ESTIMATION OF THE ENZYMIC CONDENSATION OF α-GLYCEROPHOSPHATE AND PALMITYL COENZYME A*

by

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The key role of Coenzyme A in biosyntheses has recently been emphasized[1]. That it is destined to participate in the synthesis of phospholipids seems certain[2,3]. Indeed, at the substrate level, a function for it has been clearly delineated in the esterification of α-glycerophosphate by long chain fatty acids by means of their acyl CoA derivatives[4]. The product, diacyl phosphatidic acid, may well be the precursor of the more complex phospholipids[2], possibly even of neutral fat. Because of its potential importance, a simplified assay has been developed which should accelerate studies on the properties and distribution of the condensing enzyme and its significance in lipid metabolism.

MATERIALS AND METHODS

Palmityl CoA was prepared enzymically according to KORNBERG AND PRICER[5], with minor modifications. For example, phosphate was replaced with glycylglycine, resulting in an increased yield. After processing, the bulk of the solvents (*iso*propanol and pyridine) was removed by evaporation under reduced pressure, and the concentrate diluted with water and lyophilized. The residue was taken up in hexane or petroleum ether, in which it is insoluble but readily dispersed and transferred to a centrifuge tube where the hexane was removed and the residue taken up in a small amount of water and neutralized. The solution was then repeatedly extracted with ether to remove residual pyridine since this substance interferes with the assay. Extraction was considered complete when absorption at 260 mμ was irreducible. The solution was then assayed by the hydroxamic acid method[5] and diluted to 2.5 μg per ml. Alternatively, a procedure based on the optical density at 260 mμ of an aliquot before and after acidification was also occasionally utilized for assay purposes. Radioactive palmityl CoA was similarly prepared from palmitic acid containing ^{14}C carboxyl carbon.

α-Glycerophosphate (αGP) was a commercial preparation** containing 97% α- and 3% β-glycerophosphate, confirmed by analysis.

The phosphatidic acid synthesizing enzyme was prepared according to KORNBERG AND PRICER[4].

EXPERIMENTAL

Measurement of enzymic condensation was based on the difference in the amount of palmityl CoA which disappeared in the presence and absence of αGP. The latter was assumed to be due to enzymic hydrolysis of palmityl CoA. The additional disappearance in the presence of αGP was assumed to be due to the formation of

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** "Sodium α-glycerophosphate (98% alpha)", Eastern Chemical Corporation, 34 Spring Street, Newark 2, New Jersey.

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phosphatidic acid. Both of these assumptions were substantiated by direct and indirect evidence.

The disappearance of palmityl CoA was initially measured by determining the remaining acyl CoA as hydroxamic acid. This is a useful procedure though tedious, relatively insensitive, and subject to considerable manipulative error.

The acid-insoluble nature of palmityl CoA suggested an alternative device based on the absorption at 260 mμ of the enzymic reaction mixture following precipitation of enzyme and residual palmityl CoA with perchloric acid. The absorption at 260 mμ under these circumstances is due to the CoA liberated in the course of the reaction and is, therefore, a measure of the amount of palmityl CoA utilized. With or without αGP, straight line relationships are obtained with enzyme concentration and with time under the conditions to be described.

A cogent advantage of this assay, aside from convenience and suitability for routine use, is that the amount of palmityl CoA required per assay is less than one-tenth of that required for the hydroxamic acid method. This follows from a consideration of the molar extinction of CoA on one hand and the iron complex of palmitohydroxamic acid on the other. For a 1 cm light path, the former is considered to be about 16,838[6] whereas the latter is about 1000[5].

Final conditions for routine assay were as follows: 0.05 μM palmityl CoA, 5.0 μM glycylglycine (pH 7.5), 1.0 μM glutathione, + and − 5.0 μM *dl*-αGP and enzyme were added together in a total volume of 0.1 ml. After incubation, usually 15 minutes at 25° C, the reaction was stopped by the addition of 0.9 ml of 3% perchloric acid, precipitating enzyme and residual palmityl CoA. Following centrifugation, the supernatant was transferred to a cuvette and its absorbance at 260 mμ determined against a blank consisting, preferably, of all components including acid-denatured enzyme. It is to be noted that the change in optical density which occurs as the reaction proceeds is a positive or increasing one in contrast to the hydroxamic acid method. The difference in optical density between parallel samples with and without αGP is a measure of the condensation product formed.

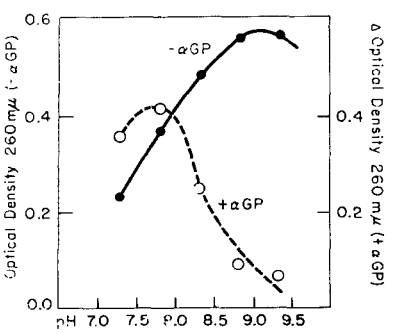

Fig. 1. Effect of pH on hydrolysis (− αGP) of palmityl CoA and on synthesis (+ αGP) of phosphatidic acid. Each tube contained per 0.1 ml, 0.05 μM palmityl CoA, 5 μM glycylglycine of appropriate pH, 1.2 mg enzyme and, in one series, 5 μM αGP of appropriate pH. Incubation, 20 minutes at 25°. Δ optical density 260 mμ signifies the difference in optical density of parallel samples with and without αGP.

As already pointed out, the assay is indirect, measuring the extra disappearance of one of the reactants as influenced by αGP. Since it was conceivable that the αGP could merely have stimulated the hydrolysis of palmityl CoA, it was necessary to validate our assumption that this additional disappearance was in fact due to a different reaction.

Two lines of indirect evidence supported such an assumption. One was the strikingly different pH maxima, illustrated in Fig. 1, of the reactions occurring in the presence and absence of αGP, indicating that the two reactions were qualitatively different. The optimum pH for hydrolysis of palmityl CoA was 8.75 whereas that of phosphatidic acid formation was about 7.5.

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Another line of indirect evidence was the specific stimulating effect of sulfhydryl compounds such as glutathione or cysteine on the reaction involving αGP, amounting to a further 50% disappearance of palmityl CoA, and again indicating a qualitative difference between the two reactions.

Direct evidence for the formation of a new product arising from palmityl CoA and αGP was sought by paper chromatography using radioactive palmityl CoA as condensing partner.

Through the generosity of Dr. ERICH BAER of the University of Toronto, a sample of synthetic α-dipalmityl phosphatidic acid was obtained as a standard of reference for chromatography. Two systems were found which, though not ideal for dipalmityl phosphatidic acid, were nevertheless adequate in establishing the existence of a new product and suggesting its nature.

System A (ter-butanol 7 ml, methanol 3 ml, formic acid 0.2 ml and picric acid 100 mg) caused dipalmityl phosphatidic acid to move as a discrete spot closely behind the picric acid front, which did not necessarily coincide with the solvent front. The degree of departure from coincidence appeared to be a function of picric acid concentration. Above 100 mg, the two fronts coincided. System B (ethyl acetate 60 ml, formamide 40 ml and pyridine 10 ml) failed to move the spot from its origin but did not cause streaking.

Radioactive phosphatidic acid was prepared as indicated in item 3 of Table I. The pilot runs (items 1 and 2) showed that αGP was responsible for the utilization of 63% of the palmityl CoA which disappeared. By extrapolation, about 0.35 μM of palmityl CoA was utilized in the preparative run for the synthesis of 0.18 μM of dipalmityl phosphatidic acid. Items 4 and 5 were controls in which no phosphatidic acid was expected.

TABLE I

SYNTHESIS OF RADIOACTIVE PHOSPHATIDIC ACID

Component	*Concn.*	*ml in tube**				
		1	*2*	*3*	*4*	*5*
Palmityl CoA ^{14}C	9.2 μM/ml	0.005	0.005	0.05	0.05	0.05
Glycylglycine, pH 7.5	0.5 M	0.01	0.01	0.1	0.1	0.1
Glutathione	0.1 M	0.01	0.01	0.1	0.1	0.1
dl-αGP	0.2 M	0.02	—	0.2	—	0.2
Enzyme	30 mg/ml	0.04	0.04	0.4	0.4	0.4**

* Tubes 1 and 2 represent a pilot run to determine the reactivity of the radioactive palmityl CoA. They were made up to 0.1 ml, incubated for 15 min at 25° C and then diluted to 1 ml with 3% $HClO_4$ for determination of absorption at 260 mμ (O.D. 260 = 0.808 and 0.303 respectively).

Tubes 3, 4 and 5 were preparative runs for chromatography. They were made up to 1 ml, incubated for 15 min at 25° C and then treated with 0.2 ml of 60% $HClO_4$. After centrifugation, the residue was washed 3 times with 2 ml of 3.5% $HClO_4$. The washed residue was extracted 3 times with 1 ml of alcohol-ether (3 to 1) and the combined extracts evaporated to dryness. The resulting material was extracted twice with 1 ml of ether, the extract evaporated to dryness and the resulting residue taken up in 0.1 ml absolute alcohol for chromatography.

** Boiled enzyme.

Radioactive products anticipated in sample 3 were phosphatidic acid and palmitic acid. Sample 4 was expected to have only radioactive palmitic acid. Sample 5 would be expected to have some palmitic acid (which occurred as a contaminant of palmityl CoA) but no phosphatidic acid.

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Due to the manner in which the above preparations were processed (see Table I) all samples turned out to be free of palmityl CoA (as determined by chromatography) and, as discussed below, only sample 3 contained a substance corresponding to dipalmityl phosphatidic acid.

Fig. 2 shows the results of the chromatography of sample 3 (the complete phosphatide-synthesizing system) together with that of palmityl CoA and palmitic acid. It is evident that area *a* of sample 3 represents a substance which is neither palmityl CoA nor palmitic acid. Its exact coincidence with synthetic phosphatidic acid suggests its identity with this substance.

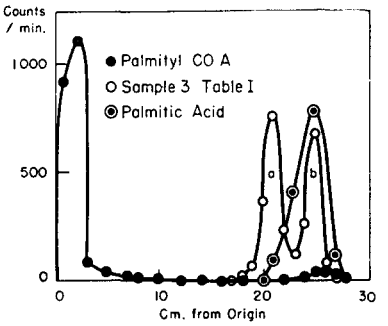

Fig. 2. Paper radio-chromatography in solvent system A (see text). 10 λ of sample 3, Table I, 4 λ (0.04 μM) of radiopalmityl CoA and 20 λ (0.04 μM) of radiopalmitic acid were respectively spotted on each of 3 Whatman No. 4 filter paper strips together with 4 λ (0.08 μM) of synthetic phosphatidic acid. After overnight development, the strips were airdried, sprayed with HANES-ISHERWOOD reagent[7] and irradiated while still moist with ultraviolet light[8] to hydrolyze the phosphatidic acid and visibilize the resulting orthophosphate. Radioactive chromatograms were cut into 1 × 1.9 cm for counting. The dipalmityl phosphatidic acid spot (R_F = 0.77) coincided with *a*. Area *b* coincided with palmitic acid (R_F = 0.97). Some contaminating palmitic acid is apparent in palmityl CoA (R_F = 0.1 or less); *a* = 1412 c.p.m. and *b* = 928 c.p.m.

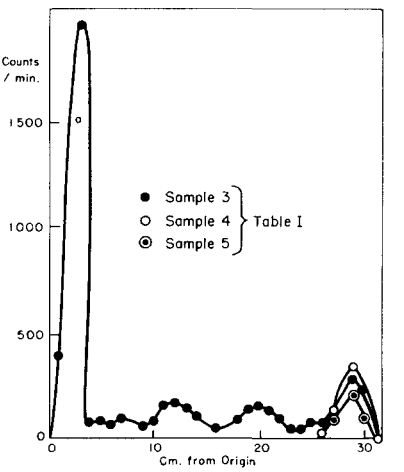

Fig. 3. Paper radio-chromatography in solvent system B. Samples 3, 4 and 5 (Table I) were respectively spotted on each of 3 paper strips together with 4 λ (0.08 μM) of dipalmityl phosphatidic acid which in each case remained at the origin in coincidence with Area *a*.

Because of the proximity of phosphatidic acid to palmitic acid in system A, system B was invoked to provide a wider separation of these two substances. In this system, dipalmityl phosphatidic acid remained discretely at the origin whereas palmitic acid moved with the solvent front. The results for samples 3, 4 and 5 are shown in Fig. 3.

It is apparent that sample 4 (without αGP) and sample 5 (boiled enzyme) show radioactivity corresponding to palmitic acid only. Neither of these controls show radioactivity at the origin, the seat of dipalmityl phosphatidic acid, or elsewhere. On the other hand, curve 3 (from sample 3) shows that the bulk of the radioactivity remained sharply at the origin of the chromatograph, coincident with the synthetic phosphatidic acid. Additional radioactivity was observed at the solvent front, corresponding, as in system A, to palmitic acid. Unexpectedly revealed were what appeared to be a series of minor products representing a significant proportion of the total radioactivity. Their nature was not investigated.

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SUMMARY

1. A simple and convenient assay is described for following the enzymic condensation of α-glycerophosphate and palmityl CoA, based on the absorption at 260 mμ of the CoA liberated during the reaction.

2. The condensation reaction is readily distinguished from the enzymic hydrolysis of palmityl CoA by its pH maxima and its stimulation by glutathione or cysteine.

3. A study of the paper chromatography of the products of the condensation reaction revealed the major product to be a substance with the properties of dipalmityl phosphatidic acid.

RÉSUMÉ

1. Les auteurs décrivent une technique simple et commode, qui permet de suivre la condensation enzymatique de l'α-glycérophosphate et du palmityl CoA. Cette technique est fondée sur l'absorption à 260 mμ du CoA libéré au cours de la réaction.

2. La réaction de condensation se distingue aisément de l'hydrolyse enzymatique du palmityl CoA par son pH optimum et par son activation par le glutathion ou la cystéine.

3. Une étude par chromatographie sur papier des produits de la réaction de condensation montre que le produit principal est une substance qui a les propriétés de l'acide dipalmityl phosphatidique.

ZUSAMMENFASSUNG

1. Eine einfache und praktische Methode für die Kontrolle der enzymatischen Kondensation von α-Glyzerophosphat und Palmityl-CoA wird beschrieben; die genannte Methode beruht auf der Absorption bei 260 mμ des während der Reaktion befreiten CoA.

2. Die Kondensationsreaktion unterscheidet sich sehr leicht durch ihren maximalen pH-Wert, sowie durch ihre mit Glutathion oder Cystein hervorgerufene Steigerung, von der enzymatischen Hydrolyse des Palmityl-CoA.

3. Durch das chromatographische Studium der Produkte der Kondensationsreaktion konnte festgestellt werden, dass das Hauptprodukt von einer Substanz dargestellt wird, welche die Eigenschaften der Dipalmitylphosphatidsäure aufweist.

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