CONVERSION OF DIGLYCERIDE TO TRIGLYCERIDE BY RAT LIVER MICROSOMES: A REQUIREMENT FOR THE 105,000 x g SUPERNATANT[1]

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SUMMARY: This paper describes a requirement for the 105,000 x g supernatant of rat liver for the synthesis of triglyceride from diglyceride and palmityl coenzyme A by rat liver microsomes. ATP and magnesium chloride are also required. The incorporation of both [1-^{14}C]-palmityl coenzyme A and [1-^{14}C]-diolein into triglyceride has been observed. The 105,000 x g supernatant has no enzymatic activity for this reaction when incubated in the absence of microsomes. The supernatant contains a soluble, essential protein which is nondialyzable, heat sensitive, and destroyed by trypsin. Net synthesis of triglyceride has been demonstrated by chemical analysis.

Work in this laboratory over the last several years has implicated several substrate specific proteins, present in the 105,000 x g supernatant[4] of rat liver, as having essential roles in the microsomal biosynthesis of sterols (1-5). These soluble proteins are required for the synthesis of sterols by the microsomal membranes, and it has been proposed that they operate as carriers for water insoluble substrates. We reasoned that similar phenomena might occur in other lipid biosynthetic pathways. The reaction we have chosen to study is the acylation of diglyceride to form triglyceride.

MATERIALS AND METHODS

Chemicals: [1-^{14}C]-Diolein was obtained from Dhom; [1-^{14}C]-palmityl-CoA from New England Nuclear; palmityl-CoA, trypsin, and trypsin inhibitor from Sigma; Silicar CC-7 Special from Mallinckrodt; 1,2-dipalmitin from Applied Science Laboratories; and 1,2-diolein from Serdary Research Laboratories, London, Ontario.

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4. Abbreviation used: S_{105}, 105,000 x g supernatant.

S_{105} and Microsome Preparation: Adult male Sprague-Dawley rats (200-300 g) were sacrificed by decapitation. Livers were immediately excised and washed in ice cold potassium phosphate buffer (0.02 M, pH 7.4) containing EDTA (0.1 mM). The livers were blotted dry, weighed and suspended in 2 ml of fresh cold buffer per gram of tissue. After mincing with scissors, the livers were homogenized with a loose fitting (1 mm clearance) Teflon pestle. The homogenate was centrifuged at 1000 x g for 10 minutes, the pellet was discarded, and the 1000 x g supernatant was then centrifuged at 20,000 x g for 15 minutes. Again the pellet was discarded, and the 20,000 x g supernatant was centrifuged at 105,000 x g for 60 minutes. The supernatant was carefully removed from the microsomal pellet and recentrifuged at 105,000 x g for 60 minutes to remove any residual light microsomes. The clear middle fraction (constituting S_{105}) was removed with a pipet. The microsomal pellet was resuspended in fresh buffer, homogenized with a tight fitting Teflon pestle, and recentrifuged at 105,000 x g for 60 minutes. This washing procedure was repeated twice more. S_{105} and triply washed microsomes were frozen separately in liquid nitrogen, and they can be stored indefinitely at -80° without loss of activity.

Incubations: All incubations were carried out in a Dubnoff shaker at 37° under an atmosphere of nitrogen in a final volume of 2 ml of potassium phosphate buffer (0.02 M, pH 7.4) containing EDTA (0.1 mM). Diglyceride was added in 5-10 µl of 2:1 dioxane-propylene glycol. Palmityl-CoA was added in 5-10 µl of sodium acetate buffer (0.1 M, pH 6.0).

Extractions: Incubations were stopped by the addition of 1:2 chloroform-methanol (7.5 ml). Subsequently chloroform (2.5 ml) and water (2.5 ml) were added as described in the extraction procedure of Bligh and Dyer (6). After a brief centrifugation the chloroform layer was removed and dried under a stream of nitrogen. The residue was taken up in toluene; the triglyceride fraction was isolated by silicic acid chromatography as described below.

Chromatography: Separations were carried out on silicic acid (Silicar CC-7 Special) columns (1 x 5 cm). Toluene was the eluting solvent, and 7 ml

fractions were collected. Cholesterol esters were completely removed from the column in Fraction 1. Triglycerides were eluted in Fractions 2 through 6. Fatty acids and diglycerides were eluted separately in much later fractions unless the solvent was changed to 1:9 diethyl ether-toluene in which case fatty acids and diglycerides were eluted together in one fraction.

Scintillation Counting: All radioactivity measurements were performed with a Packard Model 3375 liquid scintillation counter. Counting efficiency was 82%.

Chemical Determination of Esters: In the net synthesis experiment (Table 4) triglyceride ester was determined quantitatively by the hydroxamate method of Rapport and Alonzo (7).

RESULTS

As shown in Table 1 when microsomes were incubated with [1-^{14}C]-palmityl-CoA and diolein, only slight incorporation of label (3.9%) was observed in the triglyceride fraction (Flask 1). However, when S_{105}, ATP and magnesium chloride were added to the incubation mixture, substantial incorporation of label (21.4%) into the triglyceride fraction was seen (Flask 6). Omission of either S_{105} or ATP from the incubation mixture caused incorporation to fall back to low levels (Flasks 2, 3 and 4), while omission of magnesium chloride produced a lesser effect (Flask 5). No enzymatic activity was detected when S_{105} was incubated in the absence of microsomes.

Different preparations of S_{105} have given slightly different results. With one preparation added magnesium chloride was not required, and with another, neither added ATP nor added magnesium chloride was required. In any event, the ATP and magnesium chloride requirements can be induced in any preparation of S_{105} simply by dialysis for 16 hours against 20 volumes of buffer. After dialysis full activity can be restored by addition of ATP and magnesium chloride. We presume that this observation is due to variable endogenous levels of ATP and magnesium chloride in S_{105}.

Table 2 shows the results of an experiment in which the incorporation of [1-^{14}C]-diolein into triglyceride was monitored. Again S_{105} was required for

TABLE 1

INCORPORATION OF [1-^{14}C]-PALMITYL-CoA INTO TRIGLYCERIDE BY LIVER MICROSOMES: REQUIREMENT FOR S_{105}, ATP AND MAGNESIUM CHLORIDE

Flask	Additions	Incorporation of [1-^{14}C]-palmityl-CoA into triglyceride	
		%	nmoles
1	none	3.9	0.066
2	ATP + $MgCl_2$	4.6	0.078
3	S_{105}	2.2	0.037
4	S_{105} + $MgCl_2$	1.9	0.032
5	S_{105} + ATP	10.7	0.182
6	S_{105} + ATP + $MgCl_2$	21.4	0.364

All flasks contained microsomes (1.9 mg protein), diolein (8.1 nmoles) and [1-^{14}C]-palmityl-CoA (1.7 nmoles, 0.1 μCi) in a final volume of 2 ml of buffer. Flasks 2, 4 and 6 contained magnesium chloride (4 mM). Flasks 2, 5 and 6 contained ATP (4 mM). Flasks 3-6 contained S_{105} (17 mg protein).

TABLE 2

CONVERSION OF [1-^{14}C]-DIOLEIN TO TRIGLYCERIDE

Flask	Additions	Incorporation of [1-^{14}C]-diolein into triglyceride	
		%	nmoles
1	none	1.0	0.010
2	palmityl-CoA	6.7	0.067
3	S_{105}	25.9	0.259
4	S_{105} + palmityl-CoA	25.4	0.254

All flasks contained microsomes (1.9 mg protein), ATP and magnesium chloride (4 mM), and [1-^{14}C]-diolein (1 nmole, 0.1 μCi) in a final volume of 2 ml of buffer. Flasks 3 and 4 contained S_{105} (17 mg protein). Flasks 2 and 4 contained palmityl-CoA (10 nmoles).

triglyceride synthesis by microsomes. The requirement for ATP and magnesium chloride by this system was the same as shown previously in Table 1. Once again, when incubated in the absence of microsomes, S_{105} exhibited no enzymatic activity.

TABLE 3

TRYPSIN TREATMENT OF LIVER S_{105}

Flask	Additions	Incorporation of [1-^{14}C]-palmityl-CoA into triglyceride	
		%	nmoles
1	none	3.9	0.066
2	S_{105}	26.3	0.447
3	S_{105} + trypsin inhibitor	11.3	0.192
4	S_{105} + trypsin + trypsin inhibitor	1.5	0.025

All incubations and preincubations were carried out at 37° under nitrogen. Flask 1 received no preincubation. The S_{105} in Flask 2 was preincubated alone for 60 min. The S_{105} in Flask 3 was preincubated alone for 30 min at which time trypsin inhibitor (5 mg) was added and the preincubation was continued for an additional 30 min. In Flask 4 S_{105} and trypsin (5 mg) were preincubated together for 30 min at which time trypsin inhibitor (5 mg) was added and the preincubation was continued for an additional 30 min. At the end of the preincubation period, microsomes (1.9 mg protein), ATP and magnesium chloride (4 mM), dipalmitin (7 nmoles), and [1-^{14}C]-palmityl-CoA (1.7 nmoles, 0.1 μCi) were added to all flasks. The final incubation volume for all flasks was 2 ml. All flasks were then incubated for 60 min.

TABLE 4

NET SYNTHESIS OF TRIGLYCERIDE FROM [1-^{14}C]-PALMITYL-CoA AND DIOLEIN

Incubation	Triglyceride	Triglyceride synthesized		[1-^{14}C]-palmityl-CoA incorporated	
	nmoles	nmoles	%	nmoles	%
1	569	---	---	---	---
2	791	222	34.4	191	29.6

Each incubation consisted of five identical flasks. All flasks contained microsomes (1.9 mg protein), S_{105} (17 mg protein), and ATP and magnesium chloride (4 mM) in a final volume of 2 ml of buffer. Incubation 1 contained no added substrate. Incubation 2 contained a total of 645 nmoles of [1-^{14}C]-palmityl-CoA (0.645 μCi) and 405 nmoles of diolein.

Evidence for the protein nature of the component present in S_{105} responsible for the activation of microsomal triglyceride synthesis was obtained by heat and trypsin treatments. When heated in a boiling water bath (95°) for 5 minutes, S_{105} lost a substantial amount (50-75%) of its capacity to activate

the conversion of diglyceride to triglyceride by liver microsomes. Table 3 shows the results of an experiment in which S_{105} was incubated with trypsin. While trypsin inhibitor was slightly inhibitory in this reaction (Flask 3), it is clear that trypsin totally destroyed the ability of S_{105} to activate triglyceride synthesis (Flask 4).

In order to eliminate the possibility that incorporation of label was due not to net synthesis but rather to exchange of labeled palmitate with fatty acids of endogenous triglyceride, an experiment was designed to test for the net synthesis of triglyceride. Table 4 summarizes the results of such an experiment. Incubation 1 contained no exogenously added substrate. Incubation 2 contained added diolein and added [1-C^{14}]-palmityl-CoA. The net synthesis of triglyceride in Incubation 2 as determined chemically (222 nmoles or 34.4% of the added palmityl-CoA) corresponded closely with the value determined isotopically (191 nmoles or 29.6% of the added [1-^{14}C]-palmityl-CoA). These data demonstrate conclusively that this system (microsomes + S_{105} + ATP + magnesium chloride) causes the net synthesis of triglyceride from diglyceride and palmityl-CoA.

DISCUSSION

We have demonstrated a requirement for a soluble protein present in liver 105,000 x g supernatant (S_{105}) which is required for the conversion of palmityl-CoA and diglyceride to triglyceride by rat liver microsomes. In addition we have shown a requirement for ATP and magnesium chloride. Triglyceride synthesis has been monitored by incorporation of label into triglyceride using either [1-^{14}C]-palmityl-CoA or [1-^{14}C]-diolein as the source of label. Using either substrate, there is no enzymatic activity associated with S_{105} when incubated in the absence of microsomes. Net synthesis of triglyceride by the complete system has been demonstrated by chemical analysis of ester linkages.

Weiss, Kennedy and Kiyasu (8) did not report a soluble protein requirement for this reaction when they used chicken liver 20,000 x g sedimented particles as their source of enzymes.

Smith, Sedgwick, Brindley and Hübscher (9) noted a requirement for S_{105} by both rat liver mitochondria and cat intestinal mucosa microsomes in the conversion of phosphatidic acid to glycerides. Johnston, Rao, Lowe and Schwarz (10) observed a soluble protein requirement for the incorporation of either palmitic acid or L-α-glycerophosphate into triglycerides. The conclusion drawn in both papers was that the protein responsible for stimulation was L-α-phosphatidate phosphohydrolase (EC 3.1.3.4), which converts phosphatidic acid to diglyceride.

Such an explanation is not possible for the system which we have described since phosphatidic acid is not the substrate. An alternative explanation is that S_{105} contains one or more proteins which operate as carriers for the synthesis of triglycerides from water insoluble precursors by liver microsomes.

It is possible that there are substrate specific soluble proteins present in S_{105} which are essential for the synthesis of most water insoluble substrates by microsomal membranes. In fact evidence compatible with this proposal exists for the synthesis of many different types of water insoluble compounds (4). Further experiments are in progress to test this hypothesis.

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