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Acylation of Lysolecithin in the Intestinal Mucosa of Rats

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1. The presence of an active acyl-CoA–lysolecithin (1-acylglycerophosphorylcholine) acyltransferase was demonstrated in rat intestinal mucosa. 2. ATP and CoA were necessary for the incorporation of free [1-^{14}C]oleic acid into lecithin (phosphatidylcholine). 3. The reaction was about 20 times as fast with [1-^{14}C]-oleoyl-CoA as with free oleic acid, CoA and ATP. 4. With 1-acylglycerophosphorylcholine as the acceptor, both oleic acid and palmitic acid were incorporated into the β-position of lecithin; the incorporation of palmitic acid was 60% of that of oleic acid. 5. Of the various analogues of lysolecithin tested as acyl acceptors from [1-^{14}C]oleoyl CoA, a lysolecithin with a long-chain fatty acid at the 1-position was most efficient. 6. The enzyme was mostly present in the brush-border-free particulate fraction of the intestinal mucosa. 7. Of the various tissues of rats tested for the activity, intestinal mucosa was found to be the most active, with testes, liver, kidneys and spleen following it in decreasing order.

In recent years considerable progress has been made in the understanding of the mechanisms of absorption of several lipids, and the initial step appears to be hydrolysis, which is followed by micellar solubilization. From such micellar solution the lipids enter the mucosal cell by a process of microdiffusion and are re-esterified inside the cell. Such mechanisms have been reported to operate during the absorption of triglycerides (Senior, 1964) and retinyl and cholesteryl esters (Ganguly, 1967). However, very little information is available on the absorption of phospholipids. By using living rats, Scow, Stein & Stein (1967) and Nilsson (1968) provided circumstantial evidence indicating that the dietary lecithin (phosphatidylcholine, 1,2-diacyl-GPC*) is hydrolysed to lysolecithin (1-acyl-GPC), which in turn is reacylated before it enters the lymph. It would thus appear that the lecithin molecule might also be absorbed by a similar mechanism of partial hydrolysis followed by reacylation. The pancreas secretes a powerful phospholipase A that can rapidly hydrolyse dietary lecithin to lysolecithin, and a similar enzyme is also present in the brush border of intestinal mucosa of rats (Subbaiah & Ganguly, 1970). But there has been no work demonstrating the presence of an enzyme in the mucosa that can acylate lysolecithin to lecithin. In fact, during a survey of various tissues of rats for the presence of such an enzyme Webster (1965) was unable to detect this activity in the intestinal mucosa.

* Abbreviations: GP, *sn*-glycero-3-phosphate; GPC, *sn*-glycero-3-phosphorylcholine.

By using a brush-border-free particulate preparation we have demonstrated acyl-CoA–acyl-GPC acyltransferase activity in rat mucosa (Subbaiah, Sastry & Ganguly, 1969). In the present paper the results of detailed investigations on this enzyme are reported and the physiological significance of these findings in the absorption of phospholipids is discussed.

MATERIALS AND METHODS

Treatment of the animals and the methods of preparation of the various subcellular fractions of intestinal mucosa have been described in the accompanying paper (Subbaiah & Ganguly, 1970). The brush-border-free particles were suspended in 0.3M-sucrose and were used as the source of the enzymes in most of the experiments. In experiments with other tissues of rats the tissues were homogenized in 10vol. of 0.3M-sucrose. The isolation of the liver microsomes was as described by Schneider & Hogeboom (1950).

[1-^{14}C]Oleic acid was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [1-^{14}C]Palmitic acid was obtained from the Bhabha Atomic Research Centre, Bombay, India. CoA, ATP and GSH were products of Sigma Chemical Co., St Louis, Mo., U.S.A. [1-^{14}C]Oleoyl-CoA was prepared by the procedure of Kornberg & Pricer (1953) as modified by Bloomfield & Bloch (1960). The preparation had the proportions 1:1.2:3.1 for thiol ester: adenine:phosphate and its specific radioactivity was 260c.p.m./nmol of thiol ester. Lysolecithin was prepared from egg lecithin by using *Crotalus adamenteus* venom by the procedure of Long & Penny (1957). The preparation gave a single spot on t.l.c. plates, which corresponded to that of authentic lysolecithin. 1-Myristoyl-GPC was

obtained by the same procedure from synthetic 1,2-dimyristoyl-GPC, which was a gift from Dr E. Baer of the University of Toronto. Lysoplasmalogen (1-alkenyl-GPC) was prepared from ox-heart lecithin by using the method of Warner & Lands (1961). On t.l.c. plates this compound had an R_F value similar to that of lysolecithin, but it was contaminated with sphingomyelin, which accounted for about 20% of the total phosphorus. The vinyl ether content of the preparation was determined by the method of Gottfried & Rapport (1962) and the ratio of vinyl ether to phosphate in the spot corresponding to 1-alkenyl-3-*sn*-GPC was 1:1.08. 1-Alkyl-GPC was obtained from 1-alkenyl-GPC by hydrogenation in methanol with palladium as the catalyst. When tested for its acid-stability the alkyl-GPC was found to be resistant to 0.5M-HCl for 1h at room temperature, whereas the 1-alkenyl-GPC was completely destroyed under the same conditions. 2-Acyl-GPC was prepared from ox heart lecithin by the procedure of Lands & Merkl (1963). In agreement with these authors we have also noted that this preparation contains diacyl-GPC as a contaminant; it was used as rapidly as possible and without further purification. The procedure of Kates (1955) was used for the isolation of phosphatidic acid (1,2-diacyl-GP) from egg lecithin. Lysophosphatidic acid (1-acyl-GP) was obtained from phosphatidic acid by using *C. adamenteus* venom in ether medium, buffered with tris–maleate, pH7.0. The product gave a single spot on t.l.c. plates. GPC was prepared by mild alkaline hydrolysis of egg lecithin. To 70μmol of egg lecithin 0.2ml of 1M-NaOH was added and the mixture was incubated at 37°C for 30min after which 0.6ml of water was added to the mixture and the unchanged lecithin was extracted by the procedure of Bligh & Dyer (1959). The aqueous methanolic layer was removed and neutralized with Dowex 50 (H^+ form). The free fatty acids were removed by three extractions with 5ml of diethyl ether, the Dowex 50 was centrifuged off and the supernatant, which contained GPC, was neutralized with aq. ammonia.

The acyl-CoA–acyl-GPC acyltransferase activity was assayed by measuring the incorporation of [1-^{14}C]oleic acid from [1-^{14}C]oleoyl-CoA into lecithin. Unless otherwise stated the reaction mixtures contained 50nmol of [1-^{14}C]-oleoyl-CoA (260c.p.m./nmol), 0.4μmol of 1-acyl-GPC, 25μmol of NaH_2PO_4–Na_2HPO_4 buffer, pH7.4, 15μmol of $MgCl_2$ and brush-border-free particulate fraction (50–60μg of protein) in a final volume of 0.4ml. In those experiments where free fatty acids were used oleoyl-CoA was replaced by 0.1μmol of the labelled fatty acid (specific radioactivities of oleic acid and palmitic acid were 428 and 500c.p.m./nmol respectively), 0.1μmol of CoA, 5μmol of ATP and 5μmol of GSH. Unless otherwise stated the incubations (in duplicate) were carried out for 5min at 37°C. The reactions in the control experiments were stopped immediately after the addition of the enzyme. All reactions were terminated by the addition of 1ml of methanol. The lipids were extracted by the method of Bligh & Dyer (1959), after which the phospholipids were separated on silica-gel t.l.c. plates (Subbaiah, Raghavan & Ganguly, 1968) with the solvent system chloroform–methanol–water (65:25:4, by vol.). The spots corresponding to those of lecithin were scraped off and eluted (Abramson & Blecher, 1964), after which the radioactivities of the eluates were measured, as described by Murthy, David & Ganguly (1963). The specific activities of the enzymes are expressed as nmol of fatty acid incorporated into lecithin/h per mg of protein.

Phosphorus was measured by the procedure described by Bartlett (1959) as modified by Marinetti (1962). The method of Lowry, Rosebrough, Farr & Randall (1951) was used for protein determination, with bovine serum albumin as standard.

RESULTS

Time-course and cofactor requirements. In our preliminary communication (Subbaiah *et al.* 1969) we showed that a brush-border-free particulate

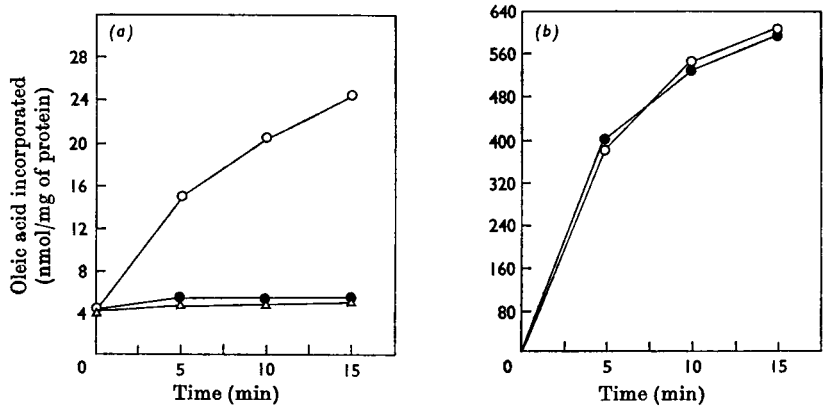

Fig. 1. Effects of ATP and CoA on the acyltransferase activity of rat intestinal mucosa. (*a*) With free oleic acid: a brush-border-free particulate preparation containing 60μg of protein was used as the enzyme source. ○, Complete system; ●, without CoA; △, without ATP. (*b*) With oleoyl-CoA: 60μg of protein from the brush-border-free particulate preparation was used as the enzyme; where indicated, ATP (5μmol) was added. ●, without ATP; ○, with ATP. Incubations were carried out at 37°C.

preparation from rat intestinal mucosa incorporated labelled oleic acid into lecithin, and that the reaction was dependent on ATP and CoA. Figs. 1(*a*) and 1(*b*) show that the reaction was linear with time up to 15min and that it was dependent upon ATP and CoA. The rate of incorporation of the label was about 20 times as fast with [1-^{14}C]oleoyl-CoA as with free [1-^{14}C]oleic acid. This is to be expected, because the activation of the fatty acid is the rate-limiting step.

Effect of protein concentration. The reaction was linearly dependent on the protein concentration up to 150μg of protein per reaction mixture with both oleic acid and oleoyl-CoA. No incorporation took place when the enzyme preparation had been boiled.

pH *optimum.* The transferase activity was optimum at pH 7.0 in tris–maleate buffer. However, phosphate buffer leads to about 20% higher activity than that obtained with tris–maleate buffer, and therefore phosphate buffer, pH 7.4 was used in all experiments. The pH optimum, as obtained here for the mucosal enzyme, closely agrees with the value reported by Van Den Bosch, Van Golde, Eibl & Van Deenen (1967) for the same enzyme from rat liver microsomes.

Effect of lysolecithin and oleoyl-CoA concentrations. There was no incorporation of the fatty acid into lecithin in the absence of added lysolecithin, and the incorporation was linear up to a concentration of 0.20μmol of lysolecithin per reaction mixture (0.5mM), which proved that the incorporation is due to acylation of the added lysolecithin to lecithin (Fig. 2). The reaction was dependent on oleoyl-CoA up to a concentration of 50nmol per reaction mixture (0.125mM), after which there was slight inhibition of the activity (Fig. 3). Such an inhibitory effect of oleoyl-CoA on acylation of lysolecithin by rat liver microsomes was also reported by Lands & Merkl (1963) and by Van Den Bosch *et al.* (1967).

Fatty acid specificity. The enzyme was not highly specific for oleic acid, since under the same conditions palmitic acid was also incorporated (Table 1). This was further substantiated by the results of experiments where equimolar amounts of labelled oleic acid and unlabelled palmitic acid, and vice versa, were used. In these trials some competition of one fatty acid for the other for incorporation into lecithin was noticed.

Substrate specificity. Several analogues of lysolecithin were tested for their relative efficiency as acyl acceptors from [1-^{14}C]oleoyl-CoA (Table 2); of all the substrates tested 1-acyl-GPC obtained from egg lecithin (which is mostly 1-palmitoyl-GPC; Tattrie, 1959) was the most active. 1-Myristoyl-GPC was about 20% as active as egg lysolecithin. Table 2 further shows that 1-alkenyl-GPC, 1-alkyl-GPC, 1-acyl-GP and 2-acyl-GPC were only 2–5% as active as egg lysolecithin. The incorporation of the labelled fatty acid was very poor when GPC was used and virtually no labelled lysolecithin or lecithin could be detected after the reaction.

Position occupied by the labelled fatty acids in the lecithin synthesized. The α-position of the lecithin molecule of biological sources is preferentially occupied by saturated fatty acids, and unsaturated fatty acids are prevalent in the β-position. However, when labelled oleic acid or palmitic acid was incorporated into the lecithin molecule by the mucosal enzyme all the radioactivity in the resulting lecithins was quantitatively liberated as free fatty acids by the action of *C. adamanteus* venom (Table 3), thereby demonstrating that both the fatty acids

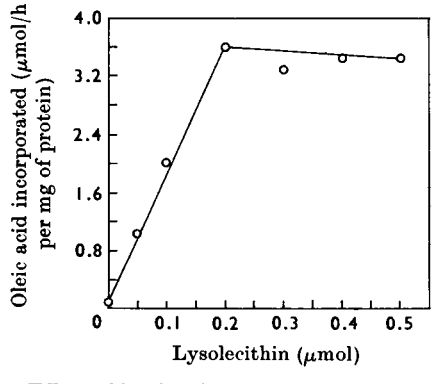

Fig. 2. Effect of lysolecithin concentration on the acyltransferase activity of brush-border-free mucosa. Incubations were carried out for 10min at 37°C.

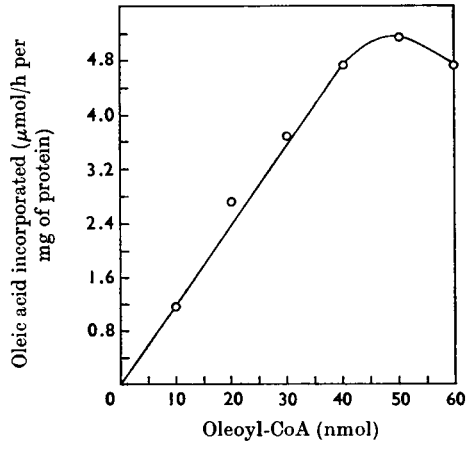

Fig. 3. Effect of oleoyl-CoA concentration on the acyltransferase activity of brush-border-free mucosa.

Table 1. *Fatty acid specificity of brush border-free intestinal mucosal acyltransferase*

The conditions of incubation were as described in the text.

Fatty acid added	Labelled fatty acid incorporated (nmol/h per mg of protein)
0.1 μmol of [1-^{14}C]oleic acid	155
0.1 μmol of [1-^{14}C]oleic acid + 0.1 μmol of unlabelled palmitic acid	120
0.1 μmol of [1-^{14}C]palmitic acid	95
0.1 μmol of [1-^{14}C]palmitic acid + 0.1 μmol of unlabelled oleic acid	90

Table 2. *Substrate specificity of brush-border-free intestinal mucosal acyltransferase*

The incubation mixtures contained [1-^{14}C]oleoyl-CoA (50 nmol; 260 c.p.m./nmol), $MgCl_2$ (15 μmol), NaH_2PO_4–Na_2HPO_4 buffer, pH 7.4 (25 μmol), brush-border-free particulate preparation (100 μg of protein) and 0.4 μmol of the given substrate. When lysophosphatidic acid was used as the substrate, the radioactivity in the spot corresponding to that of phosphatidic acid was determined. When GPC was the substrate, the total radioactivity in the spots corresponding to both lysolecithin and lecithin was measured. In all other experiments, the spot corresponding to that of lecithin only was analysed for radioactivity.

Substrate used	Labelled oleic acid incoporated into the product(s) (nmol/h per mg of protein)
None	34
1-Acyl-GPC from egg lecithin	4636
1-Myristoyl-GPC	981
1-Alkenyl-GPC	168
1-Alkyl-GPC	134
2-Acyl-GPC	144
1-Acyl-GP	230
GPC	78

Table 3. *Position occupied by the labelled fatty acids in the synthesized lecithin*

The labelled lecithin was subjected to the action of *C. adamenteus* venom. Incubation mixtures contained 1.25 μmol of labelled lecithin in 3.0 ml of diethyl ether and 2.0 mg of *C. adamenteus* venom in 0.05 ml of tris–maleate–NaOH buffer, pH 7.0, containing 10 μmol of $CaCl_2$. The reactions were carried out for 3 h at room temperature.

Conditions of incubation	Distribution of radioactivity (%)		
	Lecithin	Lysolecithin	Free fatty acid
Lecithin labelled with [1-^{14}C]oleic acid			
Without snake venom	82.3	11.1	6.6
With snake venom	6.8	3.4	89.8
Lecithin labelled with [1-^{14}C]palmitic acid			
Without snake venom	75.6	9.6	14.8
With snake venom	6.7	9.6	83.7

went into the β-position of the phospholipid molecule.

Subcellular distribution. The brush-border-free particulate fraction was the most active of all the fractions obtained from the mucosal cell (Table 4). This enzyme has been found to be associated with particulate materials in other tissues of rats, such as liver (Lands, 1960) and brain (Webster, 1965) and the present work has shown it to be of particulate nature in the mucosal cell also. However work by Eibl, Hill & Lands (1969) has indicated that it is a true microsomal enzyme in rat liver. In the present experiments attempts were not made to isolate a typical microsomal fraction and it is possible that here also it is a true microsomal enzyme. The activity in the brush-border fraction is probably due to contamination by the microsomal particles, because we have found about 15%

Table 4. *Subcellular distribution of the enzyme in the intestinal mucosa*

The amounts of protein added were 100 μg for whole homogenate, 212 μg for the brush-border fraction, and 70 μg for the brush-border-free particulate or soluble fraction.

Cell fraction	Oleic acid incorporated into lecithin (nmol/h per mg of protein)	Activity (% of total)
Whole homogenate	2080	100
Brush border	2060	20
Brush-border-free particulate	5540	65
Soluble	114	4

Table 5. *Tissue distribution of the enzyme*

In all assays 100 μg of protein per reaction mixture was used.

Tissue preparation	Oleic acid incorporated (nmol/h per mg of protein)
Intestinal mucosa	
Whole homogenate	1364
Brush-border-free particles	4800
Liver	
Whole homogenate	595
Microsomes	2252
Kidney homogenate	576
Testes homogenate	995
Brain homogenate	169
Pancreas homogenate	285
Spleen homogenate	435
Epididymal-fat-pad homogenate	303

of the total RNA of the whole mucosa in the isolated brush border (Subbaiah *et al.* 1968).

Tissue distribution. There are several reports on the acyl-CoA–acyl-GPC acyltransferase activity of many tissues of various species of animals. When the values reported in the literature for the enzyme from other tissues were compared with the present values, the activity of the mucosal enzyme appeared to be markedly higher. Whole homogenates of various tissues were compared (Table 5) and the intestinal mucosa showed the highest specific activity, followed by testes, liver, kidney and spleen. The specific activity of the brush-border-free particles was twice that of the liver microsomes.

DISCUSSION

In preliminary experiments, where the reactions were carried out for 1 h and with free [1-^{14}C]oleic acid, CoA and ATP, we did not observe any incorporation of the fatty acid into lecithin by the whole homogenates of rat mucosa (P. V. Subbaiah, P. S. Sastry & J. Ganguly, unpublished work). However, significant acyltransferase activity could be demonstrated in later experiments by using the brush-border-free particles of the mucosal homogenates (Subbaiah *et al.* 1969). It has been shown here that considerable activity can be demonstrated in the whole homogenates, as well as in the brush-border-free particles, if the reactions are carried out for 5 min. We have shown that the rat intestinal mucosa is rich in several phospholipases (Subbaiah & Ganguly, 1970), and therefore it would appear that the lecithin, which is apparently very rapidly formed, is hydrolysed on prolonged incubation with the whole homogenate; this may explain the failure in our earlier attempts, as well as in those of Webster (1965), to demonstrate the presence of this enzyme activity in the whole homogenates of rat mucosa.

The properties of the mucosal acyltransferase appear to be comparable with those of the rat liver enzyme in several respects, e.g. pH optimum, requirements for ATP and CoA and inhibition by higher concentrations of oleoyl-CoA. However, in contrast with the enzymes from other sources, the mucosal enzyme did not exhibit absolute specificity for the unsaturated fatty acid when 1-acyl-GPC was used as the acyl acceptor, since the incorporation of palmitic acid was about 60% of that of oleic acid; both fatty acids were incorporated

into the β-position of the lecithin. Hanahan & Bloomstrand (1956) have reported that dietary palmitic acid is equally incorporated into both positions of lymph lecithin in rats, and similarly, after giving 1[9,10-^{3}H]-palmitoyl-GPC to rats, Nilsson (1968) found about 20% of the label in the β-position of the lymph lecithin. These observations can readily be explained on the basis of the ability of the mucosal enzyme to acylate the β-position of lysolecithin with palmitic acid.

However, when 2-acyl-GPC was used as the acyl acceptor, the incorporation of oleic acid into lecithin was negligible. In this respect the behaviour of the mucosal enzyme is comparable with that of other tissues. Of all the acceptors tested lysolecithin with a long-chain fatty acid esterified at the 1-position was found to be most efficient, and since the phospholipase A from the pancreas or small intestine gives rise to only 1-acyl lysophosphatides, the specificity of the mucosal enzyme is for the acylation of the naturally formed lysophosphatides and thus assumes physiological significance in the absorption of phospholipids.

Acylation of lysolecithin involves at least two steps, namely activation of the fatty acid and transfer of the activated fatty acid to lysolecithin. Usually the activation step is rate-limiting in such reactions in many biological systems. According to Pande & Mead (1968), compared with other tissues rat intestinal mucosa is very weak in the fatty acid–CoA ligase activity. Therefore the use of oleoyl-CoA, as reported in the present experiments, reflected the true acyltransferase activity. By using oleoyl-CoA we have shown here that the rat intestinal mucosa is the most active of all the tissues tested, in agreement with the observations by Stein & Stein (1966), who reported that after intravenous injections lysolecithin is acylated most rapidly in the small intestine, compared with the other tissues of rats. The presence of such highly active acyl-CoA–acyl-GPC acyltransferase in the intestinal mucosa is of obvious physiological importance, because this tissue has to deal with large amounts of lysolecithin, which are formed during the digestion of dietary phospholipids.

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