Phosphatidic Acid Synthesis in Yeast

By N. J. KUHN* AND F. LYNEN

Max Planck-Institut für Zellchemie, München, Germany

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1. The presence of palmitoyl-CoA-L-glycerol 1-phosphate palmitoyltransferase (EC 2.3.1.15) has been demonstrated in a particulate fraction of baker's yeast.
2. The enzyme has been characterized, and its activity studied as a function of pH and concentration of substrates.
3. Inhibition by thiol poisons and protection by acyl-CoA have been used to obtain information on the active site.
4. By various methods of supplying acyl radicals, the species 'palmitoyl-CoA' has been shown to be the true acyl donor to the transferase.

Soon after the discovery of CoA and its role in acetate activation (for review see Decker & Lynen, 1960), Kornberg & Pricer (1953a) demonstrated a similar ATP-dependent enzyme in guinea-pig liver for the activation of long-chain fatty acids as their CoA thio esters. Measuring the incorporation of L-glycerol 1[³²P]-phosphate into an acid-insoluble ethanol-soluble form, Kornberg & Pricer (1953b) described its esterification by acyl-CoA to give phosphatidic acid. Stansly (1955) later developed a more rapid optical assay based on the acid-insolubility of palmitoyl-CoA and the solubility of the free CoA released by palmitoyl transfer to the glycerol 1-phosphate.

Several other compounds have since been shown to be enzymically acylated by long-chain acyl-CoA, including monoglyceride (Senior & Isselbacher, 1962), diglyceride (Weiss, Kennedy & Kiyasu, 1960), α - and β -lysocleithin (Lands & Merkl, 1962), α - and β -lysoethanolamine phosphatide (Merkl & Lands, 1962) and lysophosphatidylinositol (Keenan & Hokin, 1962). Despite the importance of the 'monoglyceride pathway' in certain tissues, it remains probable that the formation of complex lipids requires phosphatidic acid as an intermediate, arising in the manner shown by Kornberg & Pricer (1953b).

In the studies cited, acyl groups were supplied either as their CoA thio esters or as the free acid together with ATP, Mg²⁺ ions and CoA. In view of the importance of the formation of fatty acids by fatty acid synthetase(s), we have investigated whether their use in glyceride synthesis requires their CoA thio esters as intermediates, or whether there could be direct acyl transfer from the fatty acid synthetase (referred to below as 'synthetase')

to the glyceride-synthesizing enzyme. The choice of a yeast system appeared suitable since yeast fatty acid synthetase forms a high-energy thio ester with its product before a reversible transacylation of the latter on to CoA.

We report below the isolation from baker's yeast of an acyl-CoA-L-glycerol 1-phosphate acyltransferase (EC 2.3.1.15), referred to below as 'transferase', together with a study of its properties and the exact form of the acyl group accepted as substrate.

METHODS

Yeast preparation. Pressed baker's yeast (10 g.) was shaken for 1½–2 min. at 4° with 30 g. of Ballotini beads and 25 ml. of 0.1 M-sodium phosphate buffer, pH 6.5, containing EDTA (2 mM), in the apparatus described by Merken-schlager, Schlossmann & Kurz (1957). The homogenate was used either directly after decantation from the beads or after fractional centrifugation.

Assay methods. Incorporation of glycerol 1[³²P]-phosphate was measured by the method of Kornberg & Pricer (1953b), the final extracts being dried on aluminium planchets and counted in a gas-flow counter. In all cases where only a small amount of enzyme preparation was used, 5 mg. of crystalline bovine serum albumin was added to the incubation mixture.

Release of free CoA from palmitoyl-CoA was measured optically by the method of Stansly (1955). The material absorbing at 260 m μ gave an absorption spectrum identical with that of commercially obtained CoA.

Thin-layer chromatography. Plates were prepared by shaking 20 g. of Kieselgel G (E. Merck A.-G., Darmstadt, Germany) with 40 ml. of water for 1 min. and distributing the slurry on glass plates with the apparatus marketed by Desaga (Heidelberg, Germany). After a few minutes the plates were placed in an oven at 110° for 20 min. After cooling, they were stored in a desiccator over silica gel till required. Then 10–50 μ g. of lipid, or about 100 μ g. of lipid mixture, in a volume of 1–4 μ l. of ether was pipetted on to the origin in spots 2 cm. apart. A line was scratched

* Present address: Department of Biochemistry, University of Oxford.

across the layer horizontally at a distance of 10–15 cm. from the origin to delimit the extent of solvent migration. The plates were developed vertically in closed vessels lined with solvent-soaked filter paper. The eluent mixture chloroform-methanol-aq. 30% methylamine (65:25:8, by vol.) was used to separate phosphatidic acid from palmitic acid and lecithin, these two running faster. After development, the plates were dried in an oven at about 60° for 45 min. and sprayed with a solution of 40 mg. of bromothymol blue in 100 ml. of 0.01M-KOH. Phospholipids and acidic substances appear bright yellow on a blue background.

To obtain [32 P]phosphatidic acid for chromatography, the enzymic reaction mixture was precipitated and washed with 3% (w/v) perchloric acid and extracted once with 0.5 ml. of 95% (v/v) ethanol. The extract was evaporated to dryness under a stream of nitrogen and the residue was extracted with 0.1 ml. of ether; this extract was placed on the thin-layer chromatogram, together with carrier phosphatidic acid.

Preparation of chemicals. (a) L-Glycerol 1-[32 P]-phosphate. This was prepared in two enzymic steps, via [32 P]ATP. By using rat-liver mitochondria, [32 P]ATP was prepared from 25 μ moles of AMP and 50 μ moles of inorganic [32 P]phosphate, as described by Colowick & Kaplan (1957). (AMP may be replaced by ADP.) Samples were removed to follow the disappearance of non-esterified phosphate. Finally the reaction mixture was made 3% with respect to perchloric acid, the precipitate removed by centrifugation and the pellet re-extracted twice with 1.0 ml. volumes of 3% perchloric acid. The combined extracts were adjusted to pH 7 with KOH solution and the precipitate of potassium perchlorate was removed by centrifugation. To the supernatant were then added 1.0 ml. of glycine-hydrazine buffer, pH 9.8, containing MgCl_2 (Wieland, 1963), 0.5 ml. of 10 M-glycerol, 0.1 ml. of 5% (w/v) serum albumin and 0.1 ml. of glycerokinase. The contents of the tube were mixed and incubated overnight at 30°.

After the addition of 450 μ moles of DL-glycerophosphate as carrier, protein, phosphate and adenine nucleotides were precipitated by the addition of slight excess of 1M-barium acetate and removed by centrifugation. Then 100 μ moles of sodium phosphate were added to the supernatant and reprecipitated in the same way. To the final supernatant were added rather more than an equal volume of ethanol and a slight excess of barium acetate solution. The solution was adjusted to a slightly alkaline pH and kept in ice for several hours during the precipitation of barium glycerophosphate. The latter was removed by centrifugation, washed with 50% (v/v) ethanol and dissolved in 5 ml. of water with the help of a drop of conc. HCl.

Contamination by labelled ATP or inorganic phosphate was tested for by high-voltage paper electrophoresis: 0.01 ml. of the glycerol[32 P]phosphate soln. was mixed with 0.25 mg. each of carrier ATP and phosphate and placed on Whatman no. 1 filter paper moistened with buffer (acetic acid-pyridine-water; 1:10:890, by vol.). The migration time was 3 hr. in a voltage gradient of 40 v/cm. The paper was supported on a glass plate cooled to -4°. The paper was dried at 60° for 30 min. and the spots were made visible by spraying with bromocresol green soln. (0.04% in 95% ethanol, made just alkaline with 1N-NaOH). Inorganic phosphate, glycerophosphate and adenine nucleotides were all well separated, migrating in that order.

The paper was scanned in an end-window strip counter and showed a single peak coinciding with the glycerophosphate spot. The soln. of barium glycerol[32 P]phosphate was passed through a column of Dowex 50 resin (K^+ form) to replace Ba^+ ions by K^+ . The resulting soln. of potassium glycerol[32 P]phosphate was assayed enzymically for L-glycerol 1-phosphate (Hohorst, 1963), and used directly for incorporation studies.

(b) Phosphatidic acid. Egg lecithin, prepared by the method of Pangborn (1951), was hydrolysed with phospholipase D, purified from Savoy cabbage (Davidson & Long, 1958) as far as the acetone precipitation stage. A reaction mixture containing lecithin (200 mg.), phospholipase D (20 ml.), diethyl ether (20 ml.), 1M-sodium acetate buffer, pH 6.5 (5 ml.), and 1M- CaCl_2 (5 ml.) was shaken in a dark glass-stoppered bottle for 4 hr. at room temperature. Subsequent extraction of the phosphatidic acid followed the description of Kates (1954), and yielded 177 mg. of dry disodium phosphatidate. Analysis for ester content (Stern & Shapiro, 1953), organic phosphorus (Allen, 1940) and L-glycerol 1-phosphate (alkaline hydrolysis followed by enzymic determination) gave in all cases the values expected for disodium 2-linoleoyl-1-stearoylglycerol 3-phosphate. In the system of thin-layer chromatography described above, phosphatidic acid from egg lecithin migrates as an elongated spot with approximate R_F 0.25 or 0.50, depending on whether lined or unlined chambers are used. All preparations have also been found to contain a second minor component with smaller R_F . Since lysophospholipids generally move behind their parent phospholipids on silicic acid and the lecithin was known to contain a lysolecithin contaminant, this second spot is likely to be lyso-phosphatidic acid. Attempts to prepare pure lysophosphatidic acid were unsuccessful. We were unable to degrade the diacyl derivative completely either with snake-venom phospholipase A or by acid treatment. However, a partial deacylation was achieved, as evidenced by a fall in ester value, and a concomitant increase in the size of the second component was observed. Like Lands & Hart (1964) we were able to phosphorylate 1-monoglyceride with phosphorus oxychloride and isolate an ethanol-insoluble sodium derivative with the theoretical ester and phosphorus values. However, analysis by thin-layer chromatography revealed, apart from several trace components, two major components migrating respectively faster and slower than phosphatidic acid. The slower component moved parallel with the presumed lysophosphatidic acid. It is possible that the faster component is a cyclic phosphate derivative.

(c) Acetyl-CoA. This was prepared and assayed as described by Ochoa (1955).

(d) Malonyl-CoA. This was made from *S*-malonyl-*N*-octanoylcysteamine, kindly provided by Dr Eggerer, according to the method of Eggerer & Lynen (1962). It was assayed both with purified synthetase, measuring NADPH disappearance, and by the hydroxamate method.

(e) Palmitoyl-CoA. This was prepared by the method of Seubert (1959) and assayed by the absorption of the adenine ring at 260 m μ .

(f) Oleoyl-CoA. This was prepared by a slight modification of the method for palmitoyl-CoA, in the absence of thioglycollic acid. After esterification of all thiol groups, the chilled alkaline suspension was extracted twice with diethyl ether, to remove tetrahydrofuran, and the aqueous phase was acidified with HCl and extracted five times with

light petroleum. The solvent was removed under a stream of nitrogen and the aqueous phase was adjusted to pH 6 with KOH. The oleoyl-CoA was assayed either by the hydroxamate method or by determining the CoA content (by its absorption at 260 $m\mu$) before and after acidification with 3% perchloric acid in the presence of serum albumin. After acidification, the oleoyl-CoA was precipitated with the protein and could be removed by centrifugation.

(g) NADPH. This was used for synthetase assays, and was prepared by reduction of NADP by acetaldehyde in the presence of purified yeast acetaldehyde dehydrogenase (Seegmiller, 1955).

Enzymes. Glycerokinase, glycerophosphate dehydrogenase and *Zwischenferment* were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Fatty acid synthetase and acetaldehyde dehydrogenase, both purified from yeast, were kindly provided by C. Daba. The synthetase was purified as described by Lynen (1962).

RESULTS

Isolation and characterization of yeast transferase

Cellular distribution. The method of cell rupture used allowed no detailed assignment of activity to particular cell organelles. However, the particulate fraction sedimented by centrifugation at 100 000 g for 60 min., and subfractions of this, were active, whereas the supernatant was virtually inactive. For subsequent routine studies the crude homogenate was centrifuged at 500 g for 90 min. to remove whole cells and cell debris, the supernatant being decanted off and centrifuged for 60 min. at 100 000 g in the Spinco model L preparative ultracentrifuge. The clear supernatant was discarded and the sediment was twice washed by suspension and reprecipitation in the same manner. It was finally resuspended in the original volume of 0.1 M-sodium phosphate buffer, pH 6.5, containing EDTA (2 mM).

The ultraviolet-absorption spectrum of a washed particle suspension showed a broad shoulder spanning 280 $m\mu$, but with little suggestion of absorption at 260 $m\mu$.

Ultrasonic treatment solubilized 40% of the organic matter, as assayed by the method of Johnson (1949), but not the transferase.

Absence of cofactor requirement. As with the mammalian enzyme, no cofactors seem to be required. In experiments where palmitoyl-CoA was supplied by means of the synthetase, the presence of magnesium chloride, manganese chloride or calcium chloride gave no significant stimulation. Either sodium or potassium buffers could be used. To protect thiol groups, cysteine was added as a routine.

Dependence on substrate concentration. Having established that the incorporation of glycerophosphate was a linear function of both incubation time and the amount of enzyme used,

Fig. 1. Effect of palmitoyl-CoA concentration on the incorporation of glycerophosphate into lipid by washed yeast particles. The reaction mixtures were incubated at 30° for 10 min. The reagents used were: yeast preparation (0.01 ml.); sodium phosphate buffer, pH 6.5 (50 μ moles); cysteine (1.5 μ moles); serum albumin (5 mg.); glycerophosphate (1.5 μ moles); palmitoyl-CoA (59–590 $m\mu$ -moles); the total volume was 0.5 ml.

Fig. 2. Effect of glycerophosphate concentration on the release of CoA from palmitoyl-CoA. The reaction mixtures were incubated at 30° for 10 min. The reagents used were: yeast preparation (0.01 ml.); sodium phosphate buffer, pH 6.5 (50 μ moles); cysteine (4 μ moles); serum albumin (5 mg.); palmitoyl-CoA (200 $m\mu$ moles); glycerophosphate (0.5–17.5 μ moles of L-isomer); the total volume was 0.5 ml.

we investigated the kinetics with respect to the two substrates. Fig. 1 shows the effect of varying palmitoyl-CoA concentration. A linear relation holds up to about 0.36 mM-substrate, after which sharp inhibition sets in. Oleoyl-CoA, which also stimulated incorporation of glycerophosphate, gave

exactly the same effect with a peak at 0.53 mM concentration.

The dependence on glycerophosphate concentration is shown in Fig. 2. Stansly's (1955) assay was used, and concentration is expressed in terms of L-glycerol 1-phosphate. Graphical evaluation of the Michaelis constant by the Lineweaver-Burk method gave K_m 10 mM. This very high K_m is in contrast with the clearly greater affinity of the enzyme for acyl-CoA compounds.

The specific activity at optimum concentrations of both substrates was found to be 0.041 μ mole of CoA released/min./mg. of organic matter at pH 6.5. The oxidation equivalent of the yeast preparation was determined by Johnson's (1949) method, and his conversion factor for yeast taken for conversion to a weight basis.

Dependence on pH. With Stansly's (1955) assay, the release of CoA from palmitoyl-CoA by the enzyme was measured as a function of pH in the presence and absence of glycerophosphate. In Fig. 3 the curve obtained in the absence of glycerophosphate shows the expected increase of substrate hydrolysis with rising pH. Absorption at the lowest pH is due to some material extracted from the yeast particles by the 3% perchloric acid used in the assay. Although the values at high pH are probably due to a mainly non-enzymic hydrolysis, the rising values below pH 7 are better accounted for by a deacylase, which may or may not be identical with the transferase.

The curve in Fig. 3 showing the extra CoA release in the presence of glycerophosphate exhibits a broad optimum around neutrality, with rather slow falling off on the acid side. A comparable curve,

with a shift to higher pH, was obtained by Stansly (1955) with the mammalian enzyme. Two buffers were used to obtain the curve, overlapping at pH 5.7. Correction by a factor of 0.76 was needed to bring values for one buffer on to the scale of the other.

Inhibition by thiol poisons. As may be expected for this enzyme, it is sensitive to specific thiol poisons. Prior incubation of the enzyme with the poison for 15 min. at 30°, followed by removal of excess of poison with cysteine, resulted in a marked loss of enzyme activity with *N*-ethylmaleimide (see Table 1); 0.01 M-iodoacetamide at the same pH of 6.5 (which is not ideal for this reagent) gave 25% inhibition. As also shown in Table 1, the addition of palmitoyl-CoA to the enzyme before the addition of poison results in a high degree of protection of the enzyme. In a similar manner there was complete protection against 0.01 M-iodoacetamide.

These results support, but do not prove, a simple scheme for the reactions catalysed by the transferase:

The oleoyl-CoA-dependent reaction was inhibited to about the same extent by *N*-ethylmaleimide, and was similarly protected by preincubation with oleoyl-CoA.

Fig. 3. pH-dependence of CoA release from palmitoyl-CoA in the presence (O) and absence (●) of glycerophosphate. The reaction mixtures were incubated at 30° for 10 min. The reagents used were: yeast preparation (0.01 ml.); sodium phosphate buffer, pH 5.7–8.0 (50 μ moles), or sodium acetate buffer, pH 4.0–5.7 (50 μ moles); cysteine (1.5 μ moles); serum albumin (5 mg.); glycerophosphate (12.5 μ moles of L-isomer); palmitoyl-CoA (190 μ moles); the total volume was 0.5 ml.

Table 1. Inhibition of transferase by *N*-ethylmaleimide, and protection by preincubation with palmitoyl-CoA

Preincubation times were: palmitoyl-CoA (palm.-CoA), 5 min.; *N*-ethylmaleimide (NEM), 15 min.; cysteine (Cys), 5 min.; glycerophosphate (GP), 5 min. All incubations were at 30°. After the enzymic reaction had been started with either palmitoyl-CoA or glycerophosphate, the vessels were incubated for 10 min. The reagents used were: yeast preparation (0.02 ml.); sodium phosphate buffer, pH 6.5 (50 μ moles); serum albumin (5 mg.); palmitoyl-CoA (177 μ moles); cysteine (an excess of 5 μ moles); *N*-ethylmaleimide (0.5 or 5.0 μ moles); glycerophosphate (1.5 μ moles); the total volume was 0.53 ml.

Simplified order of reagent addition	Concn. of <i>N</i> -ethylmaleimide (M)	Transferase activity (%)
[³² P]GP; Cys; palm.-CoA	0	100
[³² P]GP; NEM; Cys; palm.-CoA	0.01	7
[³² P]GP; NEM; Cys; palm.-CoA	0.001	27
Palm.-CoA; NEM; Cys; [³² P]GP	0.01	70
Palm.-CoA; NEM; Cys; [³² P]GP	0.001	98

Table 2. *Protection of palmitoyl-CoA-dependent incorporation of glycerol[³²P]phosphate by pre-incubation with oleoyl-CoA*

Preincubation times and abbreviations are as given in Table 1. The final reaction mixtures were incubated at 30° for 30 min. The reagents used were: yeast preparation (0.05 ml.); sodium phosphate buffer, pH 6.5 (50 μ moles); cysteine (10 μ moles); *N*-ethylmaleimide (5 μ moles); glycerol[³²P]phosphate (1.8 μ moles); serum albumin (5 mg.); palmitoyl-CoA (216 m μ moles); oleoyl-CoA (approx. 120 m μ moles); the total volume was 0.53 ml.

Simplified order of reagent addition	Radioactivity incorporated (counts/min.)	Tube
[³² P]GP; Cys; palm.-CoA	769	(a)
[³² P]GP; NEM; Cys; palm.-CoA	74	(b)
Oleoyl-CoA; NEM; Cys; [³² P]GP	203	(c)
Oleoyl-CoA; NEM; Cys; palm.-CoA; [³² P]GP	1165	(d)

Owing to the inhibition by excess of acyl-CoA, competition experiments with these two substrates could not be done to determine whether one or two enzymes are involved. However, with sub-optimum acyl-CoA concentrations it was shown that preincubation of the particles with oleoyl-CoA protected the palmitoyl-CoA-dependent incorporation of glycerol[³²P]phosphate from *N*-ethylmaleimide poisoning and vice versa. The results for protection of palmitoyl-CoA-dependent activity are given in Table 2. Though the sum of the values for tubes (a) and (c) is slightly lower than the value for tube (d), it is clear that a preincubation of the yeast particles with oleoyl-CoA protects not only the oleoyl-CoA active site but also the palmitoyl-CoA active site. This would suggest that the two sites are identical, though it remains possible that each type of acyl-CoA can combine reversibly with each of two distinct sites, yet be transferred to glycerophosphate only from the one. More concrete information must await purification of the enzyme.

Specificity for substrates. As a result of using both palmitoyl-CoA and oleoyl-CoA to esterify glycerophosphate, the following comparisons can be made of these two substrates:

(a) At equal concentrations, and under the same conditions, both stimulate about the same incorporation of glycerophosphate.

(b) Both substrates cause severe inhibition in excess amounts, palmitoyl-CoA above 0.36 mM and oleoyl-CoA above 0.53 mM.

(c) Both reactions are inhibited to about the same extent by *N*-ethylmaleimide, and are protected by preincubation with acyl-CoA. The two acyl-CoA compounds exert mutual protection.

The evidence to date therefore seems to indicate that the yeast particles cannot distinguish between

Table 3. *Cofactor requirement of palmitate-activating system in cell-free yeast homogenate*

The reaction mixtures were incubated at 30° for 30 min. The complete system contained: yeast preparation (0.1 ml.); sodium phosphate buffer, pH 7.5 (50 μ moles); cysteine (2.5 μ moles); glycerol[³²P]phosphate (1.8 μ moles); serum albumin (5 mg.); palmitate (1.0 μ mole); ATP (1.0 μ mole); MgCl₂ (5 μ moles); CoA (1.0 μ mole); the total volume was 0.62 ml.

Omission(s) from complete system	Radioactivity incorporated	
	(counts/min.)	(%)
None	1126	100
ATP + MgCl ₂	78	7
CoA	65	6
ATP + MgCl ₂ + CoA + palmitate	4	0

these two substrates. As shown below, myristoyl-CoA is also utilized, though no direct comparison of activity was made.

No substrate has yet been found to replace the glycerophosphate. Glycerol, cholesterol, ergosterol, D-glucosamine, D-galactosamine and ethanolamine were all inactive in Stansly's (1955) assay. However, the sterols might not have been properly in solution.

Nature of product. From the nature of the assay, the reaction studied cannot have involved any dephosphorylated product of esterified glycerophosphate. Two experiments were done to see whether a mono- or a di-acylated glycerophosphate was the product. First, the time-course was followed, the extent of reaction at several times being measured simultaneously, in the same vessel, by incorporation of glycerophosphate and by glycerophosphate-stimulated CoA release. In both of two identical experiments, the CoA/glycerophosphate ratio was 1.5. This compares with the value (1.3) found in rat liver by Brandes, Olley & Shapiro (1963), who concluded that a mixture of mono- and di-acylated glycerophosphate was indicated.

However, chromatography of our labelled product on a thin layer of Kieselgel G in the presence of carrier phosphatidic acid revealed only a single radioactive component, migrating with the spot tentatively identified as lysophosphatidic acid.

Experiments with enzymically generated acyl groups

CoA derivative as obligatory acyl donor. It was shown that the activated palmitoyl group could be provided by either of two enzymic systems *in situ*. As a demonstration of fatty acid activation, Table 3 shows that incorporation of glycerol[³²P]phosphate with a crude yeast homogenate occurs when palmitate, ATP, magnesium chloride and CoA are

provided. The yeast preparation was the supernatant after centrifugation at 1000 *g* for 15 min. The omission of CoA leads to nearly complete loss of incorporation. This suggests that the immediate acyl donor is not an AMP derivative as with amino acids, but the CoA thio ester, as for acetate.

The use of myristate instead of palmitate in the above system gave 37% incorporation of glycerophosphate (palmitate 100%).

The same crude yeast homogenate also contains an active fatty acid synthetase, and glycerophosphate incorporation could be induced by the addition of acetyl-CoA, malonyl-CoA and an NADPH-generating system. The addition of purified synthetase gave no further stimulation, showing that the endogenous concentrations were already in excess. With a washed particle preparation, good dependence on the several substrates of this enzyme was obtained, confirming the origin of the palmitoyl groups.

Studies on the purified synthetase (Schweizer, 1963; Hagen, 1963) have shown that, in the reversible exchange of malonyl and acetyl groups between CoA and the synthetase, several model coenzymes, including pantetheine, can replace CoA. In the similar exchange of a palmitoyl group, however, pantetheine is completely inactive. This

difference in the specificities of different functions of the synthetase was used to set up a system whereby the synthetase could be charged with acetyl and malonyl groups, yet could not produce palmitoyl-CoA unless free CoA was further added. With such substrates, the assay of the synthetase by measuring NADPH disappearance shows that it continues to function, and unpublished experiments by I. Hopper-Kessel indicate that free palmitate is released. Fig. 4 shows that, when fatty acids are supplied to the transferase by such a system, the incorporation of glycerophosphate is entirely dependent on added CoA. The maximum incorporation approached the value obtained when acetyl-CoA and malonyl-CoA were supplied at the same concentrations.

The CoA requirement confirms that the species 'palmitoyl-CoA' is an obligatory intermediate in the transfer of a palmitoyl group from fatty acid synthetase to the transferase, and that 'palmitoyl-synthetase' is not the immediate donor to the transferase.

DISCUSSION

The exclusion of fatty acid synthetase as direct acyl donor to the transferase that produces phosphatidic acid throws open the question of the spatial distribution of these two enzymes within the cell. Since the mediation of low-molecular-weight easily-diffusible CoA guarantees the transfer of activated fatty acids from one protein to the other, even over a relatively large distance, no absolute need seems to exist for these two enzymes to be spatially adjacent to one another in the living yeast cell. Whether, despite this, such an orientation does exist *in vivo* cannot yet be decided. However, centrifugation of the broken cells separates the enzymes into distinct fractions: the synthetase remains in the soluble supernatant, whereas the transferase is bound to the particulate matter sedimenting at 100 000 *g*.

The apparent inability of the transferase to distinguish between saturated and unsaturated acyl groups is consistent with work by Lands & Hart (1964) on the mammalian enzyme. Although Colodzin, Bachur, Weissbach & Udenfriend (1963) have reported the esterification of ethanolamine with palmitoyl-CoA in the presence of rat-liver microsomes, yeast particles are unable to catalyse this reaction under the conditions used.

Although we did not assay phosphatase, we noted no loss of phosphatidic acid through its action. It is possible, as appears to be the case for the mammalian enzyme, that phosphatidic acid phosphatase has low activity towards substrates containing only saturated acyl groups.

The inhibition of the transferase by excess of acyl-CoA is considered to be of no physiological

Fig. 4. CoA requirement for glycerophosphate incorporation into lipid by the synthetase-transferase multi-enzyme system when acetyl and malonyl groups are supplied as pantetheine derivatives. The reaction mixtures were incubated at 30° for 10 min. The reagents used were: yeast preparation (0.01 ml.); sodium phosphate buffer, pH 6.5 (50 μmoles); cysteine (1.5 μmoles); acetylpantheine (0.25 μmole); malonylpantetheine (0.11 μmole); glycerophosphate (1.48 μmoles of L-isomer); NADP (0.26 μmole); glucose 6-phosphate (3.0 μmoles); *Zwischenferment* (0.01 ml.); synthetase (200 milliunits); serum albumin (5 mg.); CoA (12.5–300 mμmoles); the total volume was 0.5 ml. The upper horizontal line indicates the value obtained with acetyl-CoA and malonyl-CoA.

importance since the synthetase is inhibited by roughly tenfold lower concentrations.

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