MONOACYLGLYCEROL ACYLATION AND DEACYLATION IN RAT SMALL INTESTINAL EPITHELIAL CELLS

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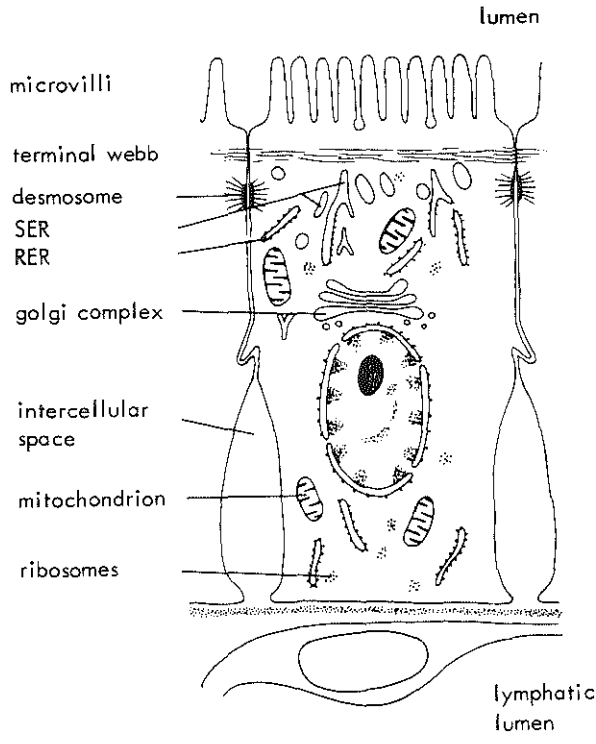

Fig. 1.1 The epithelial villus cell

CHAPTER I INTRODUCTION

1.1 GENERAL

Acylation and deacylation of monoacylglycerols in rat small intestinal cells play a role in the intestinal absorption of dietary triacylglycerols. One of the first steps in this absorption process is the breakdown of the ingested material inside the intestinal lumen. Triacylglycerols are partially hydrolyzed by the action of pancreatic lipase, resulting in the formation of monoacylglycerols and fatty acids. These reaction products then are taken up by the epithelial cells, which cover the villi of the intestine (Fig. 1.1). Inside these cells triacylglycerols are resynthesized from absorbed materials in energy requiring processes. Finally, the triacylglycerols are surrounded with phospholipids and protein during the formation of chylomicrons, which are released into the chyle for transport (for a review, see: refs. 1-5).

The monoacylglycerol generated in the lumen and absorbed into the epithelial cells can be used for two important reactions. In a transacylation reaction diacylglycerol is synthesized from monoacylglycerol. The reaction is part of a reaction sequence for triacylglycerol synthesis. A second reaction of monoacylglycerol is the hydrolysis into glycerol and fatty acids. These two reactions form the subject of this thesis. Attention will be paid to the importance of both reactions, the mechanism by which they are regulated and, in particular, to the inhibition of the hydrolytic activity.

In this Chapter some of the processes involved in triacylglycerol absorption and of the reactions which convert the monoacylglycerol will be discussed. These are schematically drawn in Fig. 1.2.

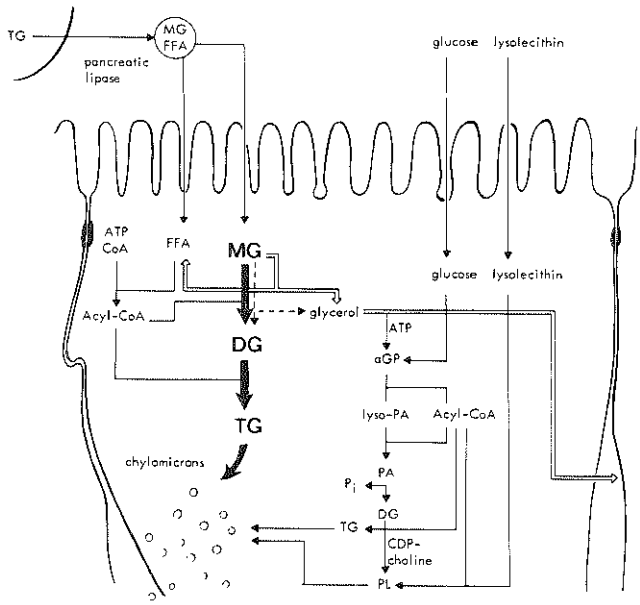

Fig. 1.2 Schematic drawing of pathways involved in lipid metabolism in the small intestinal cells.

1.2. THE FORMATION OF MONOACYLGLYCEROL AND FATTY ACIDS

Emulsified dietary triacylglycerol enters the duodenum where it is mixed with bile salts and pancreatic lipase. The surface active properties of the bile salts help in the emulsification of the triacylglycerol. They are able to form mixed micelles with the products of triacylglycerol hydrolysis, catalyzed by pancreatic lipase (EC 3.2.1.3). With the aid of colipase [6,7] and bile salts [8], the pancreatic lipase is bound to the oil/water interface of the emulsions, so that hydrolysis of triacylglycerol occurs. Only the ester bonds of the 1 and 3 positions are cleaved [9] and thus 2-monoacylglycerol is generated, together with fatty

acids. The 2-monoacylglycerol is a amphiphilic molecule, i.e. it is water insoluble and has surface active properties, and therefore helps in the emulsification of triacylglycerols. Most importantly, it is also able to form mixed micelles with bile salts [10]. It has been shown that these micelles contain fatty acids but very little di- and triacylglycerols [11], so they can be regarded as the form in which the reaction products of triacylglycerol hydrolysis are transported.

1.3. THE ABSORPTION OF 2-MONOACYLGLYCEROL AND FATTY ACID BY EPITHELIAL CELLS

Monoacylglycerols rather than di- or triacylglycerols can enter the epithelial cells without further hydrolysis [12,13], but only recently there is an increasing agreement about the mechanism of monoacylglycerol and fatty acids absorption. The lipids diffuse from a monodisperse solution [14] through the unstirred layer [15] to the cell membrane. The idea that whole micelles might be taken up by pinocytosis [16] or by other mechanism [17], has been disproven by the demonstration that several micellar constituents have different rates of uptake [18]. The micellar solubilization of fatty acids and monoacylglycerols promotes the uptake into the cell by maintaining a high monodisperse concentration outside the cell, because the micelles serve as a reservoir [19]. The absorption can be regarded as a passive diffusion process, which is energy independent [20].

It has been shown by electronmicroscopy and autoradiography [21] that at $0^{\circ}C$ monoacylglycerol and fatty acids can penetrate the cell below the terminal web. After incubation at $37^{\circ}C$ both were used for triacylglycerol synthesis, since only after the temperature increase lipid droplets, containing triacylglycerol, were seen associated with the

endoplasmic reticulum. It was concluded therefore, that a transport must take place inside the epithelial cells of both the monoacylglycerols and fatty acids from the site of absorption in the microvilli to the endoplasmic reticulum where they are used. It has been suggested that fatty acids can be transported by a protein with a low molecular weight [22] - the "fatty acid binding protein" (FABP) - which seems to be identical with Z-protein in liver [23]. This protein is important because by blocking the function of FABP, the triacylglycerol synthesis is inhibited [24,25]. An analogous protein for the transport of monoacylglycerol has not been described. FABP also does not bind monoacylglycerol [26].

1.4. TRIACYLGLYCEROL FORMATION

1.4.1. ACTIVATION OF THE FATTY ACIDS

The formation of an ester bond involves the investment of energy. Therefore the fatty acids are first activated by the formation of the CoA derivatives. The synthesis of acyl-CoA is catalyzed by fatty acid-CoA synthetase (EC 6.2.1.3) [27]. This reaction is driven by the hydrolysis of ATP. The fatty acyl-CoA is used in transacylation reactions to form ester bonds with the hydroxyls of glycerol derivatives. Several transacylation steps appear to be involved in the pathways for triacylglycerol synthesis.

1.4.2. MONOACYLGLYCEROL PATHWAY

The monoacylglycerol pathway for the synthesis of triacylglycerol was first demonstrated in epithelial cells of the intestine of rabbits [28,29] and later in rats [30,31] and other animals [comp. ref. 4]. It was also demonstrated in other tissues, such as adipose tissue [32] and mammary gland [33]. This pathway involves two transacylation reactions:

monoacylglycerol + acyl-CoA → diacylglycerol + CoA
diacylglycerol + acyl-CoA → triacylglycerol + CoA

The enzymes catalyzing these reactions, acyl-CoA: monoacylglycerol acyltransferase (EC 2.3.1.?) and acyl-CoA: diacylglycerol acyltransferase (EC 2.3.1.20) are not identical [34].

In vivo the natural substrate for the first acylation step is 2-monoacylglycerol but the enzyme can acylate also the 1-isomer [29,31] or the ether analogue [35,36]. The maximal reaction velocity with 1- and 2-monoacylglycerol is similar but the enzyme appears to have a lower K_m for the 2-isomer [31]. With 1(3)-monoacylglycerol the acylation occurs preferentially at the 3(1)-position of the glycerol. A small but variable percentage of 1,2-diglyceride can be formed depending on the nature of the acyl group of the acyl-CoA used [34,37-39]. In rat the 1,2-diacylglycerol is preferentially used for triacylglycerol synthesis, because it has been shown that triacylglycerol is the end product of acylations of 2-monoacylglycerol and 1,3-diacylglycerol is the end product of the 1-isomer [38,40].

The enzymes involved in the monoacylglycerol pathway have been partially purified [41]. The enzymes acyl-CoA synthetase and both transacylating enzymes could not be separated during purification, suggesting a multi-enzyme

complex for triacylglycerol synthesis.

1.4.3. GLYCEROLPHOSPHATE PATHWAY

The glycerolphosphate pathway is the dominant pathway for triacylglycerol synthesis in tissues such as the liver, where it was first discovered [42]. It operates also in the intestine [43]. This pathway involves the following reactions:

3-phosphoglycerol + acyl-CoA → lysophosphatidate + CoA
lysophosphatidate + acyl-CoA → phosphatidate + CoA
phosphatidate → *sn*-1,2-diacylglycerol + P_i
sn-1,2-diacylglycerol + acyl-CoA → triacylglycerol + CoA

The primer in this reaction sequence, the 3-phosphoglycerol, can be generated from glycolysis by the reduction of dihydroxyacetonephosphate, or by the phosphorylation of glycerol by glycerol kinase [44,45]. The latter reaction is of minor importance because of the low levels of the enzyme activity.

The lysophosphatidate formed by the acylation of 3-phosphoglycerol can also be generated by a parallel route: dihydroxyacetonephosphate can be acylated, resulting in the formation of an acyldihydroxyacetonephosphate, which is then subsequently reduced to lysophosphatidate [46]. The importance of this alternative pathway is not yet established.

Most reactions of this reaction sequence serve a dual function, because they can also lead to the formation of phospholipids. The branch-point is after the formation of 1,2-diacylglycerol, which is a substrate for both the acyltransferase in the formation of triacylglycerol and for CDP-choline: *sn*-1,2-diacylglycerol cholinephosphotransferase (EC 2.7.8.2) in the formation of lecithin [47].

1.4.4. RELATIVE IMPORTANCE OF INDIVIDUAL PATHWAYS

The cholinephosphotransferase can only use the substrate formed by the glycerolphosphate route [48], but cannot use diacylglycerol formed in the monoacylglycerol pathway. Therefore it may be assumed that two pools of the diacylglycerol exist, and that both pathways are physically separated. Several laboratories reported the contribution of both routes to overall triacylglycerol synthesis [49-51]. Approximately 80% of the triacylglycerol is formed via the monoacylglycerol pathway. The preference for this route has been contributed to the inhibition of the glycerolphosphate pathway by 2-monoacylglycerol and the ether analogue, because it was shown [52] that the phosphatidate synthesis is impaired in the presence of these precursors for the monoacylglycerol route. However, later it was shown [53] that both pathways are not competitive. The preference for the monoacylglycerol route may only be due to higher enzyme activities [53]. The phosphatidate phosphohydrolase reaction may limit the glycerolphosphate pathway.

The absorption of monoacylglycerol and fatty acids is generally considered to be the rate-limiting step of the triacylglycerol synthesis *in vivo* [15,19]. Inside the cell or in broken cell preparations the rate of this reaction seems to be determined by the activities of the transferases. It also has been suggested that fatty acid activation may be the rate-limiting step, but it has been shown that the activity of this enzyme is in excess of that of the rate-limiting enzyme of the glycerolphosphate pathway [4,53,54] and of the monoacylglyceroltransferase [53,54].

1.4.5. SUBROUTE OF THE MONOACYLGLYCEROL PATHWAY

Preliminary experiments have shown another possibility for the synthesis of diacylglycerol. From a molecule monoacylglycerol the acyl moiety can be transferred to another monoacylglycerol, resulting in the formation of a diacylglycerol and glycerol. This reaction is catalyzed by an enzyme, which might be called monoacylglycerol: monoacylglycerol transacylase (EC 2.3.1.?). The enzyme is fairly active, the maximal rate of activity is about a quarter of that of the acyl-CoA: monoacylglycerol acyltransferase, but the role of the enzyme in the synthesis of triacylglycerol *in vivo* remains to be elucidated.

1.5. DEACYLATION OF MONOACYLGLYCEROL

The ability of intestinal mucosa cells to hydrolyze monoacylglycerol was first described by Schmidt [55] and raised much attention at the time that the monoacylglycerol pathway was discovered, because the hydrolysis interfered with the acylation of the substrate [56]. The hydrolytic activity was shown to be different from that of pancreatic lipase [57] and was the subject of several studies [58-64]. The existence of the hydrolytic activity was demonstrated also in other tissues and much attention was paid to the activity in adipose tissue [65-70], liver [70-73] and in serum [74].

The hydrolytic activity has been attributed to monoacylglycerol lipase(s) but it is known that non-specific esterases can also hydrolyze monoacylglycerols [75,76]. The substrate specificities of monoacylglycerol lipases and esterases are very similar [compare refs. 64 and 75], and a distinction cannot always be made. However, the

activities can be distinguished as has been shown by the purification of both esterases and monoacylglycerol lipase [72,76, Chapter V].

Precursors for the synthesis of triacylglycerol are removed via hydrolysis of monoacylglycerols. The products can be used again for triacylglycerol synthesis, by the action of glycerol kinase, acyl-CoA synthetase and the operation of the glycerolphosphate pathway, but this route is inefficient, because two extra moles ATP are used per mole of triacylglycerol. Furthermore, it is not very likely that this occurs, because of the low glycerol kinase activity. The hydrolysis *in vitro* can be quite substantial as was shown in transacylation studies [56]. Studies of monoacylglycerol absorption [12] reported that up to 50% of the monoacylglycerol can be hydrolyzed. Therefore, the hydrolysis may be an important factor in the metabolism of monoacylglycerols. However, *in vivo* most of the monoacylglycerol is saved for triacylglycerol synthesis [77]. Therefore a mechanism should exist *in vivo* that directs monoacylglycerol into the triacylglycerol pathway. Because the maximal activity of the hydrolases *in vitro* is about 5-10 times higher than the activity of the transacylases, it is to be expected that the mechanism may only work when the hydrolase activities are inhibited. Another possibility is the compartmentalization of the hydrolyzing and acylating enzymes.

The hydrolyzing and acylating enzymes are acting on the same substrate and the function of the hydrolases has been correlated with triacylglycerol synthesis. Because it had been shown that the hydrolysis can be inhibited by large amounts of palmitoyl-CoA [56], it has been suggested [63] that the hydrolysis should work only when the fatty acid concentration is relatively low with respect to that of monoacylglycerol. By the action of the hydrolases, fatty acids are generated, which can be used for the triacylglycerol synthesis, while accumulation of the

amphiphilic monoacylglycerol molecules may be avoided.

1.6. SCOPE OF THIS THESIS

The goal of the study presented in this thesis is to gain more insight in the mechanism by which the monoacylglycerol is saved for the triacylglycerol synthesis. Attention is mainly paid to hydrolyzing enzymes. Chapter II concerns the localization of these enzymes, in order to find possible effects of compartmentalization, not only intracellularly, but also intercellularly. Some experiments planned for the detection of a possible monoacylglycerol carrier in the cytosol, are described in Chapter III. In Chapter IV some properties of the monoacylglycerol hydrolase activity are described. The purification and some other properties of the hydrolyzing enzymes are further described in the next Chapter. The inhibition of the hydrolytic activities is elaborated in Chapter VI, which deals with the fate of monoacylglycerol in several preparations. Finally, some aspects of the monoacylglycerol: monoacylglycerol acyltransferase are described in the second part of that Chapter.

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CHAPTER II LOCALIZATION OF THE MONOACYLGLYCEROL HYDROLASE ACTIVITY

2.1. INTRODUCTION

In this Chapter results of studies on the localization of the monoacylglycerol hydrolase activity are described. Because the discrepancy between the relative rates of monoacylglycerol deacylation and acylation, as observed *in vivo* and *in vitro* might be caused by a compartmentation of the hydrolytic and acylating enzyme activities, the localization of these enzyme activities has been compared. This compartmentation can occur in the intestine at different levels. In addition to differences in activities between different subcellular structures, there is also a differentiation along the length of the intestine. The digestion of dietary lipids occurs preferentially in the duodenum and proximal jejunum [1], while the bile salts are actively absorbed in the distal ileum [2]. Several enzyme activities, such as alkaline phosphatase and esterase, can vary along the intestine [3]. During the development of a cell from the crypt to the villus compartment the cell obtains the enzymes required for the absorbing function. This is most dramatical for the enzymes present in the brushborders, such as alkaline phosphatase [4], but occurs also with the enzymes present in the endoplasmic reticulum, because this cell organelle also further develops during the cell maturation [5]. On this villus column two cell types can be identified. In the rat about 95% of the cells can be identified as enterocytes and only 5% are goblet cells [6].

The localization of the enzymes involved in the re-esterification of lipids has been well documented. The re-esterification of fatty acids occurs preferentially in the proximal part of the intestine [7,8] where also the highest

activity of monoacylglycerol acyltransferase was measured [9,10]. By electronmicroscopy triacylglycerol synthesis was shown to occur in the enterocytes, preferentially in the villus top cells [11]. The enzymes involved in this synthesis are more active in the villus than in the crypt. Only the *de novo* phospholipid synthesizing enzyme, the cholinephosphotransferase, is equally active in villus and crypt cells [12,13]. Inside the enterocyte the process of triacylglycerol synthesis was shown to occur in the apical part of the endoplasmic reticulum [14]. By cell fractionation studies also the enzymes involved in triacylglycerol synthesis are found in the microsomal fraction [15,16], while the endoplasmic reticulum was found as the source of the acyltransferase [17,18].

The localization of monoacylglycerol hydrolase activity, however, has been scarcely described [19,20]. The microsomal fraction has the greatest specific activity. The determination of the localization is seriously hampered by the fact that the observed activity is due to at least three different enzymes, two of which are esterases and one a more specific enzyme, the monoacylglycerol lipase (Chapter V). About 2/3 of the total hydrolytic activity towards monoacylglycerol is due to the esterases. The esterase activity can be assayed with tributyrin or p-nitrophenylacetate. The hydrolytic activities towards these substrates have been localized. More work on the localization has been done with histochemical methods. Along the intestine a gradual decrease of the activity was found from the proximal to the distal part [3,21]. During cell maturation of the enterocyte from crypt to villus the esterase activity is increasing [4,21], and is localized in the endoplasmic reticulum [4].

2.2. METHODOLOGICAL REMARKS

For the study of the enzyme activity along the intestine, intestinal cells from male Wistar rats were isolated by scraping [22]. Otherwise the cells were obtained by the vibration method of Harrison and Webster [23], which allowed the separate isolation of villus and crypt cells. Cells were collected in 0.15 M NaCl, 5 mM EDTA and 10 mM Tris-HCl pH=7.4 and centrifuged for 30 sec at 755 g. A 10% homogenate was prepared in 0.25 M sucrose, 10 mM Tris-HCl pH=7.4 and 1 mM EDTA, with a Potter-Elvehjem glass-teflon homogenizer (10 up and down strokes). For the fractionation study 1 mM dithiothreitol was added to the homogenization buffer, and the homogenate was filtered through glass-wool to remove mucous material. The centrifugation scheme used in our laboratory was followed [22].

The activities of the following enzymes are determined according to the reference given: alkaline phosphatase: Iemhof *et al.* [24], p-nitrophenylacetate hydrolase activity (esterase): Huggens and Lapides [25]; rotenone insensitive NADPH-cyt. c reductase (RINCR): De Jonge and Hülsmann [26]; ouabain sensitive Na^+/K^+-ATPase: Ferard *et al.* [27]; UDP-glucuronyl transferase: Mulders and Van Doorn [28]; monoamine oxidase (MAO): Aas [29]; pyruvate kinase: Van Berkel *et al.* [30]; glutamate dehydrogenase: Slater and Holton [31]. The method used for monoacylglycerol hydrolase measurement will be described in Chapter IV. Protein was determined by the method of Lowry *et al.* [32], using bovine serum albumin as a standard.

2.3. RESULTS

2.3.1. LOCALIZATION OF HYDROLASE ACTIVITY WITHIN THE INTESTINAL TISSUE

Enzyme activities determined in homogenates of cells isolated from different parts of the small intestine, are depicted in Fig. 2.1. Alkaline phosphatase has the highest activity in the duodenum and the lowest in the distal ileum, where the activity is less than 1/5 of the activity in the duodenum, as has been described by Harrison and Webster [3]. The esterase activity has a less steep gradient and decreases almost linearly from duodenum to ileum. A similar decline has been described for the esterase activity mea-

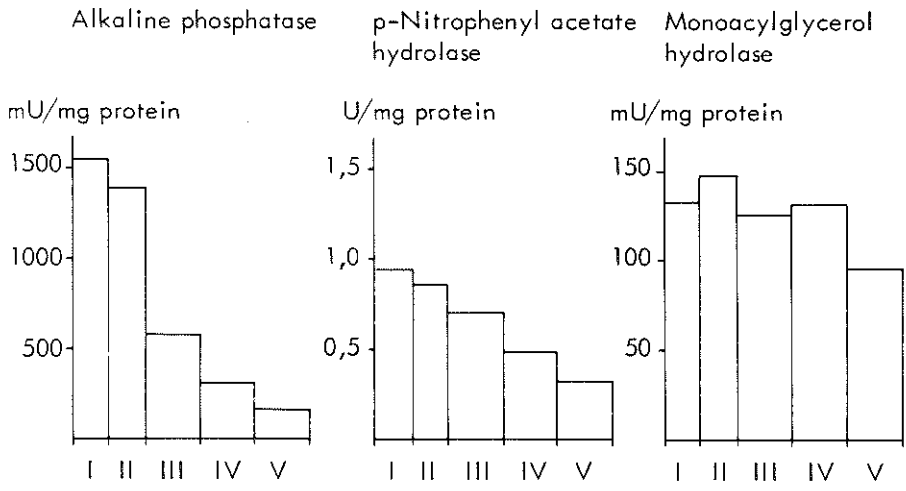

Fig. 2.1 Enzyme activities in homogenates of cells derived from different parts along the intestine.
I: duodenum II: proximal jejunum III: distal jejunum
IV: proximal ileum V: distal ileum.

sured with p-nitrophenylacetate [3] or tributyrin [21]. The monoacylglycerol hydrolase has a similar activity in all parts of the small intestine. Only in the distal ileum less activity may be present. The total amount of the hydrolytic activity towards monoacylglycerol decreases from proximal to distal parts, but is paralleled by a decrease in villus cellular protein [3]. If we assume that much of the monoacylglycerol hydrolase activity is due to the esterases (Chapter V), it can be derived from the difference between esterase and monoacylglycerol hydrolase activity that the lipase is relatively enriched in the distal parts of the ileum.

During the development of the cell from crypt to villus top, the cells acquire a.o. alkaline phosphate activity. This activity is absent in the crypt [4] and can be used as a marker for the villus cells, present in the crypt cell preparation. From Table 2.1 it can be seen that both the esterase and monoacylglycerol hydrolase activities are more active in the villus than in the crypt cells. The enrichment in the villus is comparable to that of other enzymes present in the endoplasmic reticulum, with the exception of the monoacylglycerol transferase, which is

TABLE 2.1

ENZYME ACTIVITIES IN VILLUS AND CRYPT CELL HOMOGENATES

homogenate of cell preparation	alkaline phosphate mU/mg	p-nitrophenyl-acetate hydrolase mU/mg	monoacylglycerol hydrolase mU/mg
villus top	324	500	61.8
crypt	9.5	310	44.2

The figures are averages of two experiments.

more enriched and may be absent from the crypt [13].

2.3.2. SUBCELLULAR LOCALIZATION

The enzyme activities in subcellular fractions of intestinal epithelial cells are depicted as De Duve plots in Fig. 2.2. The alkaline phosphatase is a marker for the plasma membrane and is predominantly located in the brush-borders, as can be concluded from the high relative specific activity (r.s.a.) in the N-fraction. Due to mucus present in the rat intestine, this fraction contains also much of the mitochondria and microsomes, as can be seen from the distribution of the mitochondrial markers monoamine oxidase (MAO) and glutamate dehydrogenase (GDH), and the microsomal markers rotenone insensitive NADPH-cyt. c reductase (RINCR) and UDP-glucuronyl transferase. The distribution of p-nitrophenylacetate hydrolase (esterase) and of monoacylglycerol hydrolase activities is very similar to that of the microsomal marker enzymes. About 80% of the total esterase activity is particulate and about 20% is soluble. Of the total monoacylglycerol hydrolase activity a smaller part is found in the supernatant: about 15% (compare Chapter V). This study confirms the observation of Senior and Isselbacher [19] and Pope *et al.* [20] that the highest specific activity of the monoacylglycerol hydrolase activity was found in the microsomes. However, in these studies no marker enzymes were used!

Although the distribution of the hydrolases is very similar to that of RINCR or UDP-glucuronyl transferase, it cannot be concluded from the results in Fig. 2.2 whether a small part of the enzymes is also located in the brush-borders, because of the high contamination of the microsomes in the N-fraction. Therefore, brushborders were purified by the EDTA method of Harrison and Webster [33]. No enrichment

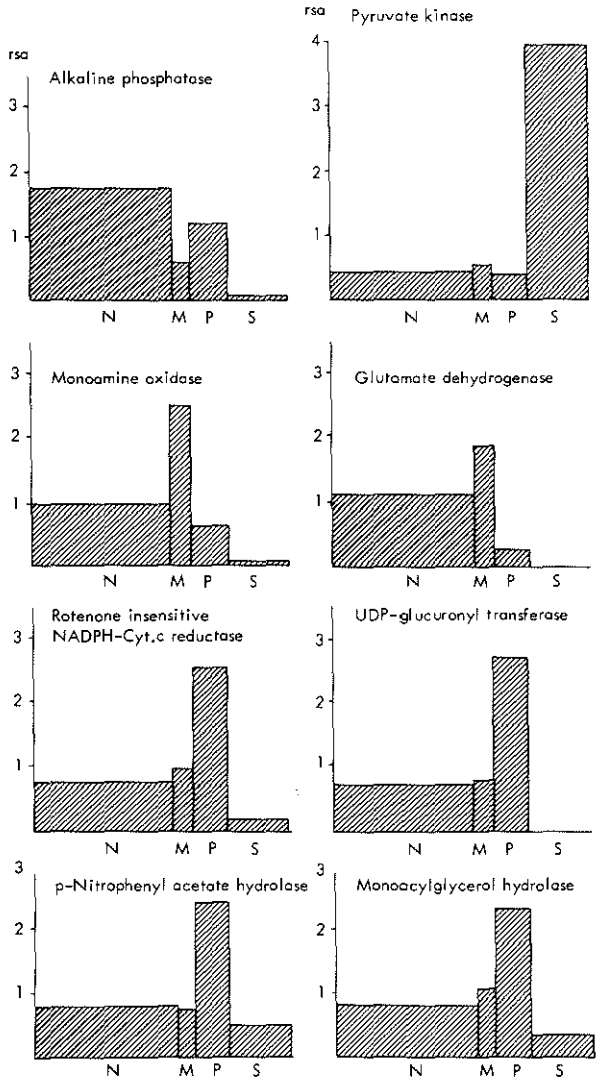

Fig. 2.2 Enzyme activities in subcellular fractions plotted according to De Duve.
N: nuclear fraction, also enriched in brushborders
M: mitochondrial fraction
P: microsomal fraction
S: soluble fraction
Relative specific activity (r.s.a.) is defined as the percentage of total activity divided by the percentage of total protein content in the homogenate fraction.

of esterase or monoacylglycerol hydrolase activity was found when compared to the RINCR activity. Therefore, it can be concluded that no hydrolase activity is present in the brushborders. By analogy an experiment was also performed with purified mitochondria, which were prepared according to Hülsmann [34]. No enrichment of the hydrolase activity in the mitochondrial fraction could be detected. Therefore, it can be concluded that about 80% of the esterase activity and 85% of the monoacylglycerol hydrolase activity is localized in the membrane fragments isolated in the microsomal fraction and that the remaining acitivity is soluble.

The microsomal fraction is derived from different cell organelles. Membrane fragments of the rough and smooth endoplasmic reticulum, the Golgi apparatus and the basal and lateral plasma membranes are sedimented in this fraction. In the plasma membrane of adipocytes a monoacylglycerol lipase activity has been described [35]. Localization of part of the hydrolase activity in the Golgi apparatus or the basal and lateral plasma membrane is not likely. In the first place treatment of isolated whole cells or isolated microsomes with heparin did not release any detectable monoacylglycerol hydrolase activity, excluding the presence of a lipoprotein lipase-like activity on the cell surface. In the second place a fractionation study of a membrane fraction on a discontinuous dextran gradient, which allowed the separation of plasma membranes from the endoplasmic reticulum [36], revealed that the monoacylglycerol hydrolase activity and the esterase activities are not localized in the plasma membrane, as can be seen in Table 2.2. The M_1 fraction is enriched in plasma membrane fragments, as can be seen from the r.s.a. of the Na^+/K^+-ATPase, while in the M_2 fraction the endoplasmic reticulum marker RICNR is concentrated. Both the esterase and monoacylglycerol hydrolase activities follow the pattern of RICNR. Thirdly, according to Redgrave [37] intestinal cells can be loaded with triacylglycerol precursors and after homogenization prechylomicron particles can be isolated, associated with Golgi

TABLE 2.2

RELATIVE SPECIFIC ACTIVITIES OF ENZYMES IN SUBCELLULAR FRACTION ENRICHED IN PLASMA MEMBRANES OR ENDOPLASMIC RETICULUM

A particulate fraction derived from a small intestinal cell homogenate was centrifuged in a discontinuous dextran gradient according to Douglas [36]. M_1 is the fraction derived from the barrier at 1.070 density and is enriched in basal and lateral plasma membranes (marker Na^+/K^+-ATPase). M_2 is the fraction below the barrier and is enriched in endoplasmic reticulum (marker rotenone insensitive NADPH reductase). Enzyme activity is expressed as the relative specific activity, relative to the activity in the homogenate.

cell fraction	rotenone insensitive NADPH-cyt.c reductase	Na^+/K^+ ATPase	p-nitrophenyl-acetate hydrolase	monooleoyl-glycerol hydrolase
M_1	0.9	3.4	0.8	0.6
M_2	8.1	1.1	7.2	10.2

membrane fragments by isolating the floating particles. Although we did not measure a Golgi membrane marker, no enrichment of hydrolytic activities was observed in these particles. Also no hydrolytic activity was found in the chylomicrons, the products of the Golgi apparatus. These were derived from the intestinal lymph and tributyrin and monoacylglycerol hydrolase activity were tested before and after delipidation (results not shown).

2.4. DISCUSSION

The presented data show that the monoacylglycerol hydrolase activity is mainly localized in the endoplasmic reticulum (at least 85%). A small part (15%) may be soluble.

The monoacylglycerol hydrolase activity is localized similar to the monoacylglycerol transferase: both activities are present in the endoplasmic reticulum, and are present in the villus top cells. The transferase activity increases more than the hydrolase activity during cell maturation from crypt to villus. Along the intestine the pattern of the transferase and the hydrolase activities may be different. The transferase activity is more active in the duodenum than in the distal ileum. This resembles the pattern of the esterase activity. The monoacylglycerol lipase activity, however, may be more active in the distal part of the intestine. The results show that the localization of the monoacylglycerol hydrolytic activities is rather similar to that of the acylating activity. Therefore, the general conclusion can be drawn that there is no basis for the idea that the discrepancy between *in vitro* and *in vivo* metabolism of monoacylglycerol is due to differences in distribution of the acylating and hydrolase activities. Another indication for this conclusion can be derived from the experiment described in Chapter VI, Table V. The intestine can be loaded *in situ* with monoacylglycerol at $0^{\circ}C$. When subsequently the microsomes are isolated and the temperature is raised to $37^{\circ}C$, the endogenous monoacylglycerol can either be hydrolyzed or, in the presence of palmitoyl-CoA, be acylated.

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CHAPTER III INTRACELLULAR TRANSPORT OF MONOACYLGLYCEROL; ABSENCE OF A BINDING PROTEIN?

3.1. INTRODUCTION

Very little is known about the transport of monoacylglycerol in the cell. Transport processes could be important because the precursors for triacylglycerol synthesis are absorbed in the microvilli and are used beyond the terminal web in the endoplasmic reticulum, where triglyceride synthesis takes place. From these precursors, the fatty acids may be transported through the cytosol by a soluble low molecular weight protein, which was simultaneously discovered by Ockner in the intestine [1,2] and by Mishkin in the liver [3], and was called fatty acid binding protein (FABP) and Z-protein, respectively. It has been shown that the binding protein plays a role in the triacylglycerol synthesis. This is based not only on theoretical aspects, but experimental evidence has also been given. Flavaspidic acid and α-bromopalmitate binds to FABP, and can inhibit the triacylglycerol synthesis [4,5]. An analogous protein for the binding of monoacylglycerol has not yet been described. The existence of such a protein would be important, because it could influence the metabolism of monoacylglycerol and thus provide an explanation for the difference of the fate of monoacylglycerol *in vivo* and *in vitro*. Such a mechanism may involve inhibition of the hydrolase activity, similar to that observed with albumin, as will be shown in Chapter IV. Therefore, it was tried to detect a soluble monoacylglycerol binding protein in the epithelial cells.

3.2. METHODOLOGICAL REMARKS

Experiment 1. The first 15 cm of a small intestine of about 60 cm length (from the ligamentum of Treitz) was removed from a rat and rinsed with cold saline. Both ends were tied and filled with 1.5 ml of a cold solution of 2.5 mM monooleoyl[2-^{3}H]glycerol (0.5 Ci/mol), 20 mM sodium taurocholate, 0.15 M NaCl and 50 mM phosphate buffer pH=7.0. After 10 min incubation at 0^{o}C, the luminal fluid was removed by rinsing with cold saline, the intestine was opened and the cells were scraped off with a glass slide. A 25% homogenate was made of the scraping in 0.25 M sucrose, 10 mM Tris-HCl pH=8.0 and 1 mM EDTA and the soluble fraction was isolated as described in Chapter II. 0.5 ml of the supernatant was placed on a Sephacryl-S 200 (Pharmacia) column (0.9 x 60 cm) and was eluted with 0.15 M NaCl, containing 10 mM Tris-HCl pH=8.0. The flow-rate was 0.3 ml/min and 2 minute fractions were collected.

Experiment 2. 0.5 ml of the supernatant, derived from a 25% w/v villus cell homogenate in the same medium as was used in the first experiment, was mixed with 25 µl acetone in which 5 x 10^{-9} mole monooleoyl[2-^{3}H]glycerol (430 Ci/mol) was dissolved. This mixture was analyzed on the Sephacryl column in the same way as described for the first experiment.

3.3. RESULTS

It was tried to demonstrate the existence of a monoacylglycerol binding protein by two techniques analogous to those used in the study on the FABP [4]. The elution pattern of the radioactive material from the column in the first experiment is shown in Fig. 3.1. The radioactivity was recovered in two peaks, of which the first one was found in the void volume.

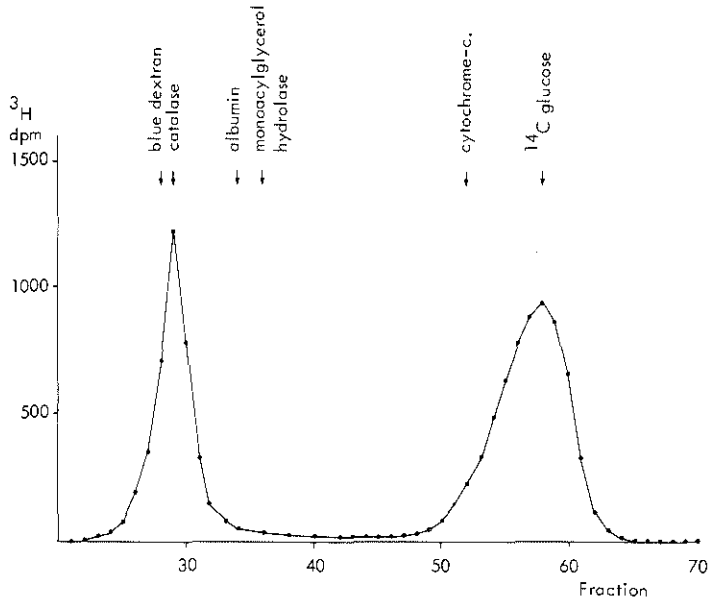

Fig. 3.1 Sephacryl-S 200 column. Elution pattern of tritiated materials contained in a 100,000 g supernatant, obtained from cells loaded in the cold with monooleoyl-[2-^{3}H]glycerol, as described under Methodological Remarks (experiment 1).

Analysis of the nature of the radioactive material by thin layer chromatography and by its solubility in water and organic solvents showed that in the first peak ^{3}H was associated with monooleoylglycerol, but in the second peak with glycerol. Moreover, by calibrating the column with proteins and [^{14}C]glucose, it was found that the elution volume of the ^{14}C label coincided with the ^{3}H label, as is indicated in Fig. 3.1. The first peak was analyzed on a Sepharose-4B column and was found to consist of several, not very discrete, subfractions, all with a molecular weight greater than that of catalase (MW: 240,000). It was therefore believed to consist of floating membranous material and particles of non specificially bound monoacylglycerol and protein. In the low molecular weight fraction

no protein was found capable to bind monooleoylglycerol. This was also found in the second experiment of which the elution pattern is shown in Fig. 3.2. In this experiment the first peak is much reduced in comparison to Fig. 3.1 considering the much higher specific activity. The radioactivity is mainly recovered in the second peak consisting of glycerol and monooleoylglycerol.

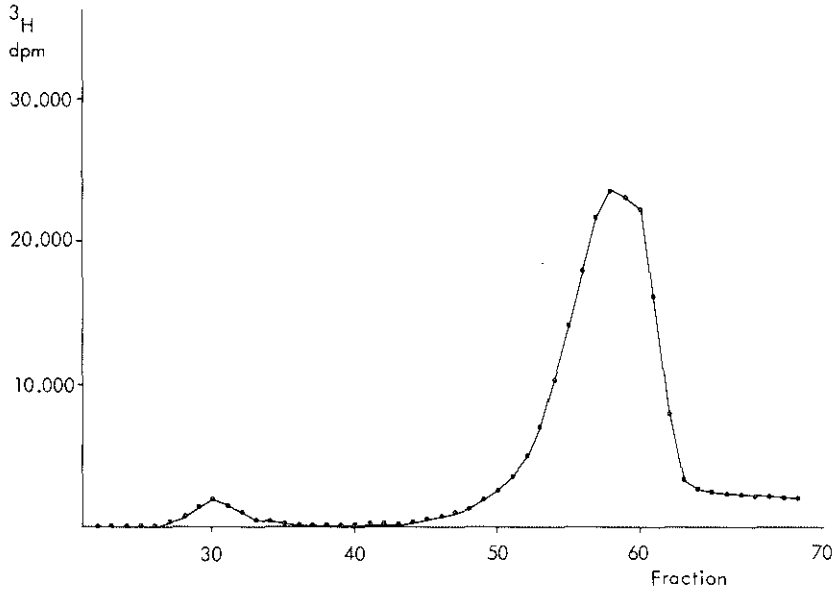

Fig. 3.2 Sephacryl-S 200 column. Elution pattern of tritiated materials in a 100,000 g supernatant after mixing with monooleoyl[2-^{3}H]glycerol in acetone. The conditions are described under Methodological Remarks (experiment 2).

3.4. DISCUSSION

These experiments show that at least by the methods used for the detection of the FABP, no analogous protein

for the binding of monoacylglycerol can be detected. Therefore, there is no experimental evidence for the assumption that a monoacylglycerol binding protein is involved in the direction of monoacylglycerol metabolism. These results show also that the FABP itself is not involved in the transport of monoacylglycerol. This conclusion was also reached by Marubbio *et al.* [6].

About the mechanism of transport of monoacylglycerol only speculations can be made. It is possible that monoacylglycerol just dissolves in the cytosol and diffuses to the endoplasmic reticulum, where it can diffuse further much more easily along the bilayer of the lipids of the membrane. The solubilization from the microvilli can be facilitated by the presence of bile salts which are absorbed in the duodenum and proximal jejunum by passive diffusion [7], albeit much more slowly than the fatty acids. Maybe such a process will hold also for the transport of the fatty acids, because the FABP may be more involved in the metabolism of acyl-CoA than of fatty acids [4,8].

The results of the experiments show that the hydrolysis of monoacylglycerol can proceed in the cold at a remarkable rate, which may become an important factor in the evaluation of experiments with loaded microsomes.

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MONOACYLGLYCEROL HYDROLASE ACTIVITY OF ISOLATED RAT SMALL INTESTINAL EPITHELIAL CELLS

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Summary

1. Albumin is the preferred stabilizer of the higher monoacylglycerol substrates, since the highest activity was measured with albumin rather than with Triton X-100 or other detergents tested.

2. The monoacylglycerol hydrolase activity may be strongly influenced by the amount of albumin used as the only emulsifier. Possible models for the physical states of the substrate are discussed.

3. The reaction rates with 1- and 2-monoacylglycerols are generally similar, but may vary according to the physical states of the substrates.

4. The same enzyme hydrolyzes both 1- and 2-isomers since the hydrolytic activities were found to be competitive rather than additive. For both isomers identical apparent K_m values <0.1 mM were obtained.

5. A comparison of the rates of hydrolysis of 1- and 2-monopalmitoylglycerol by the villus preparation at various temperatures confirmed that generally the reaction rates are similar and that the energy of activation is about 15 kcal/mol, so that the Q_{10} is about 1.8.

6. It is speculated that the microsomal level of long-chain acyl-CoA is an important determinant in the fate of the resorbed monoacylglycerol, since acyl-CoA is not only a substrate for the reacylation reactions but also an inhibitor of monoacylglycerol hydrolase.

Introduction

An enzyme that splits monoacylglycerols inside intestinal mucosa cells was first described by Schmidt et al. [1] and raised much attention at the time that Clark and Hübscher [2] discovered the monoacylglycerol pathway for triacylglycerol synthesis [3,4]. The very fact that up to 80% of the triacylglycerols in the mucosa cells are formed via the monoacylglycerol pathway [5] implies that the function of monoacylglycerol hydrolase cannot be the complete hydrolysis

of absorbed 2-monoacylglycerol, an endproduct of luminal triacylglycerol hydrolysis by pancreatic lipase [6,7]. The intestinal monoacylglycerol hydrolase was therefore thought to have an ancillary function for the hydrolysis of monoacylglycerols that are present in the cell and cannot be acylated because of fatty acid deficit. In that case the monoacylglycerols are split and generate free fatty acids for triacylglycerol synthesis [8]. However, this picture is too simple now it has been demonstrated that in vitro the enzyme removes more than some lost monoacylglycerols. In experiments designed for monoacylglycerol acylation, the monoacylglycerols were hydrolyzed up to 50% [4,5,9], a reason for utilizing the ether analogue for acylation studies instead. More information about the function of the enzyme was obtained in substrate specificity studies [10,11]. A rather broad spectrum of substrates was found, which could justify the classification of the enzyme as an esterase. However, since long-chain fatty acids in 1- or 2-ester linkage with glycerol are hydrolyzed [10,19], the enzyme may be called monoacylglycerol hydrolase. Since monodecanoylglycerol, only sparingly soluble in water, appears to be the best monoacylglycerol substrate, the enzyme may be considered as a true lipase [12].

Senior and Isselbacher [10] noted that although in dilute cellsap both 1- and 2-monopalmitoylglycerol are hydrolyzed, the rate of hydrolysis of 1-isomer in taurocholate media is slower. This is important, since 2-monoacylglycerols are preferentially used for triacylglycerol synthesis in the monoacylglycerol pathway [13]. Since bile salt resorption mainly takes place in the distal ileum, whereas fat resorption takes place mainly in duodenum and proximal jejunum, the question whether 1-monoacylglycerol is preferentially hydrolyzed, compared to the 2-isomer, also when bile salts are absent, remains important. The 1-isomers might accumulate during fatty acid shortage by transesterification of the 2-isomers. This process might be slow at neutral pH and for the higher fatty acid derivatives, but considerable for rather short fatty acid derivatives (C_8—C_{12}), which might accumulate faster by low acylation rates. Therefore the system might aid the deposition of medium-chain fatty acids in the portal vein, while higher acylglycerols are conveyed to the lymphatic system as chylomicrons.

An assay system for monoacylglycerol hydrolase has been described for the enzyme in intestinal mucosa cells [10,11] and in other tissues [14—17]. The substrate, mostly monooleoylglycerol, was dispersed with a variety of emulsifiers. For the intestinal enzyme it has been described [10,11] that the emulsifiers were all more or less inhibitory and that bovine serum albumin gave the best results [10]. Therefore in the present study albumin will mainly be used as emulsifier and 1- and 2-monopalmitoylglycerol as the substrate, since shorter and unsaturated fatty acids are known to undergo non-enzymatic transesterification much more rapidly.

Materials and Methods

Radioactive materials were purchased from the Radiochemical Centre (Amersham, England). Bovine serum albumin (fraction V), pancreatic lipase (type II) and phospholipase C from *Clostridium Welchii* (type I) were from Sigma (St. Louis, Mo., U.S.A.). All chemicals used were analytical grade.

Villus cells were isolated by the vibration method of Harrison and Webster [18] in 0.15 M NaCl, 5 mM EDTA, 10 mM Tris · HCl (pH 7.4) and 2 mM $MgCl_2$. Cells isolated this way were free from luminal contents [19]. After homogenization in a Potter-Elvehjem teflon-glass homogenizer (10 up and down strokes) in 0.25 M sucrose, 10 mM Tris · HCl (pH 7.4) and 1 mM EDTA and centrifugation for 2 min at 500 × *g*, the supernatant (postnuclear supernatant) was collected and kept frozen (−20°C) in batches until used as the enzyme source. Protein was determined by the method of Lowry et al. [20]. Bovine serum albumin was made fatty acid free by the charcoal method of Chen [21] and the pH adjusted to 7.4. Monooleoyl[2-^{3}H]glycerol or [1-^{14}C]palmitoylglycerol was prepared from [2(*n*)-^{3}H]glycerol trioleate, respectively tri[1-^{14}C]palmitate according to Mattson and Volpenhein [22] and purified by silicic acid chromatography. Thin-layer chromatography on silica G plates (Merck), impregnated by spraying with boric acid and developed with chloroform/methanol (95 : 5, v/v) as solvent, revealed a mixture of 1-monooleoyl- and 2-monooleoylglycerol (88 and 12%, respectively; ref. 25). *sn*-1-Palmitoyl 2-[9,10-^{3}H]palmitoylglycerol was prepared from *sn*-1-palmitoyl 2-[9,10-^{3}H]palmitoyl 3-phosphorylcholine glycerol by the action of phospholipase C (EC 3.1.4.3) as described by Hanahan and Vercamer [23]. The product was kept in hexane at −20°C after dilution with non-labelled material (obtained from Serdary, London, Ontario, Canada), to prevent transesterification to 1,3-dipalmitoylglycerol, until used. From it [9,10-^{3}H]palmitoylglycerol was prepared by incubation with pancreatic lipase [22], followed by extraction with chloroform/methanol as described by Bligh and Dyer [24] and chromatography on silicic acid G plates with hexane/diethyl ether/acetic acid (30 : 70 : 1, v/v) to isolate the monoglyceride (R_F 0.11). After elution the material was applied on boric acid-impregnated silicic acid G plates and kept dry. Just before use the latter chromatogram was developed with chloroform/methanol (95 : 5, v/v) to separate 1- from 2-[9,10-^{3}H]palmitoylglycerol (R_F values 0.22 and 0.46, respectively). The palmitoylglycerol isomers labelled in the glycerol portion with ^{3}H were prepared as indicated by Mattson and Volpenhein [25]: 1,3-benzylidene-[^{3}H]glycerol was made according to Hibbert and Carter [26] and was acylated using freshly distilled palmitoylchloride [27]. The blocking group was removed using trimethylborate as described by Serdarevich and Caroll [28]. The 1- and 2-isomers were again separated and purified on boric acid-impregnated silicic acid thin-layer plates [28]. After elution of the monoacylglycerols from the silicic acid by diethyl ether the solvent was removed under reduced pressure and the residue either redissolved in diethyl ether and added to bile salt or albumin solutions, as described, followed by removal of the diethyl ether in a rotating evaporator or by directly adding bile salt or albumin solutions to the dry residue. In both instances sonification (1 min per ml in a Branson S75 sonifyer set at position 1 or in a MSE sonic disintegrator at 21 kHz) followed before the substrates were used in the hydrolase assays. These were carried out in a final volume of 0.2 ml containing 50 mM Tris · HCl (pH 7.5—8.5 as indicated), 25—100 μg enzyme protein, bovine serum albumin or sodium deoxytaurocholate (Calbiochem, San Diego, U.S.A.), as indicated. After 5 and/or 10 min incubation (at 37°C unless specified otherwise) the reactions were stopped depending whether labelled glycerol or labelled free fatty acids were to

be isolated for counting. In the first case, when albumin was present, the reaction was stopped with an equal volume of 10% trichloroacetic acid which causes coprecipitation of glycerol derivatives with denatured albumin (as was worked out for trioleoylglycerol hydrolysis by Schotz and Garfinkel [29]), that can be removed by centrifugation. A sample of the aqueous supernatant was mixed directly (or after extraction with water-saturated *n*-butanol to remove traces of monoacylglycerols thereby lowering the blank values for which corrections were always made) with Instagel (Packard). In some experiments glycerol was separated from residual glycerol derivatives by stopping the reaction with 3.5 ml of a mixture of chloroform/methanol/heptane (1.25 : 1.41 : 1, v/v) followed by mixing with 1.05 ml 2% (w/v) NaCl, exactly as described by Tornqvist et al. [30], to obtain the glycerol-containing upper-phase after centrifugation. If the fatty acid moiety of the glycerol derivative was labelled instead of the glycerol portion, the reaction was stopped with chloroform/methanol/heptane, as just mentioned and the NaCl solution was replaced by 1.05 ml borate buffer (0.05 M potassium carbonate and 0.05 M boric acid brought to pH 10.5 with 6 M KOH). This buffer extracts fatty acid into the aqueous phase [31]. These monoacylglycerol hydrolase assays were compared and found to give identical results (not shown). In the assays less than one-third of the substrate added was broken down. The specific activities of the substrates were determined by counting of samples on the one hand and determination of glycerol [32] after saponification on the other hand.

Results and Discussion

Choice of incubation conditions for small intestinal monoacylglycerol hydrolase

The pH vs. activity curve obtained for postnuclear supernatant catalyzed hydrolysis of monooleoylglycerol was almost identical to that obtained by Pope et al. [11] (data not shown). Although the optimal activity was between pH 8.5 and 9.0, the enzyme activity assays in further experiments were carried out between pH 7.5 and 8.5 to limit the rate of spontaneous isomerization of 2-monoacylglycerols to 1-monoacylglycerols.

In agreement with others albumin was found to be the preferred stabilizer of the monoacylglycerol emulsion. The activity was four times higher than with emulsions made with Triton X-100 according to Tornqvist et al. [30]. Moreover, it appeared that detergents in general may be inhibitory, as will be discussed later.

Relatively high albumin concentrations, compared to the monoacylglycerol concentrations used, were found to inhibit the hydrolytic activity. This is shown for a number of experiments and different substrates in Fig. 1 and in Table I. According to Arvidsson's model [33,34] for ligand binding to albumin, monoacylglycerol can bind in two ways. When the molar ratio ($\bar{N}$) of monoacylglycerol to albumin is low monoacylglycerols are bound to the hydrophobic regions of the albumin. When $\bar{N}$ is large, monoacylglycerols are arranged on albumin in a micellar fashion. The concentration of the unbound form of monoacylglycerol is not dependent on the total monoacylglycerol concentration, but only on the molar ratio of total monoacylglycerol to albumin, $\bar{N}$ [33]. In Fig. 1 it can be seen that when $\bar{N}$ is held constant, and therefore the

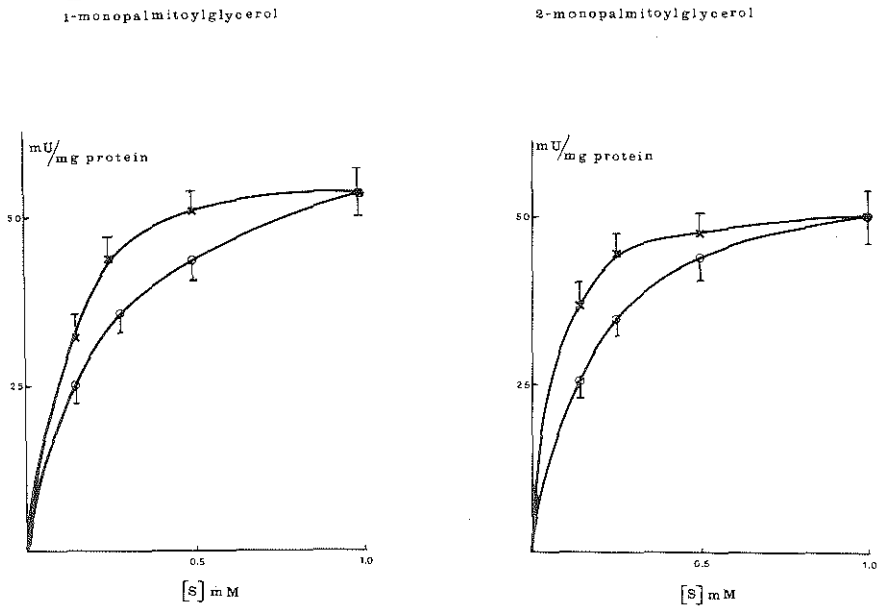

Fig. 1. Saturation curves for 1- and 2-monopalmitoylglycerol hydrolysis. Postnuclear villus supernatant was incubated at various concentrations of substrate either complexed with a constant amount of albumin (0.25 mM) (O———O) or with a variable amount of bovine serum albumin so that the molar ratio of monoacylglycerol to bovine serum albumin was constant (X———X) A, (1-monopalmitoylglycerol) ratio = 4; B, (2-monopalmitoylglycerol) ratio = 3.2. Incubations were carried out at pH 8.0; see Materials and Methods (± S.E.; $n = 4$).

concentration of the unbound form, the rate of hydrolysis varies with the total concentration, indicating that the enzyme mainly hydrolyses monoacylglycerol bound to albumin. The variation of enzyme activity with $\bar{N}$ at constant monoacylglycerol concentration (Table I) can be explained in two different ways.

TABLE I

INHIBIITION OF MONOACYLGLYCEROL HYDROLASE ACTIVITY BY EXCESS ALBUMIN

Incubation was with 50 μg postnuclear supernatant protein of homogenized isolated rat jejunal epithelial cells for 10 min at pH 8.5 and 37°C. The substrate concentration was kept constant, as indicated, while the albumin concentration was varied. $\bar{N}$ denotes the molar ratio of monoacylglycerol to bovine serum albumin.

Monooleoylglycerol $[S] = 1$ mM			1-Monopalmitoylglycerol $[S] = 0.45$ mM		
[Albumin]	$\bar{N}$	munits/mg protein ± S.E. ($n = 3$)	[Albumin]	$\bar{N}$	munits/mg protein ± S.E. ($n = 4$)
0.5	2	45 ± 3	0.45	1.0	32.6 ± 2.3
0.2	4	82 ± 5	0.30	1.5	34.3 ± 2.3
0.14	7	87 ± 5	0.15	3.0	40.6 ± 3.3
0.10	10	105 ± 7	0.075	6.0	42.4 ± 3.3
0.06	16.6	128 ± 7			
0.05	20	141 ± 15			

(1) If the first monoacylglycerol molecule were better bound to albumin than the second one etc., the average binding constant would become less at increasing $\bar{N}$, while the number and size of the micelles would increase, allowing a higher degree of saturation of the enzyme. In the v vs. S plot at constant albumin concentration (Fig. 1) no sigmoidicity is observed, which suggests that even at low $\bar{N}$ the affinity of the enzyme is higher than that of albumin for the substrate. Indeed, at least compared to the higher free fatty acids, the glycerol esters of the higher fatty acids bind less strongly to albumin [33,34], so that the product of the hydrolytic reaction is preferentially bound. (2) The enzyme might hydrolyze the monoacylglycerol while it is bound to albumin. The first molecule bound might be more hidden in the structure than the second molecule. This also would lead to a higher substrate availability at the higher $\bar{N}$ values. Also here the availability could level off again when micellar formation augments. Whatever model applies, it is clear that the Michaelis constant has only a relative significance (as applies to all lipases) and will depend largely on the physical state of the substrate. When later an apparent K_m of 0.05 mM is presented (compare also Fig. 1, which suggests a $K_m < 0.1$ mM) it is clear that the true affinity of the enzyme for the substrate is lower but may be approached best when low substrate, complexed to low albumin concentrations are used.

Although, as will be presented below, the apparent K_m for both substrates is similar, it has to be excluded that small intestinal villus postnuclear supernatant has more than one monoacylglycerol hydrolase, perhaps one that preferentially hydrolyzes the 1-isomer and one for the 2-isomer. Therefore, firstly a test was carried out to establish that there is a true 2-monoacylglycerol hydrolase activity, since transesterification of the 2- to the 1-isomer is difficult to exclude.

True 2-monoacylglycerol hydrolase activity and competition between 1- and 2-monopalmitoylglycerol

It has been amply demonstrated that pancreatic lipase has only 1,3-esterolytic activity [12] and that rat intestinal jejunum cells do not possess

TABLE II

PALMITATE RELEASED FROM THE 2-POSITION OF 1,2-DIPALMITOYLGLYCEROL

Dipalmitoylglycerol [1,2] labelled in the 2-position with [^{2}H]palmitic acid was prepared from dipalmitoyl phosphatidylcholine as described under Materials and Methods. Specific activity of the final substrate used was 0.07 Ci/mol. In Expt. 1 the final diacylglycerol concentration was 1.4 mM and 2.8 mM in Expt. 2. Further additions 0.5 mM sodium deoxytaurocholate, 0.5 mM bovine serum albumin, 0.15 mM Tris · HCl pH 8.5, 1.25 mM $CaCl_2$ and 50 μl pancreatic lipase (0.3 mg protein/ml) and/or 50 μl postnuclear supernatant of isolated rat small intestinal villus cells (1.7 mg protein/ml) in a final volume of 0.2 ml. After 10 and 20 min incubation the reactions were stopped with 3.25 ml methanol/chloroform/heptane and fatty acid extraction carried out with borate buffer as described under Materials and Methods. Results are expressed in nmol/min per ml enzyme.

	Expt. 1	Expt. 2
Pancreatic lipase	1.5	3.2
Postnuclear supernatant	0.0	0.6
Pancreatic lipase + postnuclear supernatant	5.4	10.0

TABLE III

COMPETITION BETWEEN 1- AND 2-MONOPALMITOYLGLYCEROL HYDROLYSIS IN RAT SMALL INTESTINAL VILLUS POSTNUCLEAR SUPERNATANT

Postnuclear supernatant was incubated with 2-[9,10-^{3}H]palmitoylglycerol and/or 1-[1-^{14}C]palmitoylglycerol at the indicated concentrations, complexed with 0.33 mM bovine serum at pH 8. The reactions were stopped with methanol/chloroform/heptane and fatty acids extracted by borate buffer as described under Materials and Methods.

Substrate	Palmitate release (munits/ml enzyme) ± S.E. (n = 3)	
	β-position (^{3}H release)	α-position (^{14}C release)
0.2 mM 2-[^{3}H]palmitoylglycerol	12.2 ± 1.1	—
0.4 mM 2-[^{3}H]palmitoylglycerol	13.2 ± 1.1	—
0.2 mM 1-[^{14}C]palmitoylglycerol	—	8.1 ± 0.8
0.4 mM 1-[^{14}C]palmitoylglycerol	—	9.0 ± 0.8
0.2 mM 1-[^{14}C]palmitoylglycerol + 0.2 mM 2-[^{3}H]monopalmitoylglycerol	6.2 ± 0.6	4.7 ± 0.5
0.13 mM 1-[^{14}C]palmitoylglycerol + 0.27 mM 2-[^{3}H]monopalmitoylglycerol	4.3 ± 0.6	5.7 ± 0.5

hydrolytic activity towards higher di- and triacylglycerols [10]. Therefore it was tested whether the 2-position of 1,2-dipalmitoylglycerol is attacked by the joint action of pancreatic lipase and postnuclear supernatant of small intestinal villus epithelial cells. It can be seen from Table II that pancreatic lipase has only weak activity in the release of the radioactive labelled palmitic acid fixed at the 2-position of the diacylglycerol (the small activity may be due to the presence of a small amount of 1,3-diacylglycerol formed during storage of the 1,2-isomer by transesterification (compare ref. 10). No activity is present in the postnuclear villus cell supernatant, as expected. The two activities are more than additive, which strongly supports that the two enzymes supplement each other: the pancreatic enzyme provides 2-monoacylglycerol and the villus enzyme hydrolyzes this product. The experiment of Table III suggests that the liberation of palmitic acid linked to either the 1- or the 2-position of glycerol is competitive rather than additive, suggesting the presence of one enzyme which may hydrolyze both 1- and 2-monoacylglycerols.

Preferential hydrolysis of 1- and 2-monoacylglycerols

Senior and Isselbacher [10] noticed that in taurocholate media the enzymatic hydrolysis of 2-palmitoylglycerol was slower than that of 1-palmitoylglycerol. This, for slightly different conditions, is confirmed by us (Table IV), whether albumin was present or not. This does not necessarily mean a difference in affinity for the enzyme for the two substrates, but may be the result of different physical states. Hofmann and Borgström [35] pointed out that considerable physical differences are found between the two isomers, the latter being much more soluble in bile salts than the former. Plotting of the data of Table IV as the inverse velocity vs. inverse monoacylglycerol concentration revealed identical K_m values for both 1- and 2-palmitoylglycerol, in the

TABLE IV

COMPARISON OF THE RATE OF HYDROLYSIS OF 1- AND 2-MONOPALMITOYLGLYCEROL IN THE PRESENCE OF TAURODEOXYCHOLATE

1- or 2-monopalmitoylglycerol was incubated at various concentrations complexed with 0.3 mM taurodeoxycholate in the presence or absence of 0.04 mM albumin. Incubations were carried out at pH 8.0. See Materials and Methods.

Concentration of substrate (mM)	Rate of hydrolysis (munits/mg protein ± S.E.) (n = 4)	
	+ albumin (0.04 mM)	—albumin
1-Monopalmitoylglycerol		
0.16	33.1 ± 3.1	29.5 ± 5.5
0.08	26.6 ± 2.8	23.1 ± 4.7
0.04	20.2 ± 2.1	16.1 ± 2.1
0.02	13.7 ± 2.8	11.1 ± 2.0
2-Monopalmitoylglycerol		
0.16	27.6 ± 3.1	14.8 ± 2.0
0.08	21.5 ± 4.2	12.5 ± 2.0
0.04	16.5 ± 3.8	9.2 ± 2.0
0.02	10.7 ± 3.2	5.9 ± 1.9

absence or presence of albumin: 0.05 ± 0.015 mM. That the physical state of the 1- and 2-palmitoylglycerol solutions used was different was obvious since the mM stock solution of the 1-isomer was not clear in contrast to that of the 2-isomer (at room temperature) whether sonicated in neutral mM sodium deoxytaurocholate or albumin. When the 1-monoacylglycerol solutions were heated to 50°C clarification took place. Table IV shows that albumin had little or no effect on the rate of hydrolysis of the 1-isomer while the rate of 2-palmitoylglycerol hydrolysis was stimulated, possibly by removal of 2-palmitoylglycerol from micells by albumin. No further experiments were undertaken since it was realized that the intestinal cell contains no albumin. It is not likely that the cell contains any monoacylglycerol binding protein, while we were not able to show any binding to a protein with a molecular weight <240 000 (unpublished observations). Moreover the concentration of bile salts, at least in jejunum, will be small because bile resorption preferentially takes place in the lower ileum.

Effect of temperature on the rate of reaction

Since 1-monopalmitoylglycerol compared with 2-monopalmitoylglycerol suspensions were often turbid, which could be clarified by heating to 45—50°C, the reaction temperature was varied. An Arrhenius plot (log v vs. $1/T$) revealed a practically linear relation (Fig. 2). This indicates that the observed turbidity had no influence on the reaction rate of the enzyme towards 1-monopalmitoylglycerol. Calculation of the energy of the activation and of the Q_{10} gave average values of 15.2 kcal/mol and of 1.8, respectively, which is rather low in comparison to the adipose tissue enzyme [36].

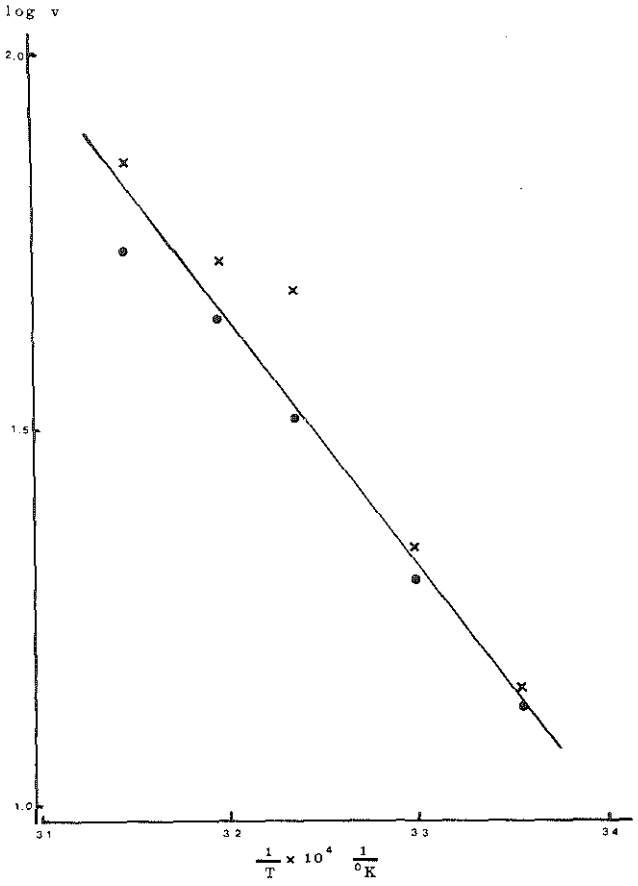

Fig. 2. Arrhenius plot of the hydrolysis of 1- and 2-monopalmitoylglycerol. 1.0 mM 1-monopalmitoylglycerol (X———X) or 0.9 mM 2-monopalmitoylglycerol (●———●), complexed with 4 mM bovine serum albumin, were incubated at pH 7.5 at various temperatures. Incubations were carried out as described under Materials and Methods.

Inhibition of monoacylglycerol hydrolase activity by naturally occurring detergents

It can be seen from Table V that a number of naturally occurring amphipatic molecules [35] strongly inhibits monoacylglycerol hydrolase activity when the substrate is bound to albumin. It is likely that the physical state of the substrate is influenced which results in an inhibition, as is the case with Triton X-100 [30]. Although the solubilities of 1- and 2-monopalmitoylglycerol in bile salts are different, the hydrolysis of both isomers bound to albumin is inhibited by detergents to the same extent. It is likely that under the experimental conditions used, the binding properties of both isomers to albumin are the same. Preliminary data (not shown) suggest that long-chain acyl esters like palmitoyl-CoA or palmitoyl-carnitine contribute to the inhibition of the hydrolase activity by interaction with the enzyme itself. Since high concentrations of acyl-CoA may be expected to accumulate in the microsomes where the bulk of fatty acid activation occurs (compare refs. 10 and 37), it is possible [8]

TABLE V

ACTIVITY OF NATURALLY OCCURRING DETERGENTS ON MONOACYLGLYCEROL HYDROLYSIS BY RAT SMALL INTESTINAL VILLUS POSTNUCLEAR SUPERNATANT

0.4 mM 2-monopalmitoylglycerol or 1-monopalmitoylglycerol complexed with 0.05 mM bovine serum albumin was incubated with 200 μg protein from a postnuclear supernatant of a rat small intestinal villus cell homogenate, in the presence of the detergents mentioned, in 50 mM Tris · HCl, pH 8.0. After 5 and 10 min the reactions were stopped by the method of Tornqvist et al. [30] as described under Materials and Methods. The velocity with 2-monopamitoylglycerol was (± S.E.) 34.9 ± 1.0 munits/mg protein 100%) and with 1-monopalmitoylglycerol 41.2 ± 1.2 munits/mg protein (100%). $n = 4$.

Detergent used (final concentration)	Relative hydrolytic activity (%)	
	2-Monopalmitoyl-glycerol	1-Monopalmitoyl-glycerol
None	100	100
0.38 mM taurodeoxycholate	74 ± 6	73 ± 3
0.38 mM palmitoyl-CoA	23 ± 2	27 ± 3
0.38 mM palmitoyl-carnitine	62 ± 3	53 ± 3
0.38 mM potassium palmitate	56 ± 6	63 ± 8
0.25 mM lysolecithin	72 ± 6	78 ± 3

that long-chain acyl-CoA promotes monoacylglycerol acylation by inhibiting its hydrolysis. This, together with the nature of the inhibition, will be discussed in detail elsewhere.

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CHAPTER V PARTIAL PURIFICATION AND PROPERTIES OF MONOACYLGLYCEROL LIPASE AND TWO ESTERASES FROM ISOLATED RAT SMALL INTESTINAL EPITHELIAL CELLS

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SUMMARY

In the intestinal epithelial cells three enzymes, possessing monoacylglycerol hydrolase activity, were found and partially purified. Two of these enzymes have properties that justify their classification as an esterase and one as a monoacylglycerol lipase. The three enzymes show similar K_m values for monooleoylglycerol and each shows similar activity towards 1- and 2-monopalmitoylglycerol. Antiserum raised in rabbit against rat liver monoacylglycerol lipase inhibits the intestinal lipase completely, suggesting that the enzymes are at least partially similar. The esterases of small intestinal villus cells are not inhibited by the antiserum against liver monoacylglycerol lipase. It was calculated that the esterases account for approximately 2/3 of the monooleoylglycerol hydrolase activity in the epithelial cells. Monoacylglycerol lipase also hydrolyzes palmitoyl-CoA, while the esterases do not. The enzymes are inhibited by micellar palmitoyl-CoA. The hypothesis that palmitoyl-CoA is an important regulator for monoacylglycerol acylation is discussed in the light of these new findings.

INTRODUCTION

Some properties of monoacylglycerol hydrolase activity were recently described [1]. Special attention was paid to the observed inhibition of the hydrolase activity by palmitoyl-CoA. Brown and Johnston [2] postulated that the hydrolysis of monoacylglycerol is important only when there is a fatty acid shortage relative to monoacylglycerol. Therefore it may be that the hydrolase activity is regulated by cellular levels of long-chain acyl-CoA: i.e. when the rate of triacylglycerol synthesis is maximal, minimal hydrolysis of monoacylglycerol is expected to occur [2].

Our assay conditions, in which monoacylglycerol is bound to albumin are very sensitive to detergent action, due to an interaction with the substrate [1]. Direct interaction of long-chain acyl-CoA with the enzyme itself was not studied. Therefore, the observed inhibition of the monoacylglycerol hydrolysis by acyl-CoA may have been artifactuous, because no albumin is present in the cell and, moreover, unphysiologically high concentrations of acyl-CoA were used. In order to investigate the possibility of a direct action of palmitoyl-CoA on the enzyme, we felt that enzyme purification was a prerequisite for such a study.

Several lipases and esterases have been described in the literature. In rat small intestinal epithelial cells, some properties of monoacylglycerol hydrolase activity were reported by Senior and Isselbacher [3]. Other laboratories described the partial purification of monoacylglycerol hydrolase activity [4] or esterase activity [5-7]. Recently, monoacylglycerol lipase from liver has been purified in our laboratory [8] and an antiserum was raised against this enzyme. Ikeda *et al.* [9] also described a monoacylglycerol lipase from liver. Most laboratories used detergents for the solubilization of the enzymes from microsomes, and stated that the activity can be preserved and eluted from

DEAE-columns by the use of detergents. Because it was our aim to study the effects of palmitoyl-CoA, which is a detergent itself, on enzyme activity, we decided to solubilize the enzyme activity by means of delipidation instead, since detergents may mutually influence each other.

MATERIALS AND METHODS

Radioactive materials were purchased from the Radiochemical Centre (Amersham, England). Monooleoyl[2-^{3}H]-glycerol and 1- and 2-monopalmitoyl[2-^{3}H]glycerol were prepared as described previously [1]. Bovine serum albumin (fraction V, Sigma) was made fatty acid-free by the method of Chen [10]. Monocaproylglycerol was a gift of Dr. U.M.T. Houtsmuller (Unilever Research Laboratories, Vlaardingen). All monoacylglycerols were purified by thin layer chromatography, using Silica gel G plates (Merck) which were developed with $CHCl_3/CH_3OH$ (96:4 v/v). Palmitoyl-CoA was made according to Stoffel *et al.* [11].

Villus cells from male Wistar rats were isolated by the vibration method of Harrison and Webster [12] in 0.15 M NaCl/5 mM EDTA/10 mM Tris-HCl (pH 7.4). Cells isolated this way were free from luminal contents [13]. Homogenization was performed in a Potter-Elvehjem Teflon-glass homogenizer (10 strokes) in 0.25 M sucrose/10 mM Tris-HCl (pH 7.4)/1 mM EDTA. After removal of the nuclei, brushborders and mitochondria, microsomes were prepared by centrifugation for 30 min at 200 000 x g.

For the partial purification of the enzyme, villus cells of the intestines of 9 rats were isolated and homogenized. A total particulate fraction was obtained by centrifugation for 1 h at 100 000 x g. The pellet was suspended in 25 ml homogenization buffer and delipidated as outlined by Morton [14]. The remaining pellet was

extracted for 1 h with 25 ml 0.05 M NH_4Cl-NH_4OH buffer (pH 8.5). Protein, which precipitated between 40 and 70% $(NH_4)_2SO_4$ saturation, was dissolved in 50 mM Tris-HCl (pH 8.0) and placed on a Sephadex-G 200 column (1.5 x 90 cm) and eluted with the same buffer. The flow rate was 0.1 ml/min, and 2 ml fractions were collected. Monoacylglycerol hydrolase activity was eluted in a single peak. The peak fractions were pooled and an aliquot was placed on a DEAE-A50 column (2.5 x 15 cm). Firstly, protein was eluted with 50 mM Tris-HCl (pH 8.0)/0.1 M NaCl, then a linear salt gradient of 0.1-0.5 M NaCl in the same buffer was applied. 2 ml fractions were collected.

Routine monoacylglycerol hydrolase assay media [1] consisted of 0.1 ml 12.5 mM monooleoyl[2-^{3}H]glycerol, complexed to 0.75 mM albumin by sonication (1 min/ml in a Branson S75 sonifier set at position 1), 0.1 ml 0.5 M Tris-HCl (pH 8.0), additions as indicated, less than 100 µg enzyme protein and water to a final volume of 0.5 ml. After 5 or 10 min incubation at 37°C the reaction was stopped with 0.5 ml 10% trichloroacetic acid, which caused coprecipitation of acylglycerols with denatured albumin [15]; this was removed by centrifugation. A sample of the aqueous supernatant was mixed with Instagel (Packard) and counted in a liquid scintillation counter. Lineweaver-Burk plots were obtained from experiments that were performed with a constant molar ratio of substrate to albumin of 16.6 [1]. For assays with 1- or 2-monopalmitoyl[2-^{3}H]glycerol, 0.1 ml of 5 mM substrate complexed to 0.75 mM albumin was used. p-Nitrophenylacetate hydrolase activity was assayed according to Huggins and Lapides [16]; palmitoyl-CoA hydrolase activity as described by Jansen and Hülsmann [17], using 20 µM palmitoyl-CoA; 4-methylumbellipherylnanonate hydrolase activity according to Cortner *et al.* [18] and tributyrin hydrolase activity was measured titrimetrically at pH 8.0 in a Radiometer (Copenhagen), pH-stat with 0.0085 M NaOH. The pH-stat was also used for measuring the influence of palmitoyl-CoA on the hydrolysis of 2 mM mono-

caproylglycerol.

Protein was determined by the method of Lowry *et al.* [19], using albumin as a standard.

Polyacrylamide gel electrophoresis was performed according to Maurer [20] in 7.5% polyacrylamide at pH 9.5 with 3 mA per rod. Esterase activity was detected by staining with α-naphthylacetate according to Allen *et al.* [21] and protein was stained with Coomassie blue.

Rabbit antiserum against rat liver microsomal monoacylglycerol lipase was prepared by the method of Oerlemans *et al.* [8]. Enzyme preparations were preincubated during 3 h at 4°C, with an amount of rabbit antiserum to give maximal inhibition. Enzyme assays were performed with the supernatant after centrifugation for 4 min at 15 000 x g. Controls were tested with serum of rabbits that were not immunized. Corrections were made with respect to monoacylglycerol hydrolase activities in antiserum and control serum.

RESULTS

Enzyme purification

In Table I a summary of the purification procedure is shown. Approximately 85% of the total monoacylglycerol hydrolase activity is particulate. Although it is known that solubilized monoacylglycerol hydrolase and esterases are stabilized in the presence of Triton X-100 [18,9,7] we decided not to use any detergent, because of interference with the enzyme assays [1], and to delipidate the particulate fraction instead. 53% of the total particulate activity can be extracted with NH_4Cl-NH_4OH buffer after delipidation. The solubilized esterase activity, as measured with 4-methylumbellipherylnanonate or p-nitrophenylacetate as substrates, could be almost completely precipitated between 40 and 70% ammonium sulphate saturation. However, only 54%

TABLE I

SUMMARY OF THE PARTIAL PURIFICATION OF MONOACYLGLYCEROL HYDROLASE ACTIVITIES

	Total protein mg	Total activity U	U/mg protein	Purifi-cation	Yield %
Homogenate	1267	163	0.13	1.0	100
Particulate fraction	850	139	0.16	1.2	85
Extract of delipidated powder	177	74	0.42	3.2	46
40-70% ammonium sulphate	57	40	0.70	5.4	25
G200 pooled fractions	12.4	36	2.9	22.3	22
DEAE peak 1 fractions 35-40	1.5	11.2	7.5	58	6.8
DEAE peak 2 fractions 51-57	4.2	10.6	2.5	19	6.6
DEAE peak 3 fractions 63-68	1.5	7.4	4.9	38	4.6

of the monooleoylglycerol hydrolase activity is recovered. When subjected to Sephadex-G200 chromatography, monoacylglycerol hydrolase activity was eluted in a single peak, together with activity against 4-methylumbellipherylnanonate or p-nitrophenylacetate. In Fig. 1 the elution profile of several hydrolytic enzymes from the DEAE-column is shown. It can be seen that monoacylglycerol hydrolase activity was eluted in three peaks, peak 1 eluted at 0.125 M NaCl, peak 2 at 0.21 M and peak 3 at 0.25 M. Fractions 35-40, 51-57 and 63-68 were pooled. From the substrate specificity studies (Table II) it is clear that the enzyme activity in the first peak is due to a rather specific monoacylglycerol lipase, while the activities in the second and third peaks are due to esterases, which were denoted as esterase 1 and 2 (peaks 2 and 3, respectively). Further support for the correctness of the nomenclature is provided by the elution profile of p-nitrophenylacetate hydrolase, which was the same as 4-methylumbellipherylnanonate hydrolase (unpublished data). Peak 1 contains little of these esterase activities when compared to monoacylglycerol hydrolase activity, while the reverse is observed in peaks 2 and 3. Most of the observed protein was eluted together with peak 2, while peaks 1 and 3 contained only small amounts of protein (compare Fig. 2). The elution profile of palmitoyl-CoA hydrolase activity is also shown in Fig. 1. Two activity peaks are found: the first one, which was not absorbed on DEAE, had no hydrolase activity towards monooleoylglycerol, p-nitrophenylacetate or 4-methylumbelliperylnanonate, while the second one is cochromatographed with the monoacylglycerol lipase and will be shown to be identical. No activity was found in peaks 2 and 3.

Purity of the enzyme preparations

The purity of the enzyme preparations has been checked with polyacrylamide gel electrophoresis. The gels shown in

TABLE II

PROPERTIES OF HYDROLYTIC ACTIVITIES IN FRACTIONS SEPARATED BY DEAE-COLUMN CHROMATOGRAPHY

The conditions of the tests are described in the Materials and Methods section.

	Peak 1 (Monoacylglycerol lipase)	Peak 2 (Esterase 1)	Peak 3 (Esterase 2)
Monooleoylglycerol hydrolysis U/mg protein	7.5	2.5	4.9
p-Nitrophenylacetate hydrolysis U/mg protein	2.6	13.2	8.6
4-Methylumbellipherylnanonate hydrolysis mU/mg protein	214	1383	2996
Tributyrin hydrolysis U/mg protein	96	655	897
Monocaproylglycerol hydrolysis U/mg protein	18.8	5.4	40.1
1-Monopalmitoylglycerol hydrolysis mU/mg	650	490	456
2-Monopalmitoylglycerol hydrolysis mU/mg	579	396	325
K_m monooleoylglycerol µM	50	50	30
% Inhibition by antiserum against liver non heparin-releasable monoacylglycerol lipase [8] measured with			
monooleoylglycerol	100	4	3
palmitoyl-CoA	97	-	-
% Inhibition by E_{600} 10^{-6} M	100	100	100

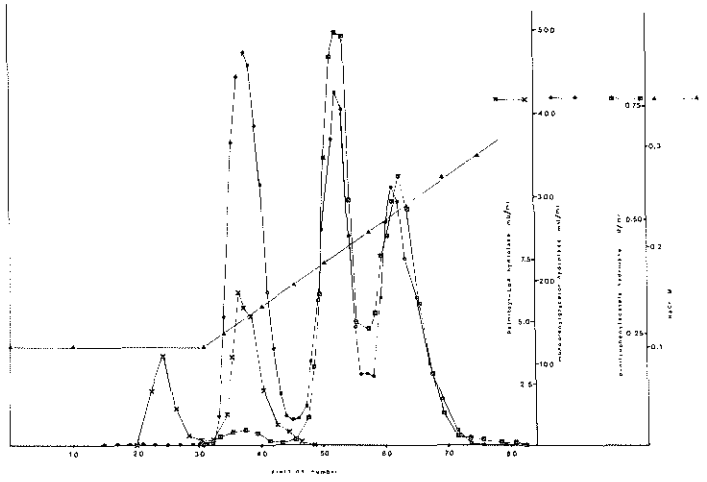

Fig. 5.1 Elution profile of DEAE column. 6.2 mg protein from the pooled G200 fractions in 50 mM Tris-HCl (pH 8.0) was applied to the DEAE column. After elution of the unbound protein with 0.1 M NaCl in the same buffer, a linear NaCl gradient was used for elution. 2 ml fractions were collected. The enzymes were assayed as described in Materials and Methods, in all fractions. ●——●, monooleoyl-glycerol hydrolase activity; ■——■, 4-methylumbellipheryl-nanonate hydrolase activity; x——x, palmitoyl-CoA hydrolase activity; ▲——▲, NaCl.

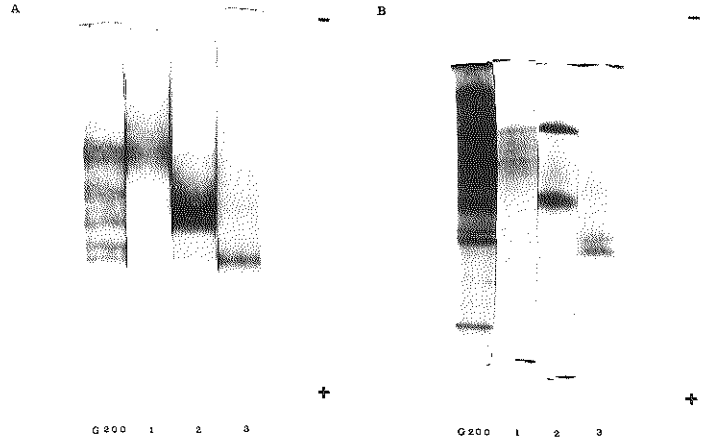

Fig. 5.2 Polyacrylamide gel electrophoresis of G200 eluate (G200) peak 1 (1), peak 2 (2) and peak 3 (3) from the DEAE column. A. Staining with α-naphthylacetate. 100 µg protein from G200 column eluate and 10-20 µg from peaks 1, 2 and 3 were applied on the gels. B. Staining with Coomassie blue. On each gel 50-100 µg protein was applied. See Materials and Methods.

Fig. 2A were stained with α-naphthylacetate, which is a non specific reagent. It is substrate for esterases and related hydrolases. The enzyme preparation derived from the Sephadex-G200 column shows three main bands. The middle and lower bands appear to be double. This is a photographic artifact due to reflection of excess chromophore. The gel was stained to show minor hydrolase activities. The main activities have been separated well on the DEAE column, as can be seen from the electrophoretic patterns of peaks 1-3. On close inspection of the pattern of peak 1, a minor α-naphthylacetate hydrolyzing band can be seen. However, both the monoacylglycerol hydrolase activity and the palmitoyl-CoA hydrolase activity were associated with the main band, while a negligible activity towards both substrates was measured in the minor band. Therefore, no further purification was performed. The enzymes were not pure with respect to protein as can be seen from the Coomassie blue staining of the gels shown in Fig. 2B. It is not certain whether the hydrolase activity will correspond to one of the protein bands.

Properties of the enzymes

As has been shown in Fig. 1, the intestinal epithelial cells contain three enzymes that are able to hydrolyze monoacylglycerol. In Table II some properties of these enzymes are described, which justify the nomenclature of the enzymes. Monoacylglycerol lipase has relatively little activity towards typical substrates for esterases like p-nitrophenylacetate, 4-methylumbellipherylnanonate and tributyrin, while esterases 1 and 2 are more than 15 times and 5 times, as active, respectively, when based on the same hydrolase activity towards monooleoylglycerol. Soluble monoacylglycerols, like monocaproylglycerol, are hydrolyzed by the lipase and both esterases; the highest activity was obtained with esterase 2. In order to evaluate the capacity of both esterases to hydrolyze monoacylglycerols, the apparent K_m for

monooleoylglycerol was determined. The enzymes showed approximately the same K_m, indicating that the affinities for this substrate were similar. No positional specificity was observed, because all enzymes showed a nearly equal rate of hydrolysis towards 1- and 2-monopalmitoylglycerol. 10^{-6} M diethyl p-nitrophenylphosphate (E_{600}) inhibited the enzyme activities of the lipase as well as of the esterases 1 and 2 (Table II).

The molecular weights of these enzymes were roughly the same as indicated by the elution profile of the Sephadex-G200 column, and were between 60 000 and 75 000 daltons, as estimated by sodium dodecyl sulphate polyacrylamide gel electrophoresis. None of these enzymes hydrolyzed long-chain di- or triacylglycerols (unpublished data).

Antibodies raised against monoacylglycerol lipase from rat liver, which was not released from liver by heparin perfusion [8], inhibited completely the monoacylglycerol lipase from small intestinal epithelium, while the two esterases were not inhibited at all (Table II). Not only was the hydrolytic activity of peak 1 (Fig. 1) towards monooleoylglycerol inhibited, but also the activity towards palmitoyl-CoA. This strongly suggested that only one enzyme was responsible for both activities, as is the case with heparin-releasable liver lipase [17].

Effects of palmitoyl-CoA

In the previous article [1] we showed that palmitoyl-CoA and other detergents were able to inhibit the hydrolysis of monoacylglycerol in a homogenate freed from nuclei, brushborders and cell debris. In this enzyme preparation the activities of the lipase and esterases were present. The effect of palmitoyl-CoA on the separate enzymes will now be reported. Peak 1 contains hydrolytic activities towards monoacylglycerol and palmitoyl-CoA. A *v vs* S curve of palmitoyl-CoA hydrolase shows that this enzyme is inhibited by excess substrate. The critical micellar concentration of palmitoyl-

CoA has been shown to be about 4 μM |22|. Because palmitoyl-CoA binds non specifically to protein, higher concentrations must sometimes be used to reach the critical micellar concentration, depending on the protein concentration used. Therefore, it seems that the lipase is inhibited by micellar palmitoyl-CoA, as is known for other palmitoyl-CoA hydrolases (compare Gatt and Bartfai, 23). The inhibition of hydrolytic activity towards monoacylglycerol and palmitoyl-CoA by the antibody used (Table II) gave a strong indication that the activities were due to one enzyme. More evidence for this was obtained from competition studies as described in Table III. The lipase has been shown to be active with monocaproylglycerol (Table II), which is soluble in water as a monomolecular dispersion to an appreciable extent. With this substrate albumin, which will interfere with substrate availability, can be omitted from the test medium and lipid-lipid interactions will be reduced as well. The effect of palmitoyl-CoA on monocaproylglycerol hydrolysis and vice versa is shown in Table III. Monocaproylglycerol hydrolysis is, indeed, inhibited by palmitoyl-CoA in true solution, as well as in micellar solution, while palmitoyl-CoA hydrolysis is inhibited by monocaproylglycerol. Competitive inhibition at low concentrations and binding to micelles at higher concentrations, was responsible for not obtaining linear Dixon plots. The hydrolysis of this substrate by esterases 1 and 2 was inhibited by micellar palmitoyl-CoA (20 μM) but not by a true solution of it (4 and 8 μM).

Albumin had a striking effect on palmitoyl-CoA hydrolase activity: it seemed to bind palmitoyl-CoA in a 1:1 molar ratio and bound palmitoyl-CoA was not available for enzyme action. Only when more palmitoyl-CoA than albumin was present could palmitoyl-CoA hydrolase activity be measured. A complex situation therefore occurs when the effect of palmitoyl-CoA on monooleoylglycerol hydrolysis is studied. The lipase and the esterases are not inhibited at all when more albumin than palmitoyl-CoA is used, but when low

TABLE III

MUTUAL INHIBITION OF PALMITOYL-CoA AND MONOCAPROYLGLYCEROL ON THEIR HYDROLYSIS BY MONOACYLGLYCEROL LIPASE

A. 2 mM monocaproylglycerol was incubated with various amounts of palmitoyl-CoA. The hydrolysis of the glycerolester was followed by titration as described in Materials and Methods.
B. 20 µM palmitoyl-CoA was incubated with various amounts of monocaproylglycerol. The hydrolysis of the CoA-ester was followed by the 5-5' dithio-bis-(2-nitrobenzoic acid) method as described by Jansen and Hülsmann [17].
The figures are averages of two experiments.

A		B	
Palmitoyl-CoA µM	Monocaproylglycerol hydrolase activity U/mg protein	Monocaproylglycerol mM	Palmitoyl-CoA hydrolase activity mU/mg protein
-	18.8	-	68.3
4	14.8	2	66.4
8	11.9	4	36.7
20	9.0	8	14.0
40	6.2		

albumin concentrations are used, inhibition does occur (Table IV). The esterases were found to be more sensitive to detergent action than monoacylglycerol lipase under the conditions of the tests. Esterase 1 especially was so sensitive that 90% was inhibited by an excess palmitoyl-CoA. The relatively low degree of inhibition of the lipase measured with monooleoylglycerol as the substrate when compared to monocaproylglycerol (Table III) can be explained by the greater affinity of this enzyme for monooleoylglycerol, as well as by a different lipid-lipid interaction of palmitoyl-CoA with the substrate used.

TABLE IV

INHIBITION OF VARIOUS MONOOLEOYLGLYCEROL HYDROLASES BY PALMITOYL-CoA

0.5 mM monooleoylglycerol, complexed to 0.03 mM albumin, was incubated with various palmitoyl-CoA concentrations. The hydrolysis by the enzymes was measured as described in Materials and Methods. Figures are rates of monooleoylglycerol hydrolysis relative to the uninhibited activity (100%).

Enzyme used	Palmitoyl-CoA				
	-	10 µM	50 µM	100 µM	250 µM
Monoacylglycerol lipase	100	98	95	87	70
Esterase 1	100	92	80	37	10
Esterase 2	100	98	80	72	44

DISCUSSION

Three enzymes from rat small intestinal epithelial cells possessing monoacylglycerol hydrolase activity have been separated by DEAE column chromatography. The enzyme that was eluted first from this column had characteristics of a lipase and was therefore called a monoacylglycerol lipase. This enzyme may be identical with the enzyme from liver that has been described by Oerlemans *et al.* [8], because antiserum against the liver enzyme inhibited the intestinal enzyme completely. The monoacylglycerol lipase from liver, purified by Ikeda *et al.* [9], shares some characteristics with the intestinal enzyme, i.e. molecular weight, indifference towards positional specificity of acyl group hydrolysis and an inability to hydrolyze long-chain triacylglycerols, as was also observed in our laboratory [24,8]. Some other characteristics are different. A different apparent K_m for monooleoylglycerol was reported, but it is known that different assay conditions will affect the K_m [1]. The liver enzyme was reported not to bind to DEAE at low ionic strength [9]. Maybe the discrepancy between that study and our findings in liver [8] and intestine is due to the different solubilization methods followed. (The liver enzyme in our studies was solubilized by delipidation instead of treatment with Triton X-100.) Ailhaud *et al.* [5-7,25] have reported on purification of an esterase from rat intestine. It is likely that this enzyme is identical with esterase 1 and 2, because it hydrolyzed p-nitrophenylacetate much faster than monooleoylglycerol and it had no activity with palmitoyl-CoA. In their studies no lipase was detected probably because of a different extraction procedure followed.

Together with the monoacylglycerol lipase a palmitoyl-CoA hydrolase activity was eluted from the DEAE column.

Like heparin-releasable liver lipase [17], intestinal monoacylglycerol lipase possessed both hydrolytic activities. The conclusion of the present study that the intestinal lipase hydrolyzes both palmitoyl-CoA and monoglycerides was based on the facts that antiserum against the lipase abolished both hydrolytic activities, that the electrophoretic mobility on polyacrylamide gel electrophoresis was identical and finally that palmitoyl-CoA and monocaproylglycerol mutually inhibited their hydrolysis. This common identity may be functionally important because *in vivo* monoglyceride hydrolysis is probably inhibited as long as palmitoyl-CoA is rapidly generated for triacylglycerol synthesis. In addition to this competitive inhibition, palmitoyl-CoA exerts another type of inhibition, the micellar inhibition. This occurs when the concentration of palmitoyl-CoA is raised above the critical micellar concentration, which was reported to be 4 μM [22]. The activity will then be limited by its own substrate. If this type of inhibition occurs *in vivo*, it will inhibit palmitoyl-CoA hydrolysis, so that triacylglycerol synthesis may proceed. Micellar palmitoyl-CoA also affected esterases 1 and 2. Little is known, however, about cellular levels of long-chain acyl-CoA. Gaginella *et al.* [26] reported control values in intestinal mucosa cells of 10 μM which were raised to 30 μM after incubation with ricinoleate. If these values represent true cellular levels, it is unlikely that much micellar long-chain acyl-CoA will be present, because part of it will bind to cellular proteins, especially to the fatty acid binding protein [27]. Although the local concentrations at the microsomes could be much higher, because the bulk of long-chain acyl-CoA is synthesized [28] and used in the microsomes, the observed inhibition of the hydrolysis of monoacylglycerol bound to albumin by micellar palmitoyl-CoA may not occur *in vivo* where no albumin is present. Moreover, the degree of inhibition of the monoacylglycerol hydrolases by physiological concentrations of palmitoyl-CoA on albumin bound substrate is relatively low

(Table IV). Therefore, it is conceivable that long-chain acyl-CoA will regulate the use of monoacylglycerol mainly by the regulation of the monoacylglycerol lipase.

The contribution of the esterases to the monoacylglycerol hydrolase activity in the homogenate can be determined, if it is assumed that the hydrolytic activity of the lipase towards 4-methylumbellipherylnanonate is negligible compared to that of the esterases (Table II). From the recovery of the esterase activity in peaks 2 and 3, as determined with 4-methylumbellipherylnanonate, the contribution of the esterases to total monoacylglycerol hydrolase activity in the original homogenate was calculated to be approximately 2/3.

Therefore, only a part of the monoacylglycerol hydrolase activity may be regulated by long-chain acyl-CoA.

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CHAPTER VI

6.1. MONOACYLGLYCEROL METABOLISM IN RAT SMALL INTESTINAL EPITHELIAL CELLS

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SUMMARY

1. In various preparations of rat small intestinal epithelial cells the acylation and deacylation of monoacylglycerol were studied. In the *in vitro* vascularly perfused intestine, of which the lumen was loaded with monoacylglycerol with or without fatty acids, acylation exceeded deacylation. In contrast, deacylation was much faster in isolated microsomes and in isolated whole cells.
2. In vascularly perfused intestine, without long-chain fatty acids present in the lumen, the amount of di- and triacylglycerol formed was found to be a half of that formed in perfusion with long-chain fatty acids in the lumen, while the glycerol formation was increased 1.4-fold.
3. The concentration of monoacylglycerol is an important factor in determining the relative rates of monoacylglycerol acylation and deacylation in microsomes: the ratio acylation/deacylation decreases with increasing monoacylglycerol concentrations.
4. The function of the monoacylglycerol lipase in fat resorption is discussed.

INTRODUCTION

It is known that monoacylglycerol is utilized in the intestinal epithelial cells for triacylglycerol synthesis [1], and it is also known that monoacylglycerol can be degraded by enzymatic hydrolysis [2-4]. *In vivo* most of the monoacylglycerol is acylated. *In vitro*, however, the hydrolytic activity is much faster than the rate of acylation [5,6] for which we found a value of 70 mU/mg microsomal protein at pH 7.4 with monooleoylglycerol as the fatty acid acceptor [6]. The hydrolytic activity is about 4-10 times higher [2-4]. Therefore, a mechanism must exist that regulates the monoacylglycerol hydrolase activity.
In a previous paper [7] we showed that at least three enzymes may be responsible for monoacylglycerol breakdown, two of which are esterases because of substrate specificity, and one a more or less specific monoacylglycerol lipase. This lipase also possesses low palmitoyl-CoA hydrolytic activity. Therefore, the lipase can be inhibited by long-chain acyl-CoA so that part of the regulation of monoacylglycerol hydrolysis is accomplished by control of the lipase. However, only 1/3 of the total hydrolytic activity of a homogenate of intestinal epithelial cells on monoacylglycerols is due to the lipase, while the esterases may contribute 2/3 of the activity. Therefore, the esterases should be regulated too. Indeed, the esterases may also be inhibited by excess (micellar) palmitoyl-CoA. It can be questioned whether this type of inhibition plays an important role *in vivo*. The critical micellar concentration of palmitoyl-CoA is 4 μM [8]. *In vivo* concentrations of acyl-CoA in the intestinal cells have been reported to be as high as 30 μM [9], part of which will be bound to proteins, such as the fatty acid binding- or Z-protein [10,11]. Although local concentrations may be much higher, the reported concentrations of acyl-CoA is too low to inhibit

the esterases efficiently [7]. We therefore felt it necessary to investigate more precisely the fate of monoacylglycerol under various conditions in order to gain insight in the mechanisms whereby the esters are preserved for triacylglycerol synthesis. Related to this question of the regulatory mechanism is that of the function of the hydrolytic enzymes. According to Fernandez-Lopez *et al.* [12], the esterases may be important in the hydrolysis of dietary short- and medium-chain triacylglycerols which may enter the mucosa cell unhydrolyzed. Brown and Johnston [13] supposed that the hydrolytic activity of mucosa cells towards long-chain monoacylglycerols may be important to generate fatty acids when these are not sufficiently available for monoacylglycerol acylation.

MATERIALS AND METHODS

Radioactive materials were purchased from the Radiochemical Centre (Amersham, England). Monooleoyl[2-^{3}H]-glycerol and 1- and 2-monopalmitoyl[2-^{3}H]glycerol were prepared as described previously [4]. Bovine serum albumin (fraction V, Sigma) was made fatty acid-free by the method of Chen [14]. 2-Monooleoylglycerol was obtained from Serdary (London, Ontario, Canada). Monoacylglycerols were purified by thin layer chromatography, using Silica G plates (Merck) developed with chloroform/methanol (96:4). For the separation of 1- and 2-monoacylglycerols, thin layers were impregnated with boric acid. Palmitoyl-CoA was made according to Stoffel *et al.* [15]. Male fed Wistar rats of about 300 g were used.

In vitro vascular perfusion of rat small intestine

The perfusion medium contained fluorocarbon F C-75 (3 M Company, St. Paul, Minn., U.S.A.) as oxygen carrier, in a Krebs-Henseleit bicarbonate buffer pH=7.4, fortified with 3.4% fatty acid-free bovine serum albumin. The fluorocarbon emulsion was prepared with the use of a non-ionic polyalkylene oxide detergent F-68 (Wyandotte Chem. Corp., Wyandotte, Mich., U.S.A.) as described by Lamers and Hülsmann [16]. The operation procedure for vascular perfusion of rat intestine was described earlier by Hülsmann [17]. After canulation of the aorta, the perfusion was started at a rate of 4 ml/min. This rate was increased to 11 ml/min after canulation of the portal vein. The temperature of the intestine and the perfusion medium were kept constant at 30°C. About 1 min after canulation of the portal vein, the lumen was filled with 4 ml 2.5 mM 2-monopalmitoyl[2-^{3}H]glycerol (36 mC/mol), 5 mM palmitic acid or octanoic acid as indicated, 20 mM sodium deoxytaurocholate, 0.15 M NaCl and 10 mM phosphate buffer pH=7.4. From the vascular perfusate 1 min samples were taken each 5 min to follow the glycerol production with the time. After 30 min the perfusion was stopped and the luminal side of the intestine was rinsed with icecold saline. to stop enzyme reactions. From the perfusate samples the fluorocarbons were removed by centrifugation (10 min at 12 000 g) and part of the supernatant was mixed with Instagel (Packard) and the radioactivity was counted. The radioactivity was shown to be water soluble by the method of Bligh and Dyer [18]. Therefore it was glycerol or a metabolite of it. For measuring diacyl- and triacylglycerol production, the chilled intestine to which the lymph vessels were attached, was homogenized and treated with chloroform and methanol according to Bligh and Dyer. After phase separation a sample of the chloroform was counted for calculating the recovery, and a known amount was applied to thin layer

Silica G plates, which were developed with chloroform/ methanol (96:4 v/v). This separated di- and triacylglycerol from monoacylglycerol effectively, so that they could be isolated by scraping from the plates and counted.

Whole cell incubations

Villus cells were isolated by the vibration method of Harrison and Webster [19] in 0.15 M NaCl, 5 mM EDTA, 5 mM $MgCl_2$ and 10 mM Tris-HCl (pH=7.4). $MgCl_2$ was added to maintain a proper energy charge [20]. Cells were spun down during 30 sec at 755 g, washed once with cold Hank's saline, fortified with 3 mM glutaminate [21] and suspended in enough of this solution of 30°C to make an approximately 10% w/v cell suspension. This preparation was immediately used in incubations in which 0.3 ml of the cell suspension was mixed with 0.2 ml 1 mM 2-monopalmitoyl[2-^{3}H]glycerol, complexed to 0.75 mM albumin in the presence or absence of 2 mM palmitic acid. After 5 and 10 min the reactions were terminated by the addition of 0.5 ml 10% trichloroacetic acid. After centrifugation a sample of the supernatant was counted for glycerol measurements. Di- and triacylglycerol production was measured by extracting the pellet with chloroform and methanol according to Bligh and Dyer [18] and processed as described above. Rates of acylation and deacylation were linear during the incubation period. The viability of the cells was tested after incubation by testing, after removal of the cells by centrifugation, the presence of lactate dehydrogenase and esterase in the medium. Not more than 5% of the total LDH activity was released and virtually no esterase activity could be detected.

Preparation of microsomes

Cells were isolated by the vibration method of Harrison and Webster [19] in 0.15 M NaCl, 5 mM EDTA and 10 mM Tris-

HCl pH=7.4. They were collected by centrifugation for 30 sec at 755 g and homogenized in a Potter-Elvehjem glass-teflon homogenizer (10 up and down strokes) in 0.25 M sucrose, 10 mM Tris-HCl pH=7.4 and 1 mM EDTA. After removal of the nuclei, brushborders and cell debris (5 min at 755 g) and mitochondria (10 min at 12 000 g), the microsomes were isolated by centrifugation during 30 min at 200 000 g, and suspended in homogenization buffer to obtain about 2 mg protein per ml.

Monoacylglycerol hydrolysis

Monoacylglycerol hydrolysis was measured as [2-^{3}H]-glycerol released from the substrate, which was complexed to albumin, in 0.1 M Tris-HCl pH=8.0 at 37^{o}C during 5 and 10 min. Linear reaction rates were measured as described previously [4,7]. The reactions were stopped with 10% trichloroacetic acid, which caused coprecipitation of acylglycerols with albumin [22]. After centrifugation a sample of the supernatant was counted. When the tri- and diacylglycerol production was measured simultaneously, the reactions were stopped with chloroform/methanol instead, according to Bligh and Dyer [18]. After phase separation a sample of the water phase was counted.

Monoacylglycerol acylation

Monoacylglycerol acylation was measured as the production of labeled di- and triacylglycerol. Incubations were done in 0.1 M Tris-HCl pH=8.0 at 37^{o}C during 5 and 50 min. Linear reaction rates were measured.
Further conditions will be given in the text. Incubations were stopped with chloroform/methanol and processed further as described above.

Incubation of microsomes loaded with 2-monooleoylglycerol

An intestine was chilled to 0°C, the lumen rinsed with saline, and filled with a cold solution of 2-monooleoylglycerol and taurodeoxycholate exactly as described for the perfusion experiments. After 20 min the lumen was rinsed with cold saline and the microsomes isolated as described above. The microsomes were suspended in a solution of 0.1 M Tris-HCl pH=8.0 and incubated at 37°C during 5 min, in the presence or absence of palmitoyl-CoA. Reactions were stopped with chloroform/methanol and processed as described earlier. The concentrations of 2-monooleoylglycerol in the incubation mixture was 1.5 ± 0.3 μM (n=4). In these experiments hydrolysis was not linear with time.

Miscellaneous

Protein was measured according to Lowry *et al.* [23] using bovine serum albumin as a standard.
For determination of significance the student t-test was used.

RESULTS AND DISCUSSION

Perfusion experiments

With the *in vitro* vascular perfusion technique we tried to evaluate the hypothesis that the function of monoacylglycerol hydrolases is to remove the monoacylglycerol when there is a relative fatty acid shortage. We therefore loaded the lumen of the rat small intestine with 2-monoapalmitoyl-[2-^{3}H]glycerol and either palmitic acid or octanoic acid (molar ratio to monoacylglycerol 2:1). It is known that octanoate is not used for triacylglycerol synthesis, but is oxidized or transported to the portal system. It was

included to serve as a possible fuel source, as palmitate may also be oxidized [17]. Subsequent experiments, however, showed no effect of omitting octanoic acid or its replacement by glucose on the rates of monoacylglycerol acylation or hydrolysis. The glycerol production was followed with time. In the first 15 min the rate of glycerol production increased and then became constant, indicating that steady state level was reached. Perfusion was continued for 30 min, because after 40-45 min glycerol production increased again, while the intestine became swollen and started to leak. After 30 min perfusion the di- and triacylglycerols were extracted from the tissue. Table I shows the amount of glycerol released in the perfusion medium and the di- and triacylglycerol production during 30 min perfusion. It can be seen that when the lumen was loaded with 2-monopalmitoylglycerol together with palmitic acid 7 times more acylation occurred than hydrolysis. This ratio dropped to 2 when palmitic acid was replaced by octanoic acid. This was due to decreased acylation as well as increased hydrolysis. When palmitic acid was absent the amount of monoacylglycerol used for the synthesis of di- and triacylglycerols was reduced, but a remarkable portion of the absorbed monoacylglycerol was still acylated. This may be explained by a store of fatty acids in the mucosa cells [24]. However, fasting overnight had no effect on the acylation. It is also possible that diglycerides were formed by a transacylation reaction involving two molecules monoacylglycerol as the substrate and diacylglycerol and glycerol as the products, as will be discussed later. During the perfusion with octanoic acid in the lumen, less monacylglycerol was taken up or bound than during perfusions with palmitic acid. Moreover, slightly less monopalmitoylglycerol could be recovered from the intestine: 1173 $\pm$ 90 (n=4) and 1409 $\pm$ 158 (n=3) respectively ($p<0.1$). From these figures the total amount of absorbed and strongly adsorbed monopalmitoylglycerol was calculated to be 2022 $\pm$ 171 and 1566 $\pm$ 93 respectively ($p<0.05$).

TABLE I

ACYLATION AND DEACYLATION OF 2-MONOPALMITOYLGLYCEROL DURING 30 MIN *IN VITRO* VASCULAR PERFUSION OF RAT SMALL INTESTINE

Rat small intestines were vascularly perfused *in vitro* as described under Materials and Methods. The amount of monopalmitoylglycerol acylated ($p<0.01$) and deacylated ($p<0.001$) as well as their ratio ($p<0.001$) and the total amount of absorbed monopalmitoylglycerol ($p<0.05$) were significantly different when palmitic acid was replaced by octanoic acid. The total amount of absorbed monopalmitoylglycerol is the sum of the amounts of monopalmitoylglycerol acylated, deacylated and recovered from the tissue.

Luminal load	Monopalmitoylglycerol			
	Total absorption nmol ± S.E.	acylation nmol ± S.E.	deacylation nmol ± S.E.	acylation/ deacylation
2-Monopalmitoylglycerol + palmitic acid (n=3)	2022 ± 171	535 ± 20	79 ± 11	6.77 ± 0.51
2-Monopalmitoylglycerol + octanoic acid (n=4)	1566 ± 93	276 ± 16	119 ± 8	2.32 ± 0.10

These experimental results do not allow a conclusion to be made about the extent to which esterases and lipase contribute to monoacylglycerol hydrolysis. Obviously, long-chain acyl-CoA has been generated during the perfusion. This may have inhibited the monoacylglycerol lipase, but it may be questioned if the esterases were inhibited, especially during the perfusions without palmitic acid. Therefore, other factors may have contributed to the low rate of hydrolysis relative to acylation, such as the presence of detergents like taurodeoxycholate and the concentration of monoacylglycerol in the epithelial cells after resorption from the lumen, which may be expected to be low. Both problems will be dealt with below.

Incubation with isolated cells

With the perfusion experiments the amounts of monoacylglycerol acylated and deacylated were studied in the presence or absence of a long-chain fatty acid. A similar type of experiment can be done with isolated whole villus cells. Since the cells desintegrate in the presence of taurodeoxycholate, the monopalmitoylglycerol was suspended with bovine serum albumin instead. In contrast with the perfusion experiments the monoacylglycerol could also enter the cells through the basal and lateral plasma membranes. From Table II it can be seen that under these conditions even in the presence of palmitic acid the rate of deacylation exceeded the rate of acylation. Omission of the fatty acid resulted in an increase in deacylation and a minute decrease in acylation rate, so that the ratio acylation/deacylation dropped as was found in the perfusion experiments. However, other features are different. Firstly, although the amount of unreacted monopalmitoylglycerol absorbed into the cell was not measured, it is possible that more substrate was transported into the cell in the absence of added palmitate than in its presence. This may

TABLE II

ACYLATION AND DEACYLATION OF 2-MONOPALMITOYLGLYCEROL BY ISOLATED EPITHELIAL CELLS

A suspension of isolated whole epithelial cells was incubated with 2-monopalmitoylglycerol bound to albumin, in the presence or absence of palmitic acid as described under Materials and Methods. The difference in the rate of deacylation and in the acylation/deacylation ratio is significant ($p<0.05$) ($n=4$).

	acylation mU/ml ± S.E.	deacylation mU/ml ± S.E.	acylation/ deacylation ± S.E.
2-Monopalmitoylglycerol + palmitic acid	2.9 ± 0.4	8.2 ± 1.9	0.35 ± 0.10
2-Monopalmitoylglycerol	2.4 ± 0.7	14.4 ± 2.0	0.17 ± 0.06

be explained by an influence of fatty acids on the binding of monopalmitoylglycerol to albumin. Therefore, the absolute rates of acylation and deacylation may not be as important as the ratio. Secondly, a greater part of the absorbed monopalmitoylglycerol is hydrolyzed so that the ratio acylation/deacylation decreased about tenfold when compared with the values obtained during perfusion (Table I). Thirdly, the total amount of monoacylglycerol converted in these experiments with isolated cells is higher than in the perfusion experiments. About 1.7 g wet weight of epithelial cells were harvested per intestine. Therefore, 17 ml of a 10% cell suspension could be made. From the figures in Table II this allows the calculation that per intestine in 30 min 1.7 µmol monoacylglycerol could be acylated and 3.7 µmol hydrolyzed by cells incubated with palmitic acid and 1.2 and 6.0 µmol in its absence, respectively. When these figures are compared with those obtained from the perfusion experiments (Table I) much more monoacylglycerol is absorbed and converted by isolated cells. The higher concentration of monoacylglycerol inside these cells may then be responsible for the lowered acylation/deacylation ratio. Other factors of importance for this lowered ratio may be the absence of bile salts and alteration of the entry side(s) of the small epithelial cells.

Microsomal acylation and deacylation

The ratio of the amounts of monoacylglycerol acylated and deacylated can also be determined with isolated microsomes. Both activities cannot be optimally determined under identical conditions because the pH optima are different and acyl-CoA required for the acylation will inhibit hydrolysis. With microsomes the optimal activities were found to be 70 mU/mg protein for the acylation [6] and 300 mU/mg protein for the hydrolysis when monooleoylglycerol was used. Somewhat lower values are obtained at pH=8.0 when both processes are

TABLE III

ACYLATION AND DEACYLATION OF MONOOLEOYLGLYCEROL BY MICROSOMES FROM ISOLATED SMALL INTESTINAL VILLUS CELLS

Microsomes were incubated at 37°C at pH=8.0 with 2.5 mM monooleoylglycerol complexed to 0.15 mM albumin. To this was added either 0.5 mM oleic acid in the presence or absence of 5 mM ATP and 0.1 mM CoA, or 20 μM palmitoyl-CoA. The reactions were stopped and processed as described under Materials and Methods. (n=3).

	acylation mU/mg ± S.E.	deacylation mU/mg ± S.E.	acylation/ deacylation ± S.E.
2-Monooleoylglycerol + oleic acid + ATP + CoA	44.1 ± 6.8	276 ± 25	0.16 ± 0.02
2-Monooleoylglycerol + oleic acid	13.2 ± 2.4	274 ± 25	0.05 ± 0.01
2-Monooleoylglycerol + palmitoyl-CoA	30.2 ± 4.1	255 ± 21	0.12 ± 0.01

saturated with monooleoylglycerol in the presence of fatty acid, ATP and CoA. It can be seen from Table III that the generated oleoyl-CoA did not influence the rate of hydrolysis, since similar values were obtained when CoA and ATP were omitted. The hydrolysis is not inhibited in this *in vitro* experiment, because the concentration of oleoyl-CoA will not rise sufficiently. The acyltransferase is saturated with monooleoylglycerol and may use the generated oleoyl-CoA very rapidly. The rate of acylation dropped when the co-factors were omitted, but increased again when oleic acid was replaced by palmitoyl-CoA. Palmitoyl-CoA in the presence of excess albumin, however, slightly inhibited hydrolysis, as was reported earlier [7]. By using microsomes instead of *in vitro* perfused small intestine or isolated cells the acylation/deacylation ratio decreased (compare the ratios in Tables I and II). If we assume that a total homogenate of villus cells obtained from 60 cm of small intestine, as was used in the perfusion studies, the intestine contains about 150 mg protein of which about 40% is microsomal [6], it can be calculated that per 60 cm intestine during 30 min 80 µmol monooleoylglycerol could be acylated and 500 µmol hydrolyzed in the presence of oleic acid, ATP and CoA, if the substrates would have been available to the endoplasmic reticulum of the cells. Although in the perfusion and whole cell experiments palmitic acid derivatives have been used as substrates instead of oleic acid derivatives for the acylation and deacylation studies, it can be concluded that *in situ* the monoacylglycerol availability is much lower than in the experiment with the microsomes. This also suggests that the concentration of monoacylglycerol within the absorbing cells is an important factor in determining the fate of this compound.

Regulation of acylation and deacylation

The conclusion that the concentration of monoacylglycerol may be important in the regulation of acylation and deacylation was tested with a microsomal preparation. Table IV gives the results of an experiment, in which acylation and deacylation were measured at various monoacylglycerol concentrations. In this experiment the albumin and the palmitoyl-CoA concentrations were also varied in order to maintain identical molar ratios (16.6:1:33.3). The rate of acylation is higher than the rate of hydrolysis which is inhibited by palmitoyl-CoA. The highest acylation/deacylation ratio is observed when the monoacylglycerol concentration is low. At the higher substrate concentrations higher rates of deacylation are observed while acylation becomes inhibited, probably because excess palmitoyl-CoA has been shown to inhibit acylation [25]. Although the variation of palmitoyl-CoA does affect both acylation and deacylation, essentially the same results were obtained when the palmitoyl-CoA concentration was kept constant at 50 μM (not shown). Therefore, it can be concluded that the acylation is indeed preferred at low monoacylglycerol concentrations. The most likely explanation is that the affinity for monoacylglycerol of the acyltransferase is higher than that of the hydrolases. However, Ailhaud *et al.* [5] reported an apparent K_m of about 100 μM for the acyltransferase with 2-monopalmitoylglycerol, while we reported an apparent K_m of 50 μM for the hydrolase activity towards the same substrate as tested in a homogenate of villus cells [4] or towards monooleoylglycerol as tested with purified esterases and monoacylglycerol lipase [7]. These figures should be interpreted with caution because the K_m's were measured in the presence of albumin, which influences the apparent K_m's of the enzymes because of binding of the substrate [4]. From the data of Table IV we conclude that acylation is favoured at low monoacylglycerol concentrations, presumably by a higher affinity of the acylating enzyme.

TABLE IV

INFLUENCE OF MONOOLEOYLGLYCEROL CONCENTRATION ON ACYLATION AND DEACYLATION

Microsomes were incubated with different amounts of monooleoylglycerol, albumin and palmitoyl-CoA, that were used at a constant ratio (16.6:1:33.3). The reactions were stopped and processed as described under Materials and Methods. (n=3)

Monooleoyl-glycerol mM	acylation mU/mg ± S.E.	deacylation mU/mg ± S.E.	acylation/ deacylation ± S.E.
0.0125	43.2 ± 3.6	4.8 ± 1.1	9.0 ± 0.8
0.025	59.3 ± 1.8	8.5 ± 1.3	7.0 ± 0.5
0.0625	68.9 ± 3.6	11.3 ± 0.5	6.1 ± 0.3
0.125	50.0 ± 1.8	14.2 ± 0.5	3.5 ± 0.1
0.25	35.0 ± 2.1	15.3 ± 0.6	2.3 ± 0.1

The experiments with whole cell preparations suggested that the presence of bile salts might also affect the fate of monoacylglycerol. Both substances are absorbed in the duodenum and jejunum by a passive diffusion process, and will be present in the cell simultaneously. The transport of labeled monoacylglycerol from the lumen to the cytosol can be followed by autoradiography. How this transport is accomplished is largely unknown. Because we have not been able to detect a low molecular weight transport- or binding protein for monoacylglycerol [4], an analogy between fatty acid transport which employs the fatty acid binding protein [11] and monoacylglycerol transport may be absent. It is therefore possible that the absorbed bile salts may be

involved instead, for instance if micellar transport of monoacylglycerol, fatty acids and bile salts were to occur. It has been shown that the hydrolysis of monoacylglycerol is inhibited by bile salts both in the presence and absence of albumin [2-4]. Therefore bile salts may contribute to preserve monoacylglycerol for triacylglycerol synthesis.
From the comparison of the perfusion experiments and the whole cell incubations it was held possible that the entry of the monoacylglycerol through the brushborder might result in preferential acylation, whereas entry through the basolateral membranes might mainly result in deacylation. A different fate of fatty acids when absorbed from the blood stream instead from the lumen has been reported by Gangl and Ockner [26]. Fatty acids from the serosal side were preferentially oxidized in the mitochondria, while fatty acids from the lumen were preferentially used for triacylglycerol synthesis. This mechanism may reflect the subnuclear localization of the mitochondria in the cell (compare ref. 6). So far, we have not been able to distinguish between the localization of the acylating and hydrolyzing enzymes. Moreover, loading of microsomes with monooleoylglycerol, absorbed from the lumen, by preincubation of the intestine at $0^{o}C$ prior to isolation of the particles, resulted in hydrolysis upon further incubation. It can be seen from Table V that 2-monooleoylglycerol absorbed from the lumen may indeed be hydrolyzed, and that a low concentration of palmitoyl-CoA (10 μM) strongly inhibited. In the presence of palmitoyl-CoA part of the monooleoylglycerol was converted to di- and triacylglycerol but rates could not be measured exactly because of high blanks. This experiment again shows strong inhibition of deacylation by palmitoyl-CoA when the monoacylglycerol concentration is low.

TABLE V

DEACYLATION IN MICROSOMES LOADED WITH LUMINAL 2-MONOOLEOYL-GLYCEROL

Microsomes were loaded with 2-monooleoylglycerol at 0-4°C and subsequently incubated at 37°C during 5 min with or without palmitoyl-CoA as described under Materials and Methods. The total amount of 2-monooleoylglycerol per incubation (0.5 ml) was 1.5 ± 0.3 µM (n=4).

palmitoyl-CoA	monooleoylglycerol hydrolysis during incubation nmol ± S.E.
-	0.33 ± 0.05
10 µM	0.09 ± 0.03
100 µM	0.03 ± 0.02

Concluding remarks

In earlier studies we showed [7] that palmitoyl-CoA is hydrolyzed by the monoacylglycerol lipase and will therefore inhibit monoacylglycerol hydrolase activity competitively at concentrations below the critical micellar concentration. At higher concentrations palmitoyl-CoA will inhibit not only the lipase but also the esterases. It has already been shown [2-4] that monoacylglycerol hydrolysis is inhibited by other detergents, such as bile salts. In the present paper we showed that monoacylglycerol acylation is favoured at low substrate concentrations. Whether the esterases contribute to overall monoglyceride hydrolysis *in situ* cannot be answered yet.

The perfusion experiments were done to test the function of the hydrolyzing enzymes as formulated by Brown and Johnston [13]. The fatty acid deprivation used in the

present study is rather extreme when compared to *in vivo* conditions. Although the amount of monoacylglycerol that was hydrolyzed, indeed, increased by omitting fatty acid, it was still lower than the amount that was acylated. Therefore, although it was confirmed that with fatty acid shortage more monoacylglycerol is hydrolyzed, it can be questioned whether the main function of the monoacylglycerol lipase is to hydrolyze monoacylglycerol absorbed from the lumen. Perhaps the enzyme has a main function in hydrolyzing substrate entering the enterocyte from the blood side. In this compartment monoacylglycerol can be generated by lipoprotein lipase action on very low density lipoproteins and chylomicra. The generated fatty acids could be used as fuel, or used for triacylglycerol synthesis during the formation of lipoproteins.

Finally, the hydrolases could contribute to transacylation between monoacylglycerol substrates. A monoacylglycerol: monoacylglycerol transacylase reaction has been described for a liver plasma membrane enzyme [27]. In earlier studies [6] we observed in small intestinal villus microsomes a palmitoyl-CoA independent diacylglycerol formation from monoacylglycerol (unpublished). The present paper (Table III) shows that in the absence of CoA and ATP microsomes catalyze the formation of diacylglycerol from monoacylglycerol, indeed. The mechanism of this reaction requires further investigation.

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6.2. MONOACYLGLYCEROL: MONOACYLGLYCEROL TRANSACYLASE

INTRODUCTION

In rat small intestinal cells the following reaction has been found to occur:

monoacylglycerol + monoacylglycerol → diacylglycerol + glycerol.

Such a transacylation reaction may be a third route contributing to triacylglycerol synthesis in the intestinal cells. The observations which led to the demonstration of this enzyme activity have been dealt with in the preceeding paragraph.

ENZYME ASSAY

The diacylglycerol synthesis was measured with 3.1 mM monooleoylglycerol complexed to 0.19 mM fatty acid free albumin (molar ratio 1:16.6) in 0.125 mM Tris-HCl pH=8.0 and microsomal protein (up to 100 µg protein) in a total volume of 0.4 ml. In the blank a boiled enzyme preparation was used. After incubation for 10 min at 37°C, the reaction was stopped with 1.5 ml chloroform/methanol (1:2). After phase separation by adding 0.5 ml chloroform and 0.5 ml water [1], a part of the water phase was mixed with Instagel and counted for the determination of glycerol production. Mono-, di- and triacylglycerols from the chloroform phase were separated with thin layer chromatography on Silica gel G plates, which were developed in hexane:diethylether:acetic acid (30:70:1 v/v). The glycerol derivatives were scraped from the plates, mixed with water and Instagel, and counted.

RESULTS

The rate of the enzyme reaction increases linearly with the amount of protein used and is constant under the conditions mentioned. The specific activity of the palmitoyl-CoA independent transacylase is about 15 mU/mg microsomal protein. The rate of glycerol formation can be measured simultaneously. It was found that up to 8% of the liberated glycerol is accounted for in the transacylation reaction (Table VI). Since more than 90% of the glycerol is generated in the hydrolytic reaction(s) these are of major importance for the glycerol formation observed *in vitro*.

TABLE VI

v vs S OF MONOOLEOYLGLYCEROL HYDROLASE ACTIVITY AND MONOACYL: MONOACYL TRANSACYLASE

10 µg of microsomal protein was incubated with different monooleoylglycerol concentrations, with a constant ratio of monooleoylglycerol to albumin (1:16.6). Incubations were performed as described in the methodological section. In this experiment much of the substrate is used during the experiment.

monooleoyl-glycerol µM	glycerol formed mU/mg protein	diacylglycerol synthesis mU/mg protein	% glycerol originated from the synthetic reaction
25	77	0.62	0.8
62.5	125	1.8	1.4
125	157	5.8	3.7
250	172	10.8	6.3

The apparent K_m of the transacylase reaction for monoacylglycerol was estimated to be approximately 0.5 mM. This is about 10 times higher than the K_m of the hydrolytic enzymes, as has been described in Chapter IV and V. The difference in K_m can also be concluded from Table VI. At very low substrate concentrations the percentage of glycerol originated from the transacylase reaction, is much lower than at the higher substrate concentrations. Due to the hydrolytic activity much of the substrate is used during the reaction periods, which results in an underestimation of the transacylase reaction at low monoacylglycerol concentrations.

DISCUSSION

The existence of a monoacylglycerol: monoacylglycerol transacylase reaction has been demonstrated, but the significance of this reaction *in vivo* is not yet understood. In comparison with the hydrolytic activity towards monoacylglycerol *in vitro*, the glycerol formed in the transacylase reaction is of minor importance. This enzyme may contribute to the diacylglycerol formation. The data of Hülsmann and Kurspershoek-Davidov [2] for enzyme activities in microsomes allow the calculation that the acyl-CoA independent transacylase is 4 times less active than the acyl-CoA dependent reaction, and twice less active than the glycerolphosphate acyltransferase. Whether these *in vitro* activities can be taken as a measure for the *in vivo* activities is not likely, because the K_m's of both transferases are different. It has been shown in the preceeding paragraph that in the presence of palmitoyl-CoA more monoacylglycerol is acylated than deacylated at the lower substrate concentrations. This may be a reflection of the lower K_m of the acyl-CoA acyltransferase. The K_m of the monoacylglycerol transacylase is higher than

the K_m of the hydrolytic reactions (Table VI). Thus the K_m of the palmitoyl-CoA acyltransferase is lower than that of the monoacylglycerol transacylase. Therefore, the latter reaction seems to be efficient for the removal of monoacylglycerol when the cell is overloaded with this amphiphilic molecule. This function has been ascribed to the hydrolytic enzymes, but the perfusion experiments described in the preceeding paragraph did not show a very high rate of hydrolysis, and rather point to the formation of diacylglycerol.

The formation of glycerol in reactions other than the hydrolytic reactions described in Chapter V, reduces the value of the calculation for the portion of the hydrolytic activity in the whole cell due to the monoacylglycerol lipase. However, it is possible that one of the monoacylglycerol hydrolases described may be responsible for the monoacylglycerol transacylase activity, because only glycerol formation was measured. It is possible that the enzyme(s) may have properties in common with the enzyme described by Waite and Sisson [3-5]. This enzyme is a transacylase localized in the liver plasma membrane. The acyl acceptor of this reaction may be the hydroxyl moiety of water (hydrolysis) or of monoacylglycerol (transfer). The acyl donor may be mono- and diacylglycerol, and 1,2-diacyl- and 1-acylglycerolphosphoethanolamine. What factors influence the hydrolytic and transacylating properties is not known. It is possible that the lipid environment is of importance [5]. The difference in K_m for both reactions may be explained by the fact that more monoacylglycerol is required to reduce the amount of water in the vicinity of the enzyme-substrate complex. Moreover, delipidation of an enzyme may alter the enzymatic properties. Okuda and Fujii [6] showed that liver esterase acquired the ability to hydrolyze Ediol substrate by sonicating the enzyme with a liver lipid extract, and that a liver lipase lost this ability by delipidation. Whether changes in properties occurred during the isolation of the

intestinal monoacylglycerol hydrolases cannot be answered at this moment.

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S U M M A R Y

The metabolism of monoacylglycerol in rat small intestinal epithelial cells has been studied. Monoacylglycerol can be acylated, a reaction in the major pathway for triacylglycerol synthesis in small intestine, or deacylated. *In vivo* most of the monoacylglycerol is used for the synthesis of triacylglycerol, but *in vitro* the hydrolytic enzymes are far more active, resulting in the formation of glycerol and fatty acid. Therefore, a mechanism must exist *in vivo* that saves monoacylglycerol for triacylglycerol synthesis.

A study of the localization of the hydrolytic activity showed that there was no basis for the idea that a different localization of the hydrolyzing and transacylating activities could be responsible for the differences in the metabolism of monoacylglycerol observed *in vivo* and *in vitro*. A soluble monoacylglycerol binding protein that might influence the metabolic fate, was not detected. These observations made it likely that the regulatory mechanism was to be found in the properties of the enzymes involved. Because the hydrolytic activity *in vitro* is 5-10 times higher than the transacylating activity, this mechanism is likely to occur by an inhibition of the hydrolytic activity.

Monoacylglycerol can be degraded to fatty acids and glycerol by the action of at least three enzymes: two esterases and a more specific enzyme, which is called monoacylglycerol lipase. These enzymes are localized mainly in the endoplasmic reticulum of the endothelial cells. The esterases are present mainly in the proximal part of the intestine, while the lipase may be more active in the distal part.

The physical state of the substrate is an important factor for the hydrolase activity, because the highest

activity was measured with monoacylglycerol complexed to albumin, while solubilization of the substrate with detergents, such as Triton X-100 and bile salts, resulted in lower enzyme activity. Detergents, as well as albumin, inhibited the rate of hydrolysis of albumin-bound monoacylglycerol. A physiologically important detergent is palmitoyl-CoA, which is used as a substrate in the acylating reaction. It has been found that this molecule can be hydrolyzed by the monoacylglycerol lipase. This reaction is inhibited by excess substrate: when the palmitoyl-CoA concentration is higher than the critical micellar concentration. The hydrolysis of monoacylglycerol by the lipase is therefore inhibited competitively at concentrations below the critical micellar concentration and non-competitively by the detergent action of concentrations of palmitoyl-CoA above the critical micellar concentration. The esterases are inhibited only by micellar palmitoyl-CoA. Although this inhibition is rather effective for one of the esterases, it may be questioned whether this type of inhibition will occur within the cells, because the reported concentration of palmitoyl-CoA is rather low and much of it will be bound to protein. Therefore, the inhibition of the monoacylglycerol hydrolase activity by long-chain acyl-CoA occurs only by the inhibition of the monoacylglycerol lipase, which may be responsible for only 1/3 of the observed hydrolytic activity in a cell homogenate.

Another factor in determining the relative rates of acylation and deacylation is the concentration of the monoacylglycerol. This factor is important not only for the esterases but also for the lipase, since the hydrolases had similar apparent K_m values of about 50 μM. Although the apparent K_m of the acylating enzyme was reported to be about 100 μM, it was found that the ratio acylation/deacylation depended on the monoacylglycerol concentrations and that low concentrations favour acylation.

In the *in vitro* vascularly perfused intestine acylation exceeded deacylation, while in an isolated whole cell preparation deacylation was much faster. This probably reflects the low steady state concentration of monoacylglycerol in the intact intestine. Moreover, in experiments with perfused intestine the brushborder side of the epithelial cells is exposed to the monoacylglycerol substrate, while in experiments with isolated cells also basolateral plasma membranes are exposed.

These experiments were performed in order to test the hypothesis that the function of the monoacylglycerol hydrolases is to remove the amphiphilic molecule when there is a relative fatty acid shortage. In vascularly perfused intestine omission of fatty acids from the lumen containing monoacylglycerol resulted in only a moderate increase of the deacylation activity, while the rate of reacylation was affected to a greater extent. Yet the acylation rate was higher than the deacylation rate. Because this fatty acid deprivation was rather extreme and probably does not occur *in vivo*, it can be questioned whether the main function of the hydrolytic activity is to hydrolyze monoacylglycerol absorbed from the lumen.

Perhaps the enzyme may act also with substrate entering the enterocyte from the blood side. An additional function for the hydrolases may be the catalysis of a transacylation reaction between two monoacylglycerol molecules, resulting in the generation of diglyceride and glycerol. This reaction has been shown to occur with a microsomal preparation. The quantitative importance of this reaction *in vivo* is not yet known.

S A M E N V A T T I N G

In dit proefschrift is een studie beschreven over het metabolisme van monoglycerides in de epitheelcellen van de dunne darm van de rat. Monoglycerides ontstaan in het darmlumen door partiële hydrolyse van triglycerides onder invloed van pancreas lipase. Na opname in de epitheelcellen kan het monoglyceride geacyleerd of gehydrolyseerd worden. De acyleringsreactie is de eerste stap van een route die leidt tot de synthese van triglycerides. *In vivo* wordt het merendeel van de geabsorbeerde monoglycerides gebruikt voor de triglyceride synthese. Dit in tegenstelling tot de *in vitro* situatie waar de activiteit van de hydrolytische enzymen groter is dan de activiteit van het monoacylglyceride acyltransferase. Deze discrepantie tussen de *in vivo* en de *in vitro* situatie duidt erop dat er *in vivo* een mechanisme is, dat ervoor zorg draagt dat monoglycerides niet worden afgebroken, maar worden gebruikt voor de synthese van triglycerides.

De localisatie van de hydrolase activiteit in de darm werd bepaald en vergeleken met de reeds bekende localisatie van het acyleringsenzym. Hieruit bleek dat het idee dat een verschil in de localisatie van deze activiteiten verantwoordelijk zou kunnen zijn voor het waargenomen verschil in het metabolisme van monoglycerides *in vivo* en *in vitro* niet gegrond is. Een andere mogelijke verklaring voor dit verschil is dat er een eiwit zou kunnen bestaan dat monoglycerides transporteert in de cel en dat het metabolisme zou kunnen beïnvloeden. Dit monoglyceride-bindingseiwit kan echter niet worden aangetoond. Deze resultaten maken het aannemelijk dat een regulatiemechanisme inherent is aan de eigenschappen van de enzymen zelf. Omdat de activiteit van de hydrolytische enzymen *in vitro* ongeveer 5 tot 10 keer zo hoog is als dat van het monoglyceride acyltransferase, ligt het voor de hand te veronderstellen dat de

hydrolytische activiteit *in vivo* geremd zal zijn.

De hydrolyse van monoglyceride tot glycerol en vetzuur wordt gekatalyseerd door zeker drie enzymen: twee esterases en een meer specifiek enzym dat monoglyceride lipase werd genoemd. Deze enzymen zijn voornamelijk gelocaliseerd in het endoplasmatisch reticulum van de epitheelcellen. De esterases zijn actiever in het duodenum en jejunum dan in het ileum, terwijl het lipase waarschijnlijk juist actiever is in het ileum.

Belangrijk voor de activiteit van de hydrolases is de fysische toestand, waarin het substraat verkeert. De meeste activiteit werd gemeten als het monoglyceride gecomplexeerd was aan albumine, terwijl minder activiteit gevonden werd als het substraat in oplossing werd gebracht met behulp van Triton X-100 of galzouten. Deze detergentia en ook andere, waaronder albumine zelf, waren in staat de hydrolyse te remmen van het aan albumine gebonden monoglyceride. Een detergens dat fysiologisch van belang is, is palmitoyl-CoA, dat gebruikt wordt in de cel als substraat in de acyleringsreactie. Het palmitoyl-CoA zelf kan ook gehydrolyseerd worden door het monoacylglycerol lipase. Deze reactie wordt door een overmaat substraat geremd als de concentratie hoger is dan de kritsche micellaire concentratie. De hydrolyse van monoacylglycerol door het lipase wordt dan ook competitief geremd door concentraties van palmitoyl-CoA die lager zijn dan de kritische micellaire concentratie en niet competitief door concentraties hoger dan de kritische micellaire concentratie, door de detergerende werking van palmitoyl-CoA. De esterases worden alleen geremd door micellair palmitoyl-CoA. Hoewel een van de esterases effectief geremd wordt, is het toch niet zeker of dit soort remming in de cel voorkomt. De concentratie van palmitoyl-CoA in de epitheelcel is niet erg hoog, terwijl bovendien een groot deel gebonden is aan eiwit. Uit deze gegevens kan geconcludeerd worden dat de remming van de hydrolyse van monoglyceride door palmitoyl-CoA voornamelijk gebeurt door remming van de lipase, dat waarschijnlijk verantwoordelijk

is voor 1/3 van de totale hydrolytische activiteit.

Behalve de aanwezigheid van palmitoyl-CoA, is ook de concentratie van het monoglyceride belangrijk voor de mate waarin het geacyleerd of gedeacyleerd wordt. Dit geldt zowel voor de esterases als voor het lipase, omdat voor deze enzymen eenzelfde K_m is gemeten van ongeveer 50 µM. Ofschoon in de literatuur van het acyltransferase een K_m waarde van 100 µM bekend is, werd gevonden dat in aanwezigheid van palmitoyl-CoA de verhouding acylering/deacylering afhankelijk was van de concentratie van de monoglycerides. Bij lage concentraties trad relatief meer acylering op.

In de *in vitro* vasculair geperfundeerde darm trad meer acylering op dan deacylering terwijl in een preparaat van hele epitheelcellen meer monoacylglycerol werd gehydrolyseerd. Dit verschil kan waarschijnlijk ook verklaard worden door een verschil in de concentratie van het monoglyceride. Bovendien kan in de intakte darm het monoglyceride alleen de cel binnendringen door de borstelzoom, terwijl bij de geïsoleerde cellen dit ook gebeurt door het basolaterale membraan.

Deze experimenten waren opgezet om een hypothese te testen over de functie van de monoacylglycerol hydrolases. Deze zouden het monoacylglycerol verwijderen als er een relatief vetzuur tekort is in de cel. Indien vetzuren werden weggelaten uit het lumen van een vasculair geperfundeerde darm dat wel monoglyceride bevatte, werd slechts een geringe stijging van de deacylering geconstateerd, terwijl de reacylering veel meer veranderde, maar nog altijd trad meer acylering dan deacylering op. Omdat het weglaten van vetzuren een nogal extreem middel om een vetzuur tekort op te wekken is dat waarschijnlijk niet in die mate *in vivo* zal voorkomen, is het niet waarschijnlijk dat de functie van de hydrolases omschreven kan worden als het verwijderen van monoglycerides die geabsorbeerd worden uit het lumen. Het zou wel mogelijk kunnen zijn dat mono-

glycerides, die uit het bloed de cel binnenkomen, door de hydrolases gesplitst worden.

Een andere mogelijke functie van de hydrolases zou kunnen zijn dat de enzymen monoglyceride niet hydrolyseren maar gebruiken in een transacyleringsreactie. Een dergelijke reactie is ook aangetoond met microsomen: de acylgroep wordt verplaatst naar een ander molecuul monoglyceride, zodat glycerol en diglyceride geproduceerd worden. Het belang van deze reactie *in vivo* is nog niet bekend.

LIST OF ABBREVIATIONS

ATP	adenosine-5'-triphosphate
ATPase	adenosine-5'-triphosphate phosphohydrolase
CMC	critical micellar concentration
CoA	Coenzyme A
Cyt.c	cytochrome *c*
DEAE	diethylaminoethyl
E_{600}	paraoxon (diethyl-p-nitrophenylphosphate)
EDTA	ethylenediaminetetraacetic acid
FABP	fatty acid binding protein
GDH	glutamate dehydrogenase
K_m	Michaelis constant
Mao	monoamineoxidase
MUN	methylumbellipherylnanonate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
p-NPA	para-nitrophenylacetate
P_i	inorganic orthophosphate
PAGE	polyacrylamide gel electrophoresis
Q_{10}	quotient of enzyme activity at t K and at (t-10) K
RER	rough endoplasmic reticulum
RINCR	rotenone insensitive reduced nicotinamide adenine dinucleoide phosphate - cytochrome *c* reductase
rsa	relative specific activity
S	concentration of substrate
S.E.	standard error of the mean
SER	smooth endoplasmic reticulum
Tris	tris(hydroxymethyl) aminomethane
U	unit of enzyme activity defined as the amount of substrate in µmole converted per minute
UDP	uridine diphosphate
v	velocity of an (enzyme) reaction

N A W O O R D

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CURRICULUM VITAE

Op 22 december 1947 ben ik geboren te Palembang, Nederlands Indië. De lagere school en drie klassen van de middelbare school doorliep ik in Maastricht. De schoolopleiding voltooide ik aan het Aloysius College te Den Haag, waar ik in 1966 het diploma gymnasium β behaalde. In dat jaar begon ik de studie scheikunde aan de Rijksuniversiteit te Leiden. In 1973 heb ik het doctoraal examen afgelegd met als hoofdvak biochemie en als bijvakken organische chemie en wetenschapsmethodologie. Na mijn militaire dienst trad ik in augustus 1974 in dienst van de Nederlandse Organisatie voor Zuiver-Wetenschappelijk Onderzoek (Z.W.O.). Bij de afdeling Biochemie I van de Erasmus Universiteit Rotterdam verrichtte ik het onderzoek dat beschreven is in dit proefschrift.