1 activity of lipoprotein lipase is slightly stimulated when both apolipoprotein C-II and apoliporpotein C-III are present. Speculating about this phenomenon we propose that apolipoprotein C-II functions both as binding site on lipoproteins for lipoprotein lipase and as agent involved in the penetration of lipoprotein lipase through the surface film. Addition of apolipoprotein C-III would create a more tight occupation of peptides in the surface of the lipoprotein particle, interfering with the penetration of lipoprotein lipase through the surface film and by that inhibiting triglyceridase activity, while more superficial interaction between the particle and lipoprotein lipase is still possible. The inability to hydrolyze triglycerides (a complete substrate) could explain the stimulation of phospholipid hydrolysis seen under these conditions. This model, however, requires more experimental support.

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