Assay of Glycerol Phosphate Acyltransferase in Liver Particles

By RUTH BRANDES, JUNE OLLEY* and B. SHAPIRO
Department of Biochemistry, Hebrew University–Hadassah Medical School, Jerusalem, Israel

(*Received* 13 *June* 1962)

Lipid ester bonds are formed by transacylation between acyl-CoA and various acceptors. Kornberg & Pricer (1953) showed that rat-liver microsomes catalyse the acylation of L-glycerol 1-phosphate to form diacylphosphatidic acid. Weiss & Kennedy (1956) demonstrated the formation of triglycerides by the interaction of palmitoyl-CoA and D-$\alpha\beta$-diglycerides, and, according to Lands (1960), lecithin can be formed from lysolecithin and oleoyl-CoA. It is not yet clear whether a specific transacylase is required for each of these acceptors. A simple assay system for following the transacylase (acyl-coenzyme A–L-glycerol 3-phosphate acyltransferase, EC 2.3.1.15) reaction has been developed by Stansly (1955). This assay is based on the spectrophotometric determination of the acid-soluble CoA liberated by the reaction. The distinction between the CoA formed by this reaction and that resulting from the enzymic hydrolysis of palmitoyl-CoA was made possible by working at pH 7·5 in the presence of thiols. Under these conditions an increment in the CoA liberated was brought about by the addition of glycerol 1-phosphate. This increment was shown to be due to transacylation and the formation of phosphatidic acid.

Although this method presented an excellent simple way of determining the rate of the transacylase reaction in guinea-pig-liver microsomes, it was difficult to apply to other sources in which the increment of CoA formed in the presence of glycerol phosphate was very small, e.g. with rat-liver microsomes and with mitochondria of all sources tried. Further, since the hydrolase is much more stable than the transacylase, any attempts to extract the enzyme from liver particles made the hydrolytic reaction predominant and the transacylase assay more difficult.

It was therefore thought desirable to improve the assay by finding conditions under which the increment due to transacylase might be increased relative to the hydrolytic formation of CoA. Considerable advance in this direction was obtained by the addition of serum albumin to the reaction mixture. At pH 6·5 the addition of serum albumin brought about an increase in transacylase activity with a concomitant depression of the hydrolase. This made it possible to use the method for transacylase assay in various other sources in addition to guinea-pig microsomes.

The present paper gives details of this improved method and of attempts to ascertain the mechanism of the albumin action.

MATERIALS AND METHODS

Liver-particle fractions. Livers were removed from animals anaesthetized with sodium barbiturate, cooled in crushed ice and washed with ice-cold 0·25 M-sucrose. They were then homogenized with 3 vol. of a mixture of 0·25 M-sucrose and 0·5 M-tris–HCl buffer, pH 7·4 (9:1, v/v). The precipitate was removed by centrifuging at 1000*g* for 10 min. and discarded. Mitochondria were precipitated by centrifuging at 12 000*g* for 20 min. and resuspended in one-third of the original volume of 0·15 M-KCl. The supernatant was centrifuged at 100 000*g* for 30 min. and the microsomes precipitated were resuspended in half the original volume of 0·15 M-KCl. The particles were then freeze-dried and stored at −15°.

Palmitoyl-CoA. This was prepared as described by Seubert (1960) except that palmitoyl anhydride in the presence of $KHCO_3$, as described by Vignais & Zabin (1958), replaced palmitoyl chloride and continuous neutralization with NaOH.

Protein preparations. Bovine serum albumin was obtained from Pentex Inc., Kankakee, Ill., U.S.A. Human serum albumin was obtained from the Marcus Memorial Blood Institute, Jaffa, Israel. Serum γ-globulin was a product of Armour, Chicago, Ill., U.S.A.

L-[1-^{14}C]*Glycerol phosphate.* This was prepared by phosphorylation of [1-^{14}C]glycerol (from The Radiochemical Centre, Amersham, Bucks.) with glycerol kinase according to the method of Bublitz & Kennedy (1955).

Assay procedure. Unless otherwise stated, the reaction mixture contained potassium phosphate (13μmoles of phosphate; pH as indicated), glutathione (2μmoles), palmitoyl-CoA (0·1μmole) and enzyme preparation (1 mg.); then 4 mg. of serum albumin and other protein preparations, and 10μmoles of sodium glycerol 1-phosphate, were added after adjustment to the appropriate pH. The final volume was 0·24 ml.

The mixtures were incubated for 10 min. at 30° and the reaction was stopped by the addition of 1·8 ml. of 3% (w/v) perchloric acid. The mixtures were centrifuged and the extinction of the supernatant was read at 260 mμ. Perchloric acid filtrates of reaction mixtures to which the enzyme was added at the end served as blanks. Figures are presented as μm-moles of CoA liberated, calculated by the factor $\epsilon_{260\,m\mu}^{1\cdot0\,cm.} = 16\,600$.

* Present address: Torry Research Station, Department of Scientific and Industrial Research, Aberdeen, Scotland.

Table 1. *Effect of albumin on deacylation and transacylation by various enzyme preparations*

The reaction mixture and conditions were as described in the Materials and Methods section.

	CoA liberated (μm-moles)			
	Without albumin, pH 7·5		With albumin, pH 6·5	
Enzyme preparation	Deacylation	Transacylation	Deacylation	Transacylation
Rat-liver microsomes	55	12	16	37
Rat-liver mitochondria	85	−10	36	21
Guinea-pig-liver microsomes	36	58	12	80
Guinea-pig-liver mitochondria	39	4	18	57

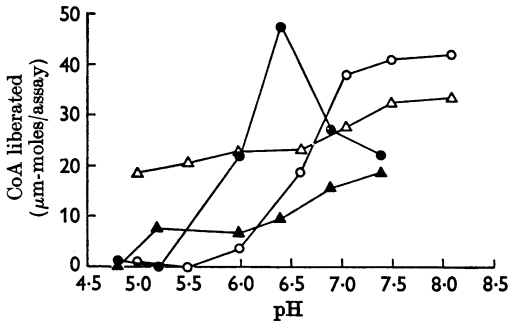

Fig. 1. Effect of albumin on the pH dependence of deacylation and transacylation by guinea-pig-liver microsomes. The reaction mixtures were as described in the Materials and Methods section except that, at pH values lower than 6, potassium citrate–phosphate buffer was used, instead of the usual potassium phosphate buffer. △, Deacylation without albumin; ○, transacylation without albumin; ▲, deacylation with albumin; ●, transacylation with albumin.

CoA liberated in the absence of glycerol 1-phosphate was taken as 'deacylation', and the increment in CoA liberated due to the presence of glycerol 1-phosphate as 'transacylation'.

RESULTS

With guinea-pig-liver microsomes serving as enzyme, the addition of glycerol 1-phosphate resulted in a considerable increase in the liberation of free CoA from palmitoyl-CoA, when tested at pH 7·5 as suggested by Stansly (1955). When, however, rat-liver microsomes or mitochondria served as enzyme, the formation of CoA in the absence of the acceptor was very rapid and the increment due to the addition of glycerol 1-phosphate was slight or not measurable. The optimum pH for transacylase activity was 7·5, and alteration of the pH of incubation beyond the range reported by Stansly (1955) did not yield any better results.

When, however, serum albumin was added, the optimum for transacylase activity was shifted to pH 6·5 (Fig. 1). At this point, serum albumin increased the increment due to glycerol 1-phosphate and at the same time the basal CoA formation due to hydrolase activity was decreased. The combination of these effects made it possible to detect transacylase activity even in preparations which are relatively rich in hydrolase, e.g. rat-liver microsomes and mitochondria from rat and guinea-pig liver (Table 1). Evidence that the enhanced CoA liberation in the presence of glycerol 1-phosphate and serum albumin was due to transacylation was provided by experiments with [1-^{14}C]glycerol 1-phosphate (Table 2). Glycerol 1-phosphate was converted into lipid compounds (Table 2): about 1·3μmoles of CoA were liberated/μmole of glycerol 1-phosphate esterified. This would indicate the formation of a mixture of mono- and di-acylphosphatidic acids.

Table 2. *Liberation of coenzyme A and incorporation of [1-^{14}C]glycerol 1-phosphate into lipid esters by guinea-pig-liver microsomes*

The reaction mixture with guinea-pig-liver microsomes was as described in the Materials and Methods section, but with [1-^{14}C]glycerol 1-phosphate. The ether-soluble glycerol compounds formed were extracted from the reaction mixture with hot ethanol–ether (3:1, v/v). By adding additional water and ether to the extracts two layers separated. A sample of the ether layer was counted. The amounts of ether-soluble glycerol compounds formed were calculated from the counts in the ether layer and the specific activity of the [1-^{14}C]glycerol 1-phosphate added (30 000 counts/μmole, counted in a windowless flow counter).

	Increment in CoA due to glycerol 1-phosphate (μm-moles)	Ether-soluble [^{14}C]glycerol compounds (μm-moles)
With serum albumin	74	59
Without serum albumin	3	5

The effect was obtained with all the different preparations of serum albumin tested. The effect with serum γ-globulin was not significant, and no effect was obtained with denatured microsomes. The activity of serum albumin persisted after dialysis but was destroyed by heating in boiling water for 2 min. (Table 3).

Serum albumin has to be present during the reaction of the enzyme with the substrate. Pretreatment of the particles by suspension in serum albumin and reprecipitation by high-speed centrifuging did not bring about any activation of the transacylase (Table 4). On the other hand, when albumin was added after incubation of the reaction mixture for 5 min., no reactivation of transacylase activity was found. When, however, both albumin and palmitoyl-CoA were absent from the reaction mixture during preincubation, the enzyme kept its activity (Table 5).

The effect of the reaction products formed during the preincubation period, i.e. fatty acids and free CoA, was tested. Palmitate, in amounts that might be formed during the reaction (0·08 μmole), was without effect, either when added in the presence of albumin at pH 6·5 or without albumin at pH 7·5. Preincubation of the reaction mixture with palmitate or CoA before the addition of albumin and substrate was also without effect. Only when the amount of fatty acid added was raised to 0·8 μmole was inactivation of the system observed (Table 6).

Table 3. *Specificity of the albumin effect on deacylation and transacylation by guinea-pig-liver microsomes*

The reaction mixture at pH 6·5 with guinea-pig-liver microsomes was used with the conditions as described in the Materials and Methods section and in Table 1, with the indicated additions (4 mg.). 'Dialysed serum albumin' was prepared by dialysis for 24 hr. of human serum albumin against three changes of 300 vol. of 0·9% (w/v) NaCl. 'Denatured serum albumin' was prepared by heating the human albumin solution added for 2 min. to 100°. Microsomes were denatured by heating the final KCl suspension for 15 min. at 100°.

Protein added	CoA liberated (μm-moles)	
	Deacylation	Transacylation
None	72	0
Human serum albumin	35	64
Dialysed serum albumin	28	48
Denatured serum albumin	45	0
Bovine serum albumin	24	56
Serum γ-globulin	30	15
Denatured guinea-pig-liver microsomes	60	0

DISCUSSION

The results show that serum albumin decreases deacylase activity and prevents the inactivation of the transacylase. In view of the well-known capacity of serum albumin to bind fatty acids, the most obvious explanation for the activation of transacylase activity by albumin would be the prevention of the inhibitory action of fatty acids liberated by the reaction. This explanation is corroborated by the finding that inactivation of

Table 4. *Effect of pretreatment with serum albumin on deacylation and transacylation by guinea-pig-liver microsomes*

The conditions used were as described in the Materials and Methods section and in Table 1. Fresh microsomes were used instead of freeze-dried preparations.

Preparation	pH of test mixture	CoA liberated (μm-moles)	
		Deacylation	Transacylation
Guinea-pig-liver microsomes	7·5	69	17
Guinea-pig-liver microsomes washed with albumin*	7·5	85	18
Guinea-pig-liver microsomes with albumin in the reaction mixture	6·5	25	59

* Fresh microsomes were suspended in 2% (w/v) serum albumin solution and reprecipitated at 100 000*g*.

Table 5. *Effect of delay in the addition of serum albumin on transacylation by guinea-pig-liver microsomes*

The incubations were carried out at pH 6·5 with guinea-pig-liver microsomes as described in the Materials and Methods section.

Expt. no.	Test mixture	CoA liberated (μm-moles)	
		Deacylation	Transacylation
1	10 min. incubation with albumin	12	35
	5 min. incubation without albumin	11	1
	5 min. incubation without albumin followed by 10 min. with albumin	21	5
2	15 min. incubation with albumin	25	67
	15 min. incubation without albumin and palmitoyl-CoA followed by 15 min. with both	21	61

Table 6. *Effect of reaction products on deacylation and transacylation by guinea-pig-liver microsomes*

The mixtures were adjusted to the indicated pH values and guinea-pig-liver microsomes were added. Fatty acids were added as aqueous emulsions, prepared by adjusting solutions of the potassium salts at pH 9 to the pH values of the assay system.

Expt. no.	Assay system	Additions and changes	CoA liberated (μm-moles) Deacylation	Transacylation
1	With serum albumin, pH 6·5	None	22	60
		80 μm-moles of palmitate	22	52
		80 μm-moles of palmitate; substrate and albumin added only after 10 min. of preincubation	26	54
		50 μm-moles of CoA; substrate and albumin added only after 10 min. of preincubation	18	67
2	With serum albumin,* pH 6·5	None	18	112
		100 μm-moles of oleate	23	70
		800 μm-moles of oleate	10	7
	Without albumin,* pH 7·5	None	48	54
		100 μm-moles of oleate	32	66
		200 μm-moles of oleate	33	63
		400 μm-moles of oleate	30	63
		800 μm-moles of oleate	20	1

* Contained 140 μm-moles of palmitoyl-CoA instead of the usual 100 μm-moles.

transacylase in the absence of albumin is brought about only when palmitoyl-CoA is present, i.e. when fatty acids are formed. On the other hand, the concentrations of fatty acids required for inactivation, when added to the reaction mixture, exceed those liberated by the enzyme about tenfold. If the assumption of fatty acid binding is to be upheld, one has to consider the possibility that added fatty acid suspensions are not equivalent to those produced on the enzyme. The attempt to show inactivation by preincubation of the enzyme with its reaction products did not substantiate this assumption. It is, however, still possible that the difficulty in getting an adequate dispersion of the fatty acid in the absence of albumin is the cause of this discrepancy.

SUMMARY

1. The assay system for glycerol 1-phosphate acyltransferase has been improved by the addition of serum albumin to the reaction system. This causes a shift in the optimum pH to 6·5, depresses palmitoyl-CoA hydrolase and accelerates transferase activity.

2. Serum albumin prevents the inactivation of the transferase brought about by its interaction with palmitoyl-CoA.

3. Free fatty acids, added to the system, inactivate the transferase only at concentrations exceeding those formed during the reaction by a factor of ten.

This investigation was supported in part by Research Grant A-3038 from the National Institute of Arthritis and Metabolic Diseases, U.S.A. Part of this work is included in a Ph.D. Thesis to be presented by R.B. to the Hebrew University.

REFERENCES

Bublitz, C. & Kennedy, E. P. (1955). *J. biol. Chem.* **211**, 951.

Kornberg, A. & Pricer, W. E., jun. (1953). *J. biol. Chem.* **204**, 345.

Lands, W. E. M. (1960). *J. biol. Chem.* **235**, 2233.

Seubert, W. (1960). *Biochem. Prep.* **7**, 80.

Stansly, P. G. (1955). *Biochim. biophys. Acta*, **18**, 411.

Vignais, P. V. & Zabin, I. (1958). *Biochim. biophys. Acta*, **29**, 263.

Weiss, S. B. & Kennedy, K. P. (1956). *J. Amer. chem. Soc.* **78**, 8550.