Dependence of microsomal lipid synthesis on added protein

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SUMMARY Lipid synthesis by rat liver microsomes from L-α-glycerophosphate-1-C^{14} in the presence of palmitic acid, ATP, CoA, and Mg^{2+} came to a standstill after 30 min while enough cofactors were still present to promote further synthesis. The extent of the reaction depended on the amount of microsomes. Doubling the microsome concentration more than doubled the extent of synthesis.

Synthesis of both neutral lipids and phospholipids could be promoted by the addition of inactivated microsomes as well as by serum albumin and by various lipoprotein preparations. A 20- to 40-fold increase of ester yield could be obtained by supplementing low concentrations of microsomes with the proteins. Ovolecithin also had a considerable activating effect.

Lipoproteins had an activating effect even in the presence of optimal albumin concentrations, pointing to different modes of action of the two proteins. The hypothesis is discussed that microsomes synthesize lipoproteins and that lipid synthesis is limited by the amount of acceptor protein available.

RAT LIVER MICROSOMES can be shown to carry out rapid synthesis of neutral fat and phospholipids, when incubated with a radioactive fatty acid, Coenzyme A, ATP, and Mg^{2+} (1). Attempts to improve the performance of this system to make it possible to measure net formation of lipid esters were unsuccessful. One limiting factor was the inhibitory effect of one of the substrates—fatty acids—when used in more than millimolar concentration. The second was the relatively short time for which the reaction proceeds at a measurable rate. The present paper deals with this last limitation and with the role of microsomal protein and other added proteins on the extent of synthesis attained.

MATERIALS

ATP (potassium salt), CoA, sodium α-glycerophosphate, and palmitic acid were commercial preparations. L-α-Glycerophosphate-1-C^{14} was prepared from glycerol-1-C^{14} (Amersham, Bucks, England) and ATP in the presence of glycerokinase (2). Bovine serum albumin (fatty acid poor) was obtained from Pentex Corp., Kankakee, Ill.

Cohn's Fractions I + III, III_0 and IV_1 were obtained from the Blood Bank of Magen David, Tel-Aviv, fractionated from human serum according to Method 6 of Cohn (3). Fractions I + III and III_0 were dissolved in sodium phosphate (0.05 M) and EDTA (0.005 M) and stored at 4°. Fraction IV_1 was obtained as lyophilized powder.

Fibrinogen and α-globulin were fractions of human plasma obtained from Armour, Chicago, Ill. β-Lactoglobulin (bovine) was a crystalline preparation. Purified β-lipoprotein was prepared from human plasma by precipitation with polyvinylpyrrolidone (PVP) according to Burstein (4) or from rat serum brought to density 1.063 with KBr and centrifuged for 16 hr at 100,000 × *g*. The floating lipoprotein was dialyzed against 0.9% NaCl. The lipid content in these two types of preparations varied between 5 and 8 μeq ester per mg protein.

METHODS

"*Native supernatant*" (NS), i.e., the mitochondria-free supernatant fraction of rat liver homogenates in KCl–Tris buffer (9 parts 0.15 M KCl, 1 part 0.5 M Tris–HCl buffer pH 7.4, 10^{-4} M in EDTA), was prepared as described previously (1).

Lyophilized Microsomes. The NS was centrifuged at 100,000 × *g* for 30 min in a "Spinco" centrifuge at 4°. The supernatant fluid was decanted and the microsomal sediment rehomogenized with the KCl–Tris–EDTA solution and centrifuged again for 30 min. The supernatant solution was discarded, the microsomes were suspended in cold double-distilled water, homogenized.

and lyophilized. The lyophilized microsomes were stored at −20°. Protein was estimated according to Lowry et al. (5).

Extraction of Lipids and Fractionation. Chloroform–methanol 2:1 (v/v) (12 ml) was added to the reaction mixtures and the lower layer was washed with 8 ml of 0.1 M KCl–methanol–chloroform 94:96:6 (v/v/v). The chloroform layer contained all the labeled lipids, free of any detectable unchanged α-glycerophosphate. It was evaporated to dryness and the residue was applied in 3 ml of chloroform to a small column of silicic acid (1 g). Neutral lipids were eluted with 50 ml of chloroform and phospholipids with 50 ml of methanol.

Delipidation of lipoproteins was performed according to the method of Avigan (6) at 4°. The lipoprotein solution was adjusted to a specific gravity of 1.063 with KBr, in order to minimize the denaturation of lipoprotein. Ten volumes of ether were added and the solution was shaken very slowly for 16 hr at 4°. The aqueous layer was then dialyzed against 0.9% NaCl in order to remove the KBr and traces of ether.

The ether solution was evaporated in the reaction tubes and the lipid dispersed in the reaction mixture by shaking at 37°.

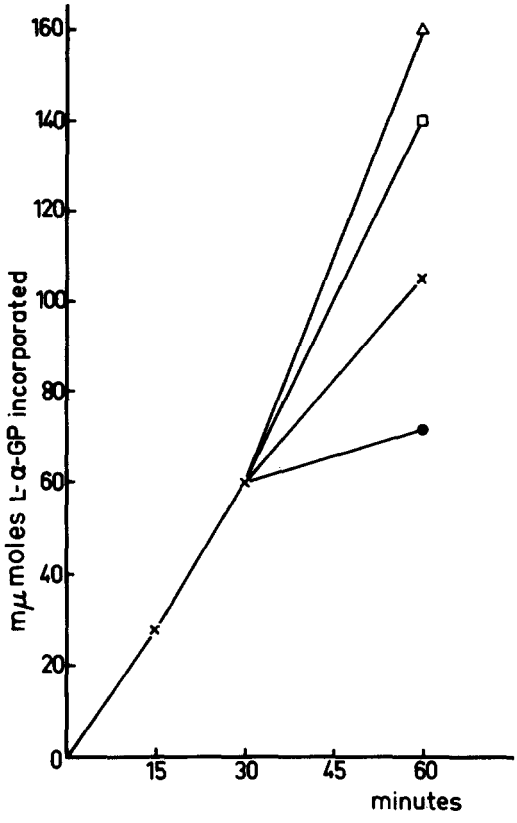

FIG. 1. Effect of additional enzyme added at the end of incubation. "Native supernatant" (NS), 0.1 ml (2.8 mg protein), was added at the beginning of incubation to all flasks, with the indicated additions after 30 min. The incubation mixture consisted of 10 μmoles of ATP, 0.1 mg of CoA, 10 μmoles of $MgCl_2$, 20 μmoles of potassium phosphate buffer pH 7.4, 3.5 μmoles of sodium L-α-glycerophosphate-1-C^{14} (30,000 cpm), 0.6 μmoles of palmitate, 50 μmoles of KF, and enzyme as indicated in a total volume of 3 ml. Incubation was carried out at 37° under air and incorporation of α-GP (L-α-glycerophosphate) calculated from the percentage incorporation of C^{14} into lipids.
● none; × 0.1 ml of NS; □ 0.2 ml of NS; Δ 0.2 ml of NS + 0.6 μmole of palmitate.

RESULTS

Experiments were performed either with the "native supernatant" or with lyophilized microsomes. The former had the advantage of simplicity and rapidity of preparation. However, considerable differences in activity were encountered from batch to batch. With lyophilized microsomes, on the other hand, experiments could be repeated with a single preparation for more than two weeks with almost identical results. After 3 weeks activity decreased markedly in these preparations. Here, too, considerable differences were found between various batches, but the activating effects reported were always highly significant. The tables and figures present representative results of at least three experiments.

The formation of fatty acid esters by rat liver "native supernatant" (NS) incubated with ATP, CoA, α-glycerophosphate, Mg^{2+}, and palmitic acid came almost completely to an end after 30 min of incubation. This cessation of activity was not due to lack of reactants, as the

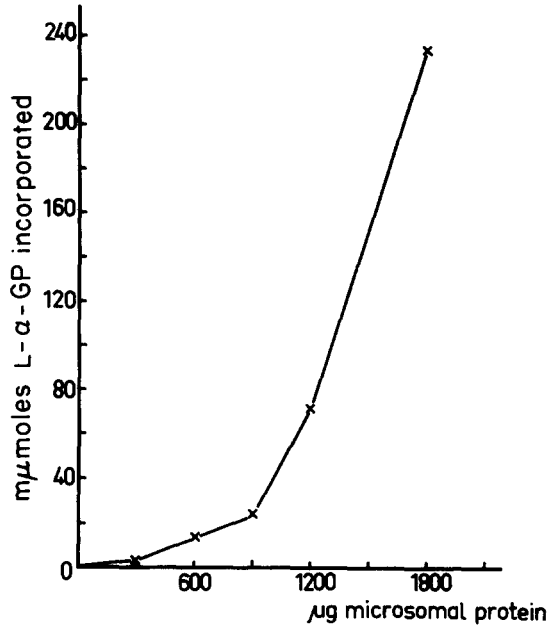

FIG. 2. Effect of varying concentrations of lyophilized microsomes on lipid synthesis. Reaction mixtures as in Fig. 1, with lyophilized microsomes added in amounts indicated. Incubation was carried out for 2 hr.

esterification was renewed upon addition of more NS (Fig. 1). With the higher enzyme concentration almost all the palmitic acid was used up and palmitate had to be added for maximal activity. The total esterification obtained after 2 hr of incubation increased with the amount of enzyme and was more than proportional to this amount (Fig. 2).

Preincubation of the NS without substrates and cofactors at 37° caused a marked reduction in activity. However, when this inactivated enzyme was supplemented with a fresh portion of NS, the system formed more than the sum of both activities and about as much as would be obtained when starting with twice the amount of unincubated enzyme (Table 1).

Small amounts of microsomes (0.9 mg of protein) produced very little lipid. The addition of various proteins increased the activity several times (Table 2). Cohn's Fraction I + III and serum albumin were highly active,

TABLE 1 ACTIVATION OF NS ("NATIVE SUPERNATANT") BY INACTIVATED NS

Expt. No.	Addition	mμmoles α-GP* Incorporated
1	0.2 ml of NS	76
2	0.2 ml of preincubated NS†	37
3	As in experiment 2 + fresh 0.2 ml of NS added with cofactors	195
4	0.4 ml of NS	162

Conditions as in Fig. 1; NS (3.5 mg of protein in 0.2 ml) was used. Incubation time, 60 min.

* α-GP = L-α-glycerophosphate.

† 0.2 ml of NS kept at 37° for 30 min. Cofactors and substrates were then added and incubation was continued for 60 min.

TABLE 2 EFFECT OF VARIOUS PROTEINS ON LIPID-SYNTHESIZING SYSTEMS IN RAT LIVER MICROSOMES

Protein Added	mμmoles α-GP Incorporated
None	30
5 mg serum albumin	125
10 mg " "	152
13 mg serum Fraction I + III	142
5 mg ovalbumin	44
10 mg "	23
5 mg fibrinogen	26
10 mg "	23
5 mg β-lactoglobulin	33
10 mg "	79
5 mg γ-globulin	46
10 mg "	49

TABLE 3 EFFECT OF VARIOUS LIPOPROTEIN PREPARATIONS ON LIPID SYNTHESIS

Expt. No.	Enzyme Preparation (NS)	Lipoprotein Added	mμmoles α-GP Incorporated
	ml		
1	0.1	None	35
	0.1	1 mg d < 1.063 lipoprotein*	154
2	0.1	None	36
	0.1	0.5 mg β-lipoprotein (precipitated with PVP)*	98
3	0.2	None	126
	0.2	4.5 mg β-lipoprotein (Cohn's Fraction III_0)	206
4	0.1	None	91
	0.1	4.5 mg human α-lipoprotein (Cohn's Fraction IV_1)	167
	0.1	6.6 mg Cohn's Fraction I + III	182

Reaction mixture as in Fig. 1.

* Lipoprotein preparations are described in "Materials."

TABLE 4 EFFECT OF VARIOUS CONCENTRATIONS OF LIPOPROTEIN AND SERUM ALBUMIN ON LIPID SYNTHESIS

Expt. No.	Protein Added	mμmoles α-GP Incorporated
1	2.6 mg lipoprotein (Fraction I + III)	45.5
	6.5 mg " "	119
	13 mg " "	185
	19.5 mg " "	164
	26 mg " "	150
2	10 mg serum albumin	200
	20 mg " "	136
	40 mg " "	77
3	5 mg " "	125
	10 mg " "	152

Reaction mixture as in Fig. 1, with 0.9 mg of lyophilized microsome protein. Incubation carried out for 120 min.

TABLE 5 COMBINED EFFECT OF LIPOPROTEIN AND ALBUMIN

	mμmoles α-GP Incorporated					
	Albumin		Lipoprotein		Albumin + Lipoprotein	
No Addition	10 mg	20 mg	13 mg	26 mg	10 mg	13 mg
16	96	98	70	96	188	
16	92	69	99	78	145	
	70		84		189	

Reaction mixture as in Fig. 1, with 0.9 mg of lyophilized microsome protein. Incubation time, 120 min. Quantities of lipoprotein (Cohn's Fraction I + III) and serum albumin refer to protein content.

TABLE 6 ACTIVATION BY ETHER EXTRACT AND BY DELIPIDATED LIPOPROTEIN

Expt. No.	Addition	mμmoles α-GP Incorporated
1	None	13.5
2	Lipoprotein (Cohn's Fraction I + III)	185
3	Ether extract of 2	45.5
4	Delipidated lipoprotein of 2	17.5

TABLE 7 EFFECT OF LIPID FACTORS ON LIPID SYNTHESIS BY MICROSOMES

Preparation	Addition	mμmoles α-GP Incorporated
1	None	37
	Triolein, 3 μeq	37
	Triolein, 9 μeq	37
	Sodium cholate, 3 mg	7
	Ediol, 2 mg	52
	Ediol, 5 mg	52
	Ovolecithin, 14 μeq	257
2	None	16
	Ovolecithin, 14 μeq	171
	Ovolecithin, 28 μeq	165

Reaction mixture as in Fig. 1, with 0.9 mg of lyophilized microsome protein. Incubation time, 120 min. Ediol = a commercial fat emulsion which contains 50% coconut oil (Schenlabs Pharmaceuticals, Inc., New York). Triolein and lecithin were added as aqueous suspensions prepared by sonication.

while γ-globulin, fibrinogen and ovalbumin were devoid of activity. An activating effect was obtained also with β-lactoglobulin. The lack of activity of fibrinogen made it likely that the effect of Fraction I + III was based on its other major component, β-lipoprotein. This could be substantiated by experiments with preparations of purified lipoproteins. All the samples of β-lipoproteins, prepared by Cohn's fractionation (Fraction VII_0), by centrifugal flotation, or by precipitation with polyvinylpyrrolidone (PVP), were highly active. Fraction IV_1 was also found to activate the synthesis (Table 3). Fraction I + III was used in most experiments, as it was the most stable.

Optimal activation was obtained with 13 mg of lipoprotein (Fraction I + III) or with 10 mg of albumin in the reaction mixture (Table 4).

The activating effect of serum albumin and lipoprotein was exerted by different mechanisms, as optimal amounts of both activators added together gave additive activating effects (Table 5).

Furthermore, while the addition of lipoprotein caused activation at all microsome concentrations tested and corrected the nonlinearity of lipid synthesis found with nonsupplemented microsomes, serum albumin was effective only at low enzyme concentrations and was even inhibitory with higher ones (Fig. 3).

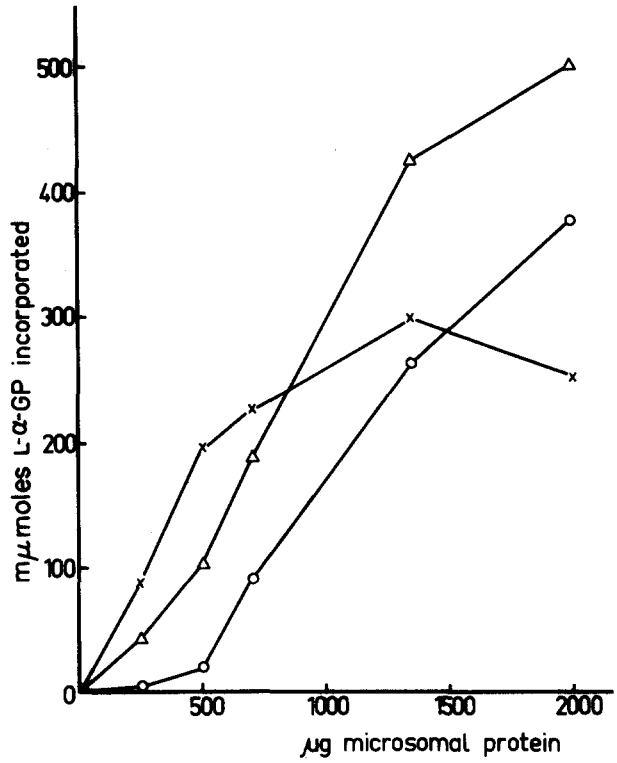

FIG. 3. Activation by lipoprotein and albumin on varying concentrations of microsomes. Reaction mixtures as in Fig. 1, with lyophilized microsomes added in amounts indicated.
O microsomes; × microsomes + 10 mg of albumin; Δ microsomes + 13 mg of Cohn's Fraction I + III.

It seemed of interest to know which part of the lipoprotein was responsible for the activation. β-Lipoprotein was extracted with cold ether as described in Methods. The ether and the protein portions were then examined for their activity. The ether extract had a significant activating effect on the microsome, but the delipidated lipoprotein proved to be inactive (Table 6).

Of the lipids tested, triglycerides, "Ediol" emulsion and sodium cholate were without effect. Ovolecithin, on the other hand, had a considerable activating effect (Table 7).

TABLE 8 ACTIVATION EFFECT OF LECITHIN

Expt. No.	No Addition	mμmoles α-GP Incorporated					
		Lecithin 14 μeq	Albumin 10 mg	Lipoprotein 13 mg	Lecithin + Albumin 14 μeq + 10 mg	Lecithin + Lipoprotein 14 μeq + 13 mg	Albumin + Lipoprotein 10 mg + 13 mg
1	16.5	46	92	70	105	125	145
2	24	49	63	52	59	80	87

Reaction mixture as in Fig. 1, with 0.9 mg of lyophilized microsome protein. Quantities of lipoprotein (Fraction I + III) and serum albumin refer to protein content. The quantity of lecithin refers to μeq fatty acid ester bonds.

TABLE 9 CENTRIFUGAL SEPARATION OF LABELED LIPIDS

	In reaction mixture with		
	10 mg Albumin	13 mg Lipoprotein	Albumin + Lipoprotein
Per cent of counts in the supernatant fraction	4	18	10
Per cent of counts sedimented with microsomes	96	82	90

Reaction mixture as in Fig. 1, with 0.9 mg of lyophilized microsome protein. The centrifugal separation was performed as indicated in text.

The activating effect of lecithin combined with serum albumin or with lipoprotein was tested (Table 8). It was found that lecithin acted additively with lipoprotein, similar to the action of albumin. On the other hand, lecithin was ineffective in increasing the albumin effect.

Centrifugal separation (at 100,000 × *g* for 1 hr) of the reaction mixtures revealed that only in the presence of lipoprotein was a considerable part of the labeled lipids found in the supernatant fraction. In mixtures incubated with albumin almost all the lipid formed precipitated with the microsomes (Table 9).

Chromatographic separation of the lipids in the reaction mixtures showed that the synthesis of both neutral lipids and phospholipids was enhanced by the addition of any of the activating factors. The additional synthesis obtained by combining albumin with lipoprotein, or egg lecithin with lipoprotein, was mainly in the neutral lipid fraction (Fig. 4).

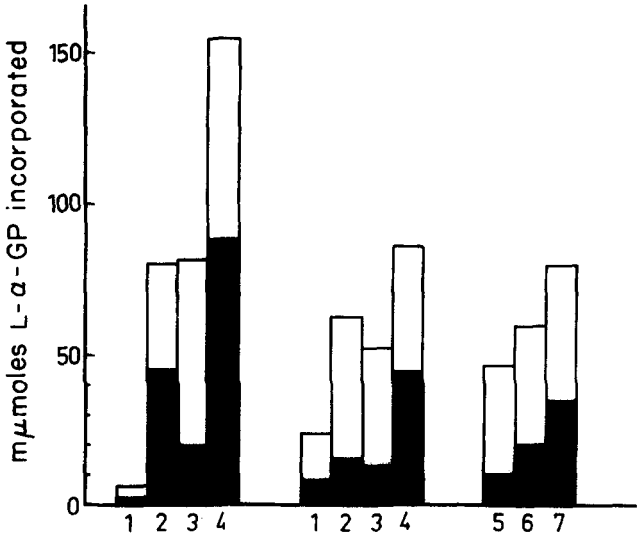

FIG. 4. Synthesis of neutral lipids and phospholipids. For details of incubation mixture, see Fig. 1. The protein content of the albumin was 10 mg and of the lipoprotein fraction I + III was 13 mg. The amount of lecithin was 14 μeq. The columns represent two experiments with two preparations: 1–4 and 1–7, with 0.9 mg of lyophilized microsome protein. The blank column represents the phospholipid fraction and the dark column the neutral lipid fraction.

1, microsome without addition; *2*, microsome + albumin; *3*, microsome + Fraction I + III; *4*, microsome + albumin + Fraction I + III; *5*, microsome + lecithin; *6*, microsome + lecithin + albumin; *7*, microsome + lecithin + Fraction I + III.

DISCUSSION

The extent of ester formation by rat liver microsomes was shown to be limited by the amount of microsomal protein available. The disproportionately large increase in ester formation when the microsomal protein concentration was increased probably resulted from a combination of factors. For example, dilute microsomal preparations were unstable, and albumin enhanced α-glycerophosphate esterification in such dilute systems. In contrast, albumin did not have this effect when microsomal concentrations were high. It is possible that albumin stabilized the dilute enzyme preparations just as it has been shown to do for palmitoyl-CoA-glycerophosphate transacylase (7). An additional stimulation of glyceride formation was provided by certain lipoproteins.

The activating effect of lipoproteins was evident even with higher concentrations of microsomes but was especially impressive with low concentrations. Its mode of action seems to differ from that of albumin, as it was elicited even in the presence of optimal albumin concentrations.

The possibility exists that the presence of a protein acceptor is obligatory for the synthesis of lipids, i.e., that the system actually forms lipoproteins rather than free lipids. If the microsomes contain a limited amount of such a protein acceptor, lipid synthesis would stop when this acceptor is saturated. The addition of external lipoproteins or of inactivated microsomes may then serve as additional acceptor or as agents for removing the synthesized lipids from the microsomal protein and allowing it to continue in its synthetic activity.

It is at present impossible to decide which of these two mechanisms prevails, although it could be shown that the added lipoprotein incorporates some of the synthesized lipids. When microsomes alone or microsomes with albumin were used, all of the radioactive lipid precipitated with the microsomes; in the presence of lipoproteins, a considerable part stayed with the lipoprotein in the supernatant fraction.

The mode of action of egg lecithin is not yet clear. It promoted the effect of the lipoprotein and not that of the albumin. Because it is a lipid, one would have expected it to replace lipoprotein as extractant of the synthesized lipids. Its activity may be due to the supplementation of the lipid moiety of the lipoprotein or the microsomal lipid acceptor.

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