ACYL CARRIER PROTEIN, V. IDENTIFICATION OF 4'-PHOSPHOPANTETHEINE BOUND TO A MAMMALIAN FATTY ACID SYNTHETASE PREPARATION

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Previous investigations from several laboratories have established the sequence of reactions leading to *de novo* synthesis of fatty acids.[1-8] Previous reports have also shown that all these reactions in *E. coli* occur with the substrates bound as thioesters to an acyl carrier protein (ACP).[7-12] Recently, the substrate binding site of ACP has been shown to be the sulfhydryl group of a prosthetic group, 4'-phosphopantetheine. 4'-Phosphopantetheine is bound to ACP through phosphodiester linkage with the hydroxyl group of a serine residue.[13, 14] Evidence has been reported which suggests the involvement in plant fatty acid synthesis of a protein similar both functionally and physically to ACP.[15, 16] Thus, in bacterial and plant fatty acid synthetase preparations which are soluble and can be fractionated, ACP has been shown to play a primary role in fatty acid synthesis. The fatty acid synthetase systems of yeast,[1, 2] pigeons,[17, 18] and mammals,[19] on the other hand, occur as multienzyme complexes which have resisted fractionation into enzymatically active components, and demonstration of ACP in these complexes has not been achieved. This report presents evidence that a fatty acid synthetase partially purified from the adipose tissue of rats contains protein-bound 4'-phosphopantetheine, suggesting that a protein similar to ACP is present in the mammalian enzyme system.

Materials and Methods.—Malonyl-CoA was synthesized by the method of Trams and Brady,[20] acetyl-CoA by the method of Simon and Shemin,[21] and C^{14}-coenzyme A was synthesized enzymatically from 4'-phosphopantetheine which was synthesized as described by Moffatt and Khorana.[22] Dephospho-CoA pyrophosphorylase and dephospho-CoA kinase were prepared by the method of Novelli[23] through the calcium phosphate gel step. Aquacide II was purchased from Calbiochem; *E. coli* alkaline phosphatase from Worthington Biochemical Co.; CoA from P-L Biochemicals; and ATP, acetyl phosphate, and *Crotalus adamenteus* venom from Sigma Chemical Co. This venom was shown to contain a pyrophosphatase that cleaves CoA to form 4'-phosphopantetheine and an adenine-containing fragment.[24] The calcium salt of 1-C^{14}-D-pantothenic acid (3 μc/μmole) was a gift from Merck & Co., Inc., through the efforts of Drs. Charles Rosenblum and Harry Robinson.

Mammalian fatty acid synthetase: The enzyme was prepared from rat epididymal adipose tissue by slight modifications of the published procedure.[25] The alumina C_γ gel step was omitted, and in one experiment the enzyme was purified only through the calcium phosphate gel step. Enzyme at this stage of purification can be stored in liquid nitrogen for several weeks without significant loss of activity. Fatty acid synthetase activity was assayed spectrophotometrically by measuring TPNH oxidation.[25]

Removal of CoA from enzyme preparation: Fatty acid synthetase, purified through the calcium phosphate gel step, is free of CoA (see *Results*). It is necessary to remove contaminating CoA in order to determine the amount of pantothenate associated with the synthetase in crude preparations. This was accomplished by filtration on Sephadex G-50. In a typical experiment, an aliquot of crude adipose tissue extract containing 57 mg of protein and 103 units of enzyme activity in 17 ml was concentrated with aquacide II to approximately 3.5 ml. The concentrate was ad-

justed to 0.10 *M* Tris-HCl, pH 8.0, 0.05 *M* 2-mercaptoethanol, and incubated at 25° for 30 min. This procedure served to reduce any mixed disulfides which had formed between sulfhydryl groups of protein and CoA. After reduction the material was poured onto a 1.2 × 21-cm column of Sephadex G-50 (fine, bead form) and eluted with 0.01 *M* Tris-HCl, pH 7.4. The protein emerging after the void volume of the column was assayed for pantothenate as described below. Chromatography of crude extracts containing added C^{14}-CoA demonstrated that the above treatment separates CoA from the fatty acid synthetase.

Chromatography of 4′-phosphopantetheine and CoA: Separation of 4′-phosphopantetheine and CoA was achieved by DEAE cellulose chromatography using a modification of the method of Moffatt and Khorana.[22] A solution containing carrier 4′-phosphopantetheine and CoA was poured on to a 1 × 5.5-cm column. After 10–30 ml of water was passed through the column, a 400-ml linear gradient elution was performed (0–0.075 *M* LiCl in 0.003 *N* HCl). Four-ml fractions were collected every 90 sec. Mixtures to be chromatographed were diluted with distilled water to a measured conductivity value which was $^1/_5$ that of 0.003 *N* HCl. This step was necessary to ensure binding of 4′-phosphopantetheine and CoA to the column. Following elution the 4′-phosphopantetheine and CoA peaks were located by measurement of the sulfhydryl group content. The CoA peak was further verified by measurement of enzymatic activity with phosphotransacetylase.[26]

Pantothenate-deficient rats: Male germ-free rats were raised in individual cages by the standard methods of a germ-free laboratory. Animals were fed a pantothenate-deficient diet consisting of 73% sucrose, 18% vitamin-free casein, 4% Wesson salt mixture (modified Osborne-Mendel), and 5% Crisco. To each 100 gm of diet were added 2 gm thiamine hydrochloride, 2 mg pyridoxine hydrochloride, 10 mg niacinamide, 200 mg choline chloride, 5 mg para-aminobenzoic acid, 1.25 mg folic acid, 3 mg riboflavin, 0.2 mg menadione, 5 μg biotin, 10 μg vitamin B_{12}, 5 mg α-tocopherol acetate, 2200 I.U. vitamin A, and 440 I.U. vitamin D. This mixture was placed in polyethylene bags (1 cm thick) and sterilized by irradiation with a total dose of 3 megarads using a Van de Graff accelerator. When desired, animal diets were supplemented with daily intraperitoneal injections of C^{14}-pantothenate (50 γ/day).

Microbiological assays: Microbiological assays for pantothenate were performed using *Lactobacillus plantarum* ATCC 8014.[27] For increased sensitivity, the volume per assay tube was reduced to 2 ml, and growth was estimated, after incubation for 3 days, by titration of the acid produced. Synthetase preparations to be assayed for pantothenate were dialyzed extensively against 0.01 *M* Tris-HCl, pH 7.4, to remove phosphate. After lyophilization the residue was suspended in 3 *N* NaOH and incubated at 105°C for 2 hr in a sealed tube. The alkaline hydrolysate was then acidified to pH 1 with Dowex-50 (hydrogen form) and relyophilized. The residue was suspended in 1.0 ml of solution containing 200 μmoles Tris-HCl, pH 9.2, 50 mμmoles $MgCl_2$, and 0.10 mg of *E. coli* alkaline phosphatase, and incubated for 1 hr at 37°. Reactions were stopped by acidification to pH 6 with trichloroacetic acid and aliquots were assayed for pantothenate.

Other assays: Protein was determined either spectrophotometrically[28] or by the method of Lowry.[29] Sulfhydryl determinations were by the procedure of Ellman.[30] Radioactive isotope determinations were made in Bray's solution[31] with a liquid scintillation spectrometer. After sufficient counting time to ensure ±6% accuracy (95% confidence), correction for quenching was made by the use of internal standards.

Results.—Previous studies showed that pantothenate could be released from bacterial ACP by alkaline hydrolysis (3 *N* NaOH) to form 4′-phosphopantothenate followed by treatment of the latter with *E. coli* alkaline phosphatase to remove the phosphate group. To determine if pantothenate might be bound in a similar manner to the mammalian fatty acid synthetase, preliminary studies were made to determine if pantothenate could be released by the above treatment. The data of Table 1 show that pooled extracts of adipose tissue from 50 rats contained, after removal of CoA, 20.6 mμg of bound pantothenate per mg protein. Preliminary evidence that the bound pantothenate is associated with the fatty acid synthetase complex was obtained by showing that the bound pantothenate concentrated along with the enzyme throughout a 49-fold purification of fatty acid synthetase activity.

TABLE 1

PURIFICATION OF FATTY ACID SYNTHETASE AND BOUND PANTOTHENATE

	Fatty Acid Synthetase					
	Total protein (mg)	Specific activity (units/mg)	Bound panto-thenate* (mμg/mg protein)	Fold Increase: Enzymatic assay	Fold Increase: Panto-thenate assay	Ratio of pantothenate to units of enzyme (mμg/unit)
Crude extract	755	1.8	20.6	—	—	11.4
DEAE fraction†						
11–12	0.57	39	344	22	17	8.8
13–14	1.25	30	306	17	15	10.2
15–16	0.34	88	653	49	32	7.4
17–18	0.42	38	281	21	14	7.4

* Pantothenate content of crude extract was determined on an aliquot treated to remove CoA, as described in *Materials and Methods*.

† The crude extract was treated with ammonium sulfate, calcium phosphate gel, and DEAE as previously described.[25]

When adjacent fractions from the last step of enzyme purification (DEAE cellulose column chromatography) are compared, it is noted that the ratio of pantothenate content to enzymatic activity remained essentially constant within the limits of the assay system (Table 1). When the purified enzyme fractions were treated with NaOH, but not alkaline phosphatase, no pantothenate could be detected upon subsequent assay. These experiments established that the mammalian fatty acid synthetase contains pantothenate and that the pantothenate moiety released from the enzyme preparation was phosphorylated. However, the finding that the crude enzyme preparation from 50 rats yielded only 15.6 μg of pantothenate discouraged direct attempts to identify the pantothenate-containing component of this enzyme complex.

Pantothenate-deficient rats: Further proof that the bound pantothenate is associated with the fatty acid synthetase complex was obtained by *in vivo* labeling of the complex with C^{14}-pantothenate. This was accomplished through administration of C^{14}-pantothenate to pantothenate-deficient animals. Since deficient rats may be more prone than normal rats to lethal infections and since the importance of coprophagy in pantothenate deficiency is not fully understood,[32] germ-free rats were employed for this study. Weanling rats raised under these conditions gained weight initially, but eventually lost weight and died exhibiting the symptoms of pantothenate deficiency (Fig. 1*A*). However, the specific activity of enzyme prepared from animals sacrificed during this period of weight loss (Fig. 1*B*) was within the limits found in healthy animals. When deficient animals were supplemented with daily injections of pantothenate, weight gain was resumed and the rate of gain was dependent upon the pantothenate dose (Fig. 1*C* and *D*). The specific activity of synthetase prepared from animals sacrificed after 7–14 days of vitamin supplementation was approximately

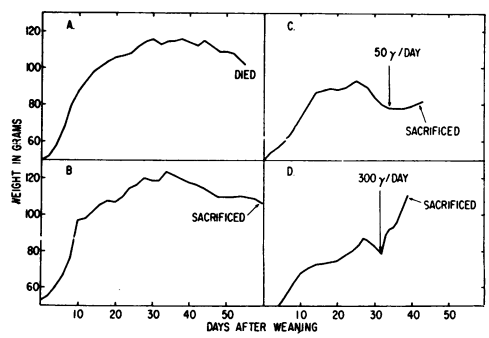

FIG. 1.—Growth curves of weanling rats raised on pantothenate-deficient diets. Animals were supplemented with 50 γ or 300 γ 1-C^{14}-D-pantothenate, where indicated.

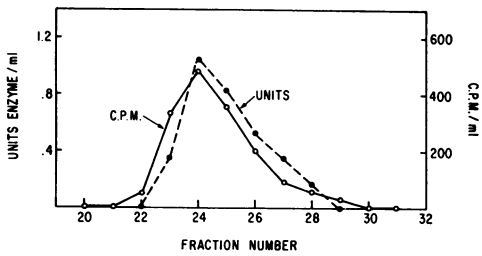

FIG. 2.—Chromatography of labeled fatty acid synthetase on DEAE cellulose after purification by ammonium sulfate precipitation and adsorption on calcium phosphate gel. Only that portion of the chromatogram which contained enzymatic activity and radioactivity is depicted.

the same as that of unsupplemented deficient rats and of normal rats. When 1-C^{14}-pantothenate was substituted for nonradioactive vitamin, radioactivity appeared in the synthetase preparation from these animals. The labeled pantothenate copurified with the enzyme activity and, as seen in Figure 2, it cochromatographed with the enzyme activity in the final step of purification on a DEAE cellulose column. Radioactive fatty acid synthetase prepared in this manner was utilized for identification of the C^{14}-pantothenate-containing component and also for studying the linkage of the radioactive component to the protein complex.

Linkage of radioactive component to enzyme preparation: Preliminary experiments tested the stability of binding of the labeled component to the enzyme preparation under conditions in which 4′-phosphopantetheine is released from CoA or from bacterial ACP. Thus at pH 12 and 70°, all of the 4′-phosphopantetheine was lost from ACP[13] and essentially all of the radioactive component was lost from the synthetase preparation, whereas CoA was not hydrolyzed to an appreciable extent (Table 2). In contrast, either 1 *N* HCl at 100° or snake venom pyrophosphatase hydrolyzed CoA, whereas neither the 4′-phosphopantetheine of ACP nor the radioactive component of the synthetase preparation were released from their respective proteins under these conditions. These experiments suggest that the radioactive component of the fatty acid synthetase preparation is bound in a manner similar to the binding of 4′-phosphopantetheine to ACP.

Identification of labeled component as 4′-phosphopantetheine: Two ml of radioactive enzyme preparation (2.88 mg, 45.6 units, and 6840 cpm), purified through the calcium phosphate gel step, were dialyzed against 6 liters of 0.01 *M* Tris-HCl, pH 7.4, for 4 hr in order to reduce the salt concentration. The dialyzed solution was adjusted to pH 12 with NaOH and incubated at 70° for 1 hr. As shown in Table 2, this treatment releases approximately 94 per cent of the bound radioactivity but does not destroy CoA. Dowex-50 (hydrogen form) was added to reduce the pH to 9, and then carrier CoA (7 mg) and 4′-phosphopantetheine (13

TABLE 2

STABILITY OF PANTOTHENATE-CONTAINING COMPOUNDS UNDER VARIOUS CONDITIONS

Treatment	CoA (% remaining)	ACP (4′-phosphopantetheine remaining on protein)	Radioactive synthetase preparation (% cpm associated with protein)
1 hr, pH 12, 70°	97	0	6
10 min, 1 *N* HCl, 100°	0	100	83
Snake venom pyrophosphatase	19	100	100

CoA remaining was assayed by the phosphotransacetylase assay.[26] 4′-Phosphopantetheine remaining on ACP was determined by acid precipitation of ACP[13] after the above treatments and analysis of the supernatant solutions for organic phosphate by modification of a procedure described by Ames and Dubin.[23] After the various treatments, the radioactive synthetase preparation was precipitated by acidification with $HClO_4$, and the radioactivity of the supernatant solutions was determined. Samples treated with snake venom pyrophosphatase were incubated in Tris-HCl buffer, pH 8.0, with 0.4–1.6 mg of protein for 1 hr at 37°.

μmoles) were added. After the addition of 280 μmoles of 2-mercaptoethanol to this mixture, a 20-min incubation at 25° served to reduce any oxidized sulfhydryl groups. The pH was then lowered to 1 by the addition of more Dowex-50. The supernatant solution was subjected to chromatography on DEAE cellulose (Fig. 3), as described in *Materials and Methods*. Aliquots were assayed for sulfhydryl groups and for radioactivity. The sulfhydryl-containing material which emerged in fractions 1 and 2 was a contaminant in the commercial CoA used as carrier. The major peak of radioactivity (fractions 16–21) containing 5120 cpm was superimposed on the peak of carrier 4′-phosphopantetheine and was widely separated from that of carrier CoA (fractions 50–70). Fractions 60–100 did not contain any radioactivity. When carrier pantothenate and pantetheine were included, these compounds emerged from the column in the initial water wash. Carrier CoA was further identified by the phosphotransacetylase assay[26] and 4′-phosphopantetheine either by phosphate analysis[33] or separate experiments where 4′-phosphopantetheine was the only carrier added.

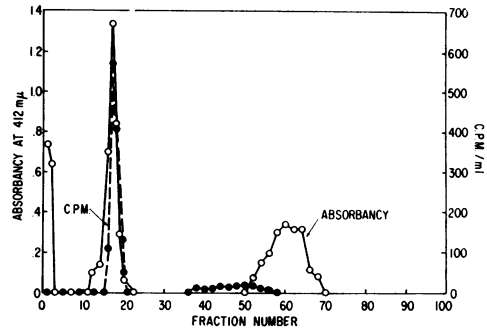

FIG. 3.—Chromatography of the products of mild alkaline hydrolysis of labeled fatty acid synthetase. All fractions were assayed for radioactivity and sulfhydryl group content. Absorbancy values less than 0.02 and radioactivity values less than 5 cpm are not plotted.

Thus it appeared that the radioactive component derived from the enzyme preparation is 4′-phosphopantetheine. To further establish this, an aliquot of fraction 17 (575 cpm) was incubated with dephospho-CoA pyrophosphorylase, dephospho-CoA kinase, and ATP as previously described.[13] This treatment converts 4′-phosphopantetheine to CoA. After the incubation, additional carrier CoA and 4′-phosphopantetheine were added, and this reaction mixture was rechromatographed in the same manner as before. Figure 4 shows that 51 per cent of the radioactivity originally eluted with carrier 4′-phosphopantetheine was now eluted with carrier CoA (fractions 32–56). The radioactive peaks found in fractions 1–2 and 22–30 were not identified. The earlier elution of both carriers, when compared to the first column, was due to a shorter initial water wash and a slightly steeper linear salt gradient. Incubation of authentic 4′-phosphopantetheine and ATP under similar conditions resulted in a 40–60 per cent conversion of the 4′-phosphopantetheine to CoA. These experiments indicate that the radioactive compound which was derived from the mammalian fatty acid synthetase preparation is 4′-phosphopantetheine.

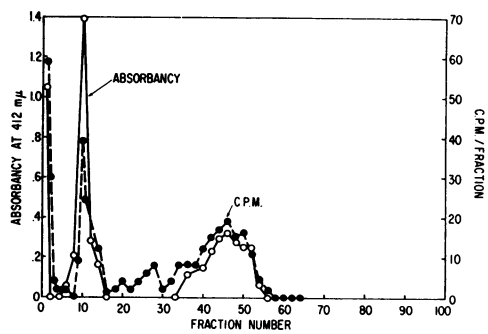

FIG. 4.—Rechromatography of radioactive 4′-phosphopantetheine (derived from experiment of Fig. 3) after incubation with dephospho-CoA pyrophosphorylase, dephospho-CoA kinase, and ATP. All fractions were assayed for sulfhydryl group content and radioactivity.

Discussion.—Mammalian fatty acid synthetase partially purified from adipose tissue contains protein-bound pantothenate. This pantothenate is present in the enzyme preparation as 4′-phosphopantetheine, and it is linked to a protein of the fatty acid synthetase preparation through a covalent bond which is split by dilute alkali but not by acid. Bacterial ACP contains 4′-phosphopantetheine as a prosthetic group which is bound to the protein through phosphodiester linkage with a hydroxyl group of a serine residue.[13, 14] The prosthetic group is hydrolyzed from bacterial ACP under the same conditions in which 4′-phosphopantetheine is released from the mammalian synthetase preparation. Thus, although it has not yet been possible to identify mammalian ACP intact, the discovery of this prosthetic group and the properties of its linkage to protein strongly suggest the presence of an acyl carrier protein in the mammalian fatty acid synthetase complex.

Earlier indirect evidence which suggested the involvement of ACP in fatty acid synthetase systems which occur as multienzyme complexes includes the finding that thioesters of *E. coli* ACP function as substrates for the mammalian enzyme complex[10] and for the pigeon liver system.[12] In addition Wakil, Pugh, and Sauer showed that boiled preparations of the pigeon liver complex or bacterial ACP stimulated fatty acid synthesis by aged preparations of the same system.[12]

The fact that pantothenate-deficient animals on the verge of death show a normal level of synthetase activity suggests that the pantothenate bound to the synthetase is maintained at the expense of other pantothenate-containing compounds or that the pantothenate-containing protein is present in excess in normal animals. Further experiments are required to delineate the mechanism of biosynthesis and catabolism of pantothenate-containing proteins in mammals.

Summary.—Protein-bound 4′-phosphopantetheine has been identified in a partially purified fatty acid synthetase preparation from adipose tissue. The binding of 4′-phosphopantetheine to this preparation is similar to the binding of this prosthetic group to bacterial ACP. These experiments suggest the existence of a mammalian acyl carrier protein similar to bacterial ACP.

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*EARLY INTRACELLULAR EVENTS IN THE REPLICATION OF T4 PHAGE DNA, I. COMPLEX FORMATION OF REPLICATIVE DNA**

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When *E. coli* B, infected with P^{32}-labeled bacteriophage T4, is disrupted with duponol or lysozyme, and the resulting lysate analyzed in a cesium chloride density gradient, a large fraction of the parental label forms a band in the gradient, while after draining CsCl solution, the residual radioactivity associated with the floating pellet can be extracted from the tube with hot alkali. If the duponol or lysozyme lysate is extracted with phenol, approximately one half of the P^{32} becomes adsorbed at the interphase between the water and the phenol. Repeated phenol extraction does not release P^{32} from the interphase pellet.[5]

This and the following paper will analyze the novel properties of T4 DNA during early stages of its replication.

Materials and Methods.—The bacterial strain used in these experiments was *E. coli* B. The bacteriophage was T_4BO_r.[1] Light, heavy, radioactive, and starvation synthetic TCG media along with the methods for purification of bacteriophage and the sucrose and CsCl density gradient techniques were described before.[2–4]

Intracellular DNA extraction procedures: (*I*) Duponol-phenol extraction: Infected bacteria were sedimented, washed once with EDTA (0.15 *M* NaCl-0.015 *M* EDTA, pH 8.0), and resuspended to a concentration of 3–6 $\times$ 10^8 bacteria per milli-