Coenzyme A Analogs. III. The Chemical Synthesis of Desulfopantetheine 4′-Phosphate and Its Enzymatic Conversion to Desulfo-coenzyme A[1]

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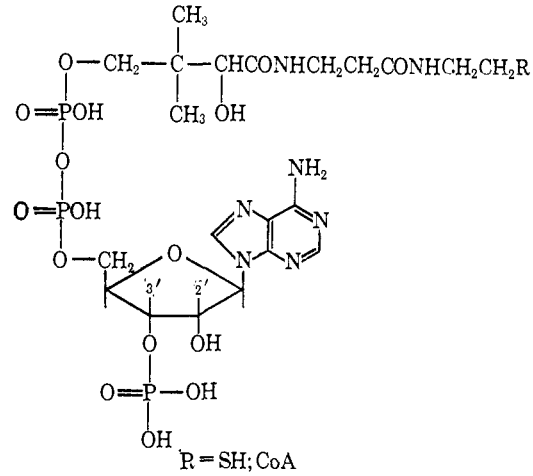
Abstract: The chemical syntheses of D-desulfopantetheine 4′-phosphate and 3′-dephosphodesulfo-coenzyme A are described. The enzymatic conversion of desulfopantetheine 4′-phosphate to desulfo-coenzyme A by the coenzyme A synthesizing enzyme system, isolated from beef liver, was shown to occur in incubation mixtures separated by DEAE-cellulose column chromatography. Dephosphodesulfo-coenzyme A was shown to be enzymatically phosphorylated to desulfo-CoA and to be enzymatically cleaved by pyrophosphate.

While the sulfhydryl group of coenzyme A (CoA) is essential for the metabolic role of CoA as an acyl carrier,[3] apparently it is not essential for the latter stages of CoA biosynthesis. D-Oxypantetheine 4′-phosphate can be enzymatically converted to oxy-coenzyme A (oxy-CoA) by the same sequence of reactions that lead from D-pantetheine 4′-phosphate to CoA.[4] Chase, *et al.*,[5] have recently prepared desulfocoenzyme A (desulfo-CoA) and have shown it to be an inhibitor of several CoA-dependent enzyme systems. It would be of interest if desulfo-CoA could be prepared enzymatically since a successful demonstration would further substantiate the noninvolvement of the sulfhydryl group in the biosynthetic conversion of pantetheine 4′-phosphate to CoA.

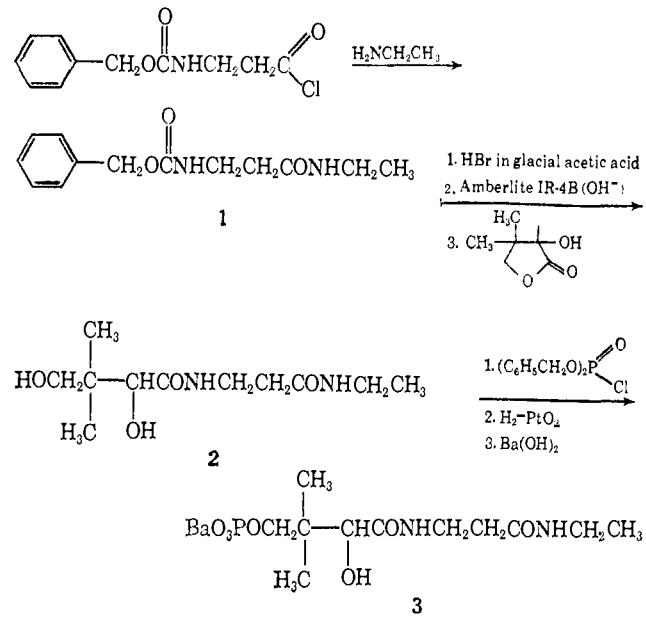


R = SH; CoA
R = OH; oxy-CoA
R = H; desulfo-CoA

This paper describes the synthesis of D-desulfopantetheine 4′-phosphate and 3′-dephosphodesulfo-coenzyme A (dephosphodesulfo-CoA, **4**) and their enzymatic conversion to desulfo-CoA by the CoA synthesizing enzyme system of Hoagland and Novelli.[6]

Chemical Synthesis. The general approach was based on the elegant synthesis of CoA by Moffatt and Khorana[7] and our previous experience with oxy-CoA.[8] The reaction sequence leading to desulfopantetheine 4′-phosphate (**3**) is shown in Scheme I.

Scheme I



N-(Carbobenzoxy-β-alanyl)aminoethane (**1**) was prepared by direct acylation of aqueous ethylamine with an ethereal solution of carbobenzoxy-β-alanyl chloride in 70% yield. Decarbobenzoxylation was achieved by treatment of **1** with HBr in glacial acetic acid.[9] The crude product, N-(β-alanyl)aminoethane hydrobromide, was extremely hygroscopic and readily formed an oil with alcohol–ether systems upon attempted crystallization. Consequently it was converted to the free base by passage through an Amberlite IR-4B(OH⁻) column and after evaporation *in vacuo*, directly fused

(1) (a) This investigation was supported by Public Health Service Grant GM-07977 from the National Institute of General Medical Sciences. (b) A portion of this work has been reported previously: C. J. Stewart, *et al.*, *Federation Proc.*, **26**, 841 (1967), and a part submitted in partial fulfillment of the requirements for the M.S. degree by A. R. A. in December 1966.

(2) National Science Foundation Undergraduate Research Participant, 1966–1967.

(3) L. Jaenicke and F. Lynen, *Enzymes*, **3B**, 3 (1960).

(4) C. J. Stewart and W. J. Ball, Jr., *Biochemistry*, **5**, 3883 (1966).

(5) J. F. A. Chase, B. Middleton, and P. K. Tubbs, *Biochem. Biophys. Res. Commun.*, **23**, 208 (1966).

(6) M. B. Hoagland and G. D. Novelli, *J. Biol. Chem.*, **207**, 767 (1954).

(7) J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 663 (1961).

(8) T. L. Miller, G. R. Rowley, and C. J. Stewart, *ibid.*, **88**, 2299 (1966).

(9) D. Ben Ishai and A. Berger, *J. Org. Chem.*, 17, 1564 (1952).

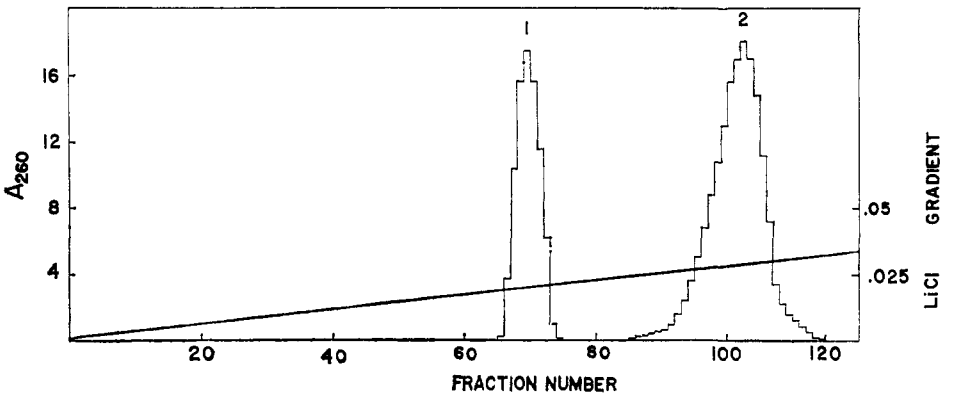


Figure 1. DEAE-cellulose column chromatography of the reaction mixture containing desulfopantetheine 4′-phosphate and adenosine 5′-phosphoromorpholidate. Peak 1, adenosine 5′-phosphate, and peak 2, dephosphodesulfo-CoA.

with D-pantolactone. The crude fusion mixture was dissolved in water and purified by passage through ion-exchange columns to yield (78%) desulfopantetheine (**2**) as a pale straw yellow viscous oil.[10]

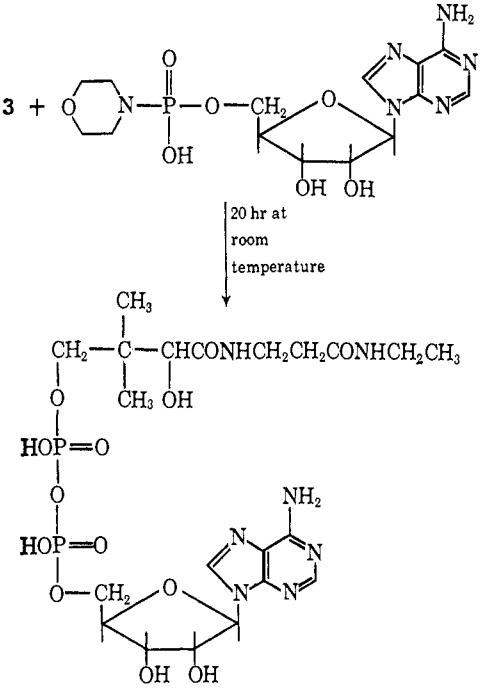
Phosphorylation of **2** was accomplished with dibenzyl phosphorochloridate, the preferred reagent for pantetheine and its analogs.[7,8,11,12] The benzyl blocking groups were removed by catalytic hydrogenation and the crude desulfopantetheine 4′-phosphate (**3**) was isolated as the barium salt. The yields were variable, 23–60%. However, variable yields were also encountered with this step in the synthesis of oxypantetheine 4′-phosphate.[8] Purification of **3** was accomplished by DEAE-cellulose column chromatography, using LiCl gradient elution. Aliquots of the column fractions were spotted on paper and sprayed for organic phosphates in order to detect[13] **3** in the column effluent.

The dilithium salt of D-desulfopantetheine 4′-phosphate obtained by this synthetic procedure exhibited $[\alpha]^{26}D$ +10.0°, compared to +8.4°[12] for D-selenopantetheine 4′-phosphate, +10.8°[11] and +14.6°[14] for D-pantetheine 4′-phosphate, and +16.7°[8] for D-oxypantethine 4′-phosphate.

3′-Dephosphodesulfo-coenzyme A (**4**) was prepared by coupling **3** with 4-morpholine N,N′-dicyclodihexylcarboxamidinium adenosine 5′-phosphoromorpholidate[15] overnight at room temperature in anhydrous pyridine, as shown in Scheme II. Dephosphodesulfo-CoA was separated from the reaction mixture by DEAE-cellulose column chromatography. The elution pattern is shown in Figure 1. A yield of 59% was obtained.

Enzymatic Syntheses. The CoA-synthesizing enzyme system of Hoagland and Novelli[6] was isolated from beef liver. This crude preparation contains two active enzymes, dephospho-CoA pyrophosphorylase and dephospho-CoA kinase.[16] These two enzymes catalyze reactions 1 and 2, respectively, to synthesize CoA from pantetheine 4′-phosphate.[4,6]

Scheme II



dephosphodesulfo-coenzyme A (**4**)

pantetheine 4′-phosphate + ATP = dephospho-CoA + PPi (1)

dephospho-CoA + ATP ⟶ CoA + ADP (2)

pantetheine 4′-phosphate + 2ATP ⟶ CoA + ADP + PPi

The ability of these enzymes to convert dephospho-desulfo-CoA and desulfopantetheine 4′-phosphate was tested by incubating the substrates in the presence of enzyme preparation and then separating the reaction mixture on a DEAE-cellulose column. Figure 2 shows the typical column elution patterns obtained from the incubation mixtures containing (A) ATP, 7.35 μmol, and dephosphodesulfo-CoA, 2.0 μmol; (B) ATP, 7.35 μmol, and desulfopantetheine 4′-phosphate, 4

(10) After storage at −20° for 6 months, white crystals have been observed to form slowly.

(11) J. Baddiley and E. M. Thain, *J. Chem. Soc.*, 1610 (1953).

(12) W. H. H. Günther and H. G. Mautner, *J. Am. Chem. Soc.*, 87, 2707 (1965).

(13) Detection of **3** in the column eluates is more difficult than either pantetheine 4′-phosphate or oxypantetheine 4′-phosphate. Both of the latter compounds will give a positive Biuret reaction while **3** does not. We are inclined to believe that the alkaline copper complexes formed with the Biuret reagent involve the terminal SH of pantetheine 4′-phosphate or the OH group of its oxy analog. Desulfopantetheine 4′-phosphate, lacking this terminal nucleophilic group, fails to produce this copper complex and hence fails to give a positive Biuret reaction.

(14) J. M. Osbond, British Patent 749,715 (1956); *Chem. Abstr.*, 51, 2853 (1957).

(15) J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, 83, 649 (1961).

(16) The systematic names for each of these two enzymes are: dephospho-CoA pyrophosphorylase, ATP:pantetheine 4′-phosphate adenyltransferase (EC 2.7.7.3); dephospho-CoA kinase, ATP:dephospho-CoA 3′-phosphotransferase (EC 2.7.1.24). The standard abbreviations for adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), and inorganic pyrophosphate (PPi) are also employed in this paper.

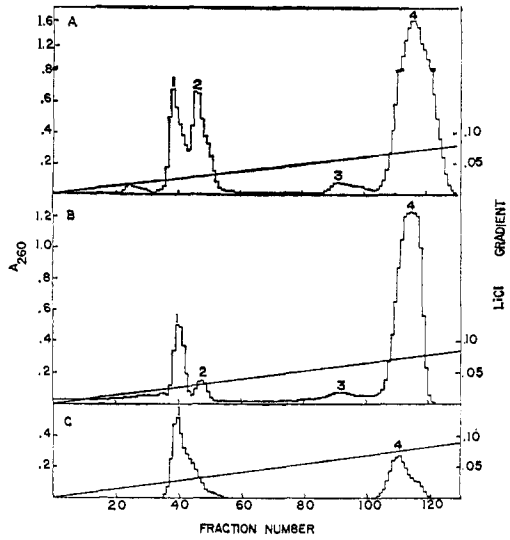


Figure 2. DEAE-cellulose column chromatography of the enzymatic incubation mixtures. (A) dephosphodesulfo-CoA incubated with ATP, (B) desulfopantetheine 4′-phosphate incubated with ATP, and (C) dephosphodesulfo-CoA incubated with inorganic pyrophosphate. Peak 1, dephosphodesulfo-CoA; peak 2, ADP; peak 3, desulfo-CoA; peak 4, ATP.

μmol; and (C) inorganic pyrophosphate, 40 μmol, and dephosphodesulfo-CoA, 2.0 μmol.

The material present in each peak of the elution patterns was characterized by paper chromatography after pooling of appropriate fractions and work-up to remove excess LiCl. The following materials were isolated from the separation shown in Figure 2A: dephosphodesulfo-CoA, 15.5 OD units (1.03 μmol); ADP, 17.3 OD units (1.15 μmol); desulfo-CoA, 4.0 OD units (0.26 μmol); and ATP, 92 OD units (6.13 μmol). The recoveries in Figure 2B were dephosphodesulfo-CoA, 11.2 OD units (0.75 μmol); ADP, 4.0 OD units (0.26 μmol); desulfo-CoA, 6.1 OD units (0.4 μmol); and ATP, 83 OD units (5.54 μmol). Figure 2C clearly demonstrates the reversibility of reaction 1 by yielding 12.8 OD units of ATP (0.85 μmol) and 17 OD units of dephosphodesulfo-CoA (1.13 μmol).

Failure to achieve exact stoichiometry between ADP and desulfo-CoA in Figures 2A and 2B was caused by the nature of our crude enzyme system.

Discussion

The results of this series of *in vitro* experiments substantiate the hypothesis that the sulfhydryl group of pantetheine phosphate is not involved as a structural feature recognized by the beef liver enzymes converting pantetheine phosphate to CoA. Since pantetheine phosphate, oxypanthetheine phosphate,[4] and desulfopantetheine phosphate may be converted to CoA, or the respective CoA analogs, the enzyme receptor sites must recognize other structural features.

Experimental Section

Methods and Materials. Paper chromatography was carried out by the descending technique on Whatman No. 1 paper. The solvent systems used were solvent A, *n*-butyl alcohol–acetic acid–water (5:2:3); solvent B, *n*-propyl alcohol–concentrated ammonia–water (55:10:35); solvent C, ethyl alcohol–1 *M* ammonium acetate, pH 7.5 (7:3).

Adenine-containing compounds were located by their ability to absorb ultraviolet light (254 mμ). Phosphate-containing compounds were located by the method of Bandurski and Axelrod.[17] The chlorine–starch–iodine method of Rydon and Smith[18] was used to locate amino- or amido-containing compounds. Table I lists the R_f values of the various compounds.

Table I. R_f Values of Compounds

Compound	Solvent A	Solvent B	Solvent C
N-(Carbobenzoxy-β-alanyl)aminoethane	0.95		
N-(β-Alanyl)aminoethane	0.68		
Desulfopantetheine	0.86		
D-Desulfopantetheine 4′-phosphate	0.56	...	0.64
Dephosphodesulfo-CoA	0.38	0.70	0.59
Desulfo-CoA	0.20	0.57	0.20
Adenosine 5′-phosphate	0.25	0.44	0.18
Adenosine 3′,5′-diphosphate	0.14	0.29	0.03
ADP	0.13	0.39	0.08
ATP	0.08	0.37	0.05

DEAE-cellulose (Selectacel standard type) was purchased from Carl Schleicher and Schuell. Sephadex G-25 was purchased from Pharmacia Fine Chemicals, Inc. Lyophilized *Clostridium kluyveri* cells, as a source of crude phosphotransacetylase, and venom phosphodiesterase (*Crotalus adamanteus*) were obtained from Worthington Biochemicals Corp. Coenzyme A was purchased from Boehringer Mannheim Corp., while acetyl-CoA was obtained from Sigma Chemical Corp. Desulfo-CoA was prepared by the procedure of Chase, *et al.*[5] Pantetheine 4′-phosphate and dephospho-CoA were synthesized by the procedure of Moffatt and Khorana.[7] 4-Morpholine N,N′-dicyclohexylcarboxyamidium adenosine 5′-phosphoromorpholidate was prepared by the procedure of Moffatt and Khorana.[15] Carbobenzoxy-β-alanine was prepared from β-alanine by the procedure of Siffered and du Vigneaud.[19] Venom phosphodiesterase digestions of desulfo-CoA and dephosphodesulfo-CoA were performed by the method previously described for CoA[7] and oxy-CoA.[8] During the preparation of the beef liver enzyme system, assays for CoA synthesizing activity were accomplished by the method of Hoagland and Novelli.[6] Protein concentrations were determined by the Biuret reaction.[20]

N-(Carbobenzoxy-β-alanyl)aminoethane (1). An ethyl ether solution of carbobenzoxy-β-alanyl chloride was prepared from carbobenzoxy-β-alanine (30 g, 0.135 mol) by the procedure of Dyer and Ballard.[21] The freshly prepared ethereal solution was then slowly added, with constant stirring, to a chilled (5°) 70% ethylamine solution (12.3 ml, 0.185 mol). During the course of addition, the reaction mixture was kept alkaline by adding 6 *N* NaOH as required, and the temperature was maintained below 10°. The product was collected by suction filtration, washed with water, and air dried to yield 23.6 g (70% yield) melting at 121–122°.[22] A 3.9-g portion was recrystallized from benzene to yield 2.7 g, 70% of fine white needles, mp 121.7–122.2°.

Anal.[23] Calcd for $C_{13}H_{18}N_2O_3$: C, 62.38; H, 7.25; N, 11.19. Found: C, 62.68; H, 7.35; N, 11.36.

Desulfopantetheine (2). N-(Carbobenzoxy-β-alanyl)aminoethane (7.5 g, 0.03 mol) was dissolved in 105 ml of 2.5 *N* HBr in glacial acetic acid. After standing at room temperature for 1 hr, the acetic acid solution was poured into 400 ml of anhydrous ether. The resultant mixture was centrifuged for 10 min at 1900 rpm. The supernatant solution was decanted, and the precipitated gum was triturated with 100 ml of anhydrous ether. The ether wash was decanted after centrifugation. The crude white hydrobromide salt was dissolved in 75 ml of water and passed through a 3.0 × 35 cm Amberlite IR-4B (OH^- form) anion-exchange column. The eluate was evaporated *in vacuo*. D-Pantolactone (4.7 g, 0.036 mol) was added to the residual oil and the tightly stoppered flask placed in a

(17) R. S. Bandurski and B. Axelrod, *J. Biol. Chem.*, **193**, 405 (1951).
(18) H. N. Rydon and P. W. G. Smith, *Nature*, **169**, 922 (1952).
(19) R. H. Siffered and V. du Vigneaud, *J. Biol. Chem.*, **108**, 753 (1935).
(20) E. Layne, *Methods Enzymol.*, **3**, 450 (1957).
(21) E. Dyer and E. Ballard, *J. Am. Chem. Soc.*, **59**, 1697 (1937).
(22) All melting points are uncorrected.
(23) Elemental analyses were performed by either C. F. Geiger, Ontario, Calif., or by M-H-W Laboratories, Garden City, Mich.

55° oven overnight. This reaction mixture was then dissolved in 50 ml of water and passed through two 1.0 × 11 cm ion-exchange columns, Amberlite IR-4B (OH^-) and Dowex 50W-X8 (H^+). The eluate was evaporated *in vacuo* to yield 5.8 g (78.3%) of a pale yellow, highly viscous oil.

For purposes of analysis, a portion of the product was dissolved in anhydrous pyridine and evaporated to dryness *in vacuo*. This process was repeated three times to remove any traces of water. Then the sample was dissolved in anhydrous pyridine and added dropwise to anhydrous ethyl ether. The mixture was centrifuged at 1000 rpm for 10 min, the ether decanted, and the precipitate dried *in vacuo* at 100° over P_2O_5 for 2 days to yield a highly viscous pale yellow syrup.

Anal. Calcd for $C_{11}H_{22}N_2O_4$: C, 53.64; H, 9.00; N, 11.37. Found: C, 53.45; H, 9.19; N, 11.50.

Desulfopantetheine 4′-Phosphate (3). Desulfopantetheine (2.88 g, 11.7 mmol) was evaporated three times with anhydrous pyridine, then dissolved in anhydrous pyridine (25 ml), and frozen in a Dry Ice–acetone bath. A solution of dibenzyl phosphorochloridate was prepared by dissolving N-chlorosuccinimide (3.33 g, 24.9 mmol) in warm, dry benzene (50 ml) and adding dibenzyl phosphite (6.53 g, 24.9 mmol) dropwise with swirling. After standing at room temperature for 2 hr, the phosphorochloridate solution was decanted into the frozen pyridine solution, and the mixture was thawed and quickly refrozen. The mixture was placed in a deep freeze (−18°) and allowed to stand 20 hr. Water (30 ml) was added, the flask was swirled, and, after 20 min at room temperature, the yellow solution was evaporated *in vacuo* with a bath temperature less than 35°. The residue was dissolved in a mixture of ethyl acetate (55 ml) and 2 *N* H_2SO_4 (30 ml) and extracted. The organic phase was extracted three times each with 2 *N* H_2SO_4 (30 ml), 10% $NaHCO_3$ (30 ml), and saturated Na_2SO_4 (30 ml), in that order. The ethyl acetate phase was dried over anhydrous sodium sulfate and the solvent evaporated *in vacuo*. The resulting syrup was dissolved in a mixture of isopropyl alcohol (60 ml) and water (15 ml), and Adams catalyst (3 g) was added. Hydrogenolysis was commenced at room temperature and atmospheric pressure and allowed to proceed until hydrogen uptake ceased (10 hr). The catalyst was removed by centrifugation, washed once with isopropyl alcohol, and recentrifuged. The wash was combined with the supernatant solution and evaporated *in vacuo* with a bath temperature less than 35° yielding a colorless syrup. The syrup was dissolved in water (75 ml) and adjusted to pH 7.5 with 1 *N* barium hydroxide. The precipitate was removed by centrifugation at 10,000 rpm, yielding a clear colorless solution. The supernatant solution was evaporated *in vacuo* to yield a white powder. The powder was dissolved in a minimum amount of methanol and traces of turbidity were removed by centrifugation. The methanol solution was then added dropwise to about 20 times its volume of acetone. The precipitated product was collected by centrifugation and dried over P_2O_5. The crude barium salt of D-desulfopantetheine 4′-phosphate (2.4 g, 40.3%) was obtained as a white powder by this procedure.

For purposes of purification, a portion of the above barium salt (500 mg) was dissolved in water and applied to a 2.7 × 75 cm DEAE-cellulose (chloride form) column. After washing well with water it was eluted with a linear gradient using 2.0 l. of 0.5 *N* lithium chloride in the reservoir and 2.0 l. of water in the mixing vessel. Fractions (15 ml) were collected. Phosphate-containing material, located by spotting fraction aliquots on paper and spraying with phosphate reagent, was found in fractions 70–79. This peak was confirmed by spotting aliquots of these fractions on paper and testing for amide-containing compounds. The peak was pooled and the solvent removed by evaporation *in vacuo*. The white solid residue was repeatedly dissolved in methanol and precipitated with acetone until a negative chloride test was obtained. The precipitate was dried *in vacuo* at 100° over P_2O_5 to yield the dilithium salt of desulfopantetheine 4′-phosphate (248 mg, 65%) in the form of a white powder. The product was found homogeneous on paper chromatography (see Table I). The specific rotation was observed to be $[\alpha]^{25}D$ +10.0°.

Anal. Calcd for $C_{11}H_{21}N_2O_7PLi_2$: C, 39.07; H, 6.25; N, 8.28; P, 9.15. Found: C, 38.83; H, 6.36; N, 7.81; P, 8.96.

3′-Dephosphodesulfo-coenzyme A (4). D-Desulfopantetheine 4′-phosphate dilithium salt (222 mg, 0.48 mmol) was converted to the pyridinium salt by passage through a 1 × 5 cm Dowex 50W-X8 (pyridinium form) column and evaporating the effluent to dryness *in vacuo*. Final traces of water were removed by several repeated additions and evaporations *in vacuo* of anhydrous pyridine. The D-desulfopantetheine 4′-phosphate in anhydrous pyridine (10 ml) was added to an anhydrous pyridine solution (3 ml) of 4-morpholine N,N′-dicyclohexylcarboxamidinium adenosine 5′-phosphoromorpholidate (222 mg, 0.28 mmol), and the mixture was evaporated to a viscous oil *in vacuo*. After an additional evaporation with anhydrous pyridine, the mixture was dissolved in anhydrous pyridine (10 ml) and permitted to react 20 hr, overnight, at room temperature in a tightly stoppered flask. Pyridine was subsequently removed by several evaporations *in vacuo* with water. The residue was dissolved in water (25 ml), the pH adjusted to 6.0 with dilute NH_4OH, and solution then applied to a 2.4 × 70 cm DEAE-cellulose (chloride form) column. The column was washed with 0.003 *N* HCl, and the adsorbed compounds were eluted with an acidic lithium chloride linear gradient, 4.0-l. reservoir of 0.15 *M* LiCl in 0.003 *M* HCl and 4.0-l. mixing vessel of 0.003 *N* HCl. Fractions (15 ml) were collected. The dephosphodesulfo-CoA peak, tubes 86–118, contained 2770 OD units at 260 mμ. Solvent was removed by evaporation *in vacuo*, and lithium chloride was removed by dissolving the white residue in a minimum amount of methanol and precipitating with 20 volumes of acetone. This process was repeated until a negative chloride test was obtained. After drying at 100° over P_2O_5 *in vacuo*, the dilithium salt of dephosphodesulfo-CoA (110 mg, 59%) was obtained as a fine white powder.

The product was homogeneous when subjected to paper chromatography. Venom phosphodiesterase digestion produced spots identical with adenosine 5′-phosphate and desulfopantetheine 4′-phosphate when chromatographed on paper.

Anal. Calcd for $C_{21}H_{33}N_7O_{13}P_2Li_2$: C, 37.79; H, 4.98; N, 14.69; P, 9.28. Found: C, 37.18; H, 5.0; N, 13.26; P, 9.29.

Preparation of CoA-Synthesizing Enzymes. Fresh beef liver was homogenized 45 sec with an equal volume of 0.15 *M* KCl and centrifuged at 40,000*g* for 1.5 hr and the supernatant solution diluted with two volumes of distilled water. Microsomes were precipitated by addition of 2% protamine sulfate followed by centrifugation at 50,000*g* for 0.5 hr as previously described for the pig liver system.[6] Then the procedure was modified. Inactive protein was removed by adjusting the protamine sulfate supernatant solution to pH 8.0 with solid Tris[24] and brought to 25% saturation by addition of solid ammonium sulfate. After centrifugation at 18,000*g* for 10 min, the pH of supernatant solution was readjusted to 8.0 and solid ammonium sulfate added to 38% saturation. The precipitate was collected by centrifugation and dissolved in distilled water to yield a crude enzyme preparation of about 30 mg protein/ml with a specific activity of 0.32 μmol of CoA synthesized/(mg hr), a 12-fold increase over the homogenate.

This crude preparation was quite stable and could be stored at −18° for 1 month without appreciable loss of activity. Consequently this solution was used as stock for further purification.

Stock enzyme solution (12 ml, 350-mg) was loaded on a Sephadex G-25 column (2.0 × 100 cm) and washed through with a 0.02 *M* Tris–HCl buffer, pH 8.0, to remove traces of ammonium sulfate. The Sephadex effluent was immediately loaded on a hydroxyapatite column[25] (2.5 × 16 cm) previously equilibrated with 0.01 *M* sodium phosphate buffer, pH 8.0. The column was washed with 0.01 *M* phosphate buffer until essentially free of inactive protein. Then 0.02 *M* phosphate buffer, pH 8.0, was applied, and 8-ml fractions were collected until the effluent was free of protein. Aliquots (0.19 ml) of the fractions were assayed for CoA-synthesizing ability.

The pooled peak was dialyzed for 6–8 hr against 0.02 *M* Tris–HCl buffer–10^{-3} *M* EDTA, at 1:20 volume ratio. The external buffer was then brought to 50% ammonium sulfate saturation over a 3-hr period. The dialysis bags were removed after an additional 3 hr. The protein suspension was removed, and the dialysis bags were washed twice with 60% saturated ammonium sulfate solution. After collection by centrifugation, the pellet was dissolved in distilled water to yield an enzyme preparation of about 12 mg/ml, specific activity 0.96 μmol of CoA/(mg hr), representing a threefold increase over the stock solution.

The enzyme system at this stage is sensitive to freezing and loses 20% of its activity even when stored at 4° for 2 days.

Enzymatic Syntheses. Incubations were carried out in a total volume of 2 ml at 38° for 1.5 hr and contained glycylglycine buffer, adjusted to pH 7.4 with Tris (80 μmol), cysteine (20 μmol), $MgSO_4$ (4 μmol), ATP (7.35 μmol), enzyme system (3 mg), and substrate.

(24) Tris = 2-amino-2-hydroxymethyl-1,3-propanediol.

(25) The best results were obtained by packing the column with the following mixture: Cellulose powder (15 g), sized twice for 10 min in 0.01 *M* phosphate buffer, pH 8.0; hydroxyapatite slurry (115 ml), freshly prepared by the method of O. Levin, *Methods Enzymol.*, 5, 27 (1962).

The substrates were D-desulfopantetheine 4′-phosphate (4 μmol) and dephosphodesulfo-CoA (2 μmol). When dephosphodesulfo-CoA was incubated with inorganic pyrophosphate (40 μmol), ATP was omitted. The reactions were stopped by placing the incubation tubes in a boiling water bath for 1 min and removing the precipitated protein by centrifugation. The supernatant solutions were diluted to a total volume of 25 ml and applied to a 1.3 × 50 cm DEAE-cellulose (chloride form) column. The column was washed with 0.003 *N* HCl. The adsorbed compounds were eluted with an acidic lithium chloride linear gradient, 350-ml reservoir of 0.12 *N* LiCl in 0.003 *N* HCl and 350-ml mixing vessel of 0.003 *N* HCl. Fractions (5 ml) were collected at a flow rate of 0.5 ml/min. Each ultraviolet-absorbing peak (260 mμ) was pooled, the pH adjusted to 4.5 with dilute LiOH, and solvent removed *in vacuo*. Lithium chloride was removed by repeated extractions with small volumes of methyl alcohol–acetone (1:15). After drying over P_2O_5 *in vacuo* at room temperature overnight, the residual material was dissolved in 1 ml of water and characterized by paper chromatography.

The concentration of each peak was calculated from the optical density units at 260 mμ, assuming an extinction coefficient of 15×10^3 for the adenosine moiety.

Nonpolar Contributions to the Rate of Nucleophilic Displacements of *p*-Nitrophenyl Esters in Micelles[1]

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Abstract: A remarkably large rate enhancement in the hydrolysis of straight-chain acyl esters of *p*-nitrophenol was observed in the presence of mixed micelles of N^{α}-myristoyl-L-histidine and cetyltrimethylammonium bromide (CTABr), as contrasted with the corresponding reaction with N^{α}-acetyl-L-histidine with or without CTABr. The micellar reaction was resolved into three steps: (1) the nonproductive rapid equilibrium binding of the esters to the CTABr regions of the micelle; (2) the reaction which leads to the acylation of the imidazolyl moiety of the N^{α}-myristoyl-L-histidine in or on the surface of the micelle, which follows second-order kinetics; and (3) the deacylation step. Both the logarithm of the apparent binding constant (step 1) and the logarithm of the second-order rate constant (step 2) increase linearly with the number of carbons in the acyl group of the *p*-nitrophenyl esters from acetate to hexanoate. A value of −630 cal/mol for the transfer of each methylene group of the acyl chain from the bulk solution to the CTABr micellar phase was calculated from the change in the apparent binding constants. In a similar manner, the observed differences in the second-order constant for the acylation reaction are compatible with a change in free energy of activation of 442 cal/mol for each methylene added to the acyl chain. These results suggest that in the micellar reaction the hydrophobic bond energy contributed by the acyl chain is used to decrease the activation energy of the catalyzed reaction.

The majority of the chemical reactions in biological systems occur in or on the vicinity of boundaries between apolar and polar regions. Thus, biological membranes are composed of phospholipid molecules associated to form lamellar or micellar aggregates. In addition, the participation of hydrophobic bonding in the stabilization of the native conformation of proteins[3] implies that in many regions the orientation of the side chains in proteins is similar to that occurring in micelles of amphipathic molecules. That is, the nonpolar amino acid side chains are directed away from the water in close van der Waals contact, whereas the polar side chains are directed so that they have maximum contact with water. This orientation would place many functional side-chain groups adjacent to or within hydrophobic regions giving them properties which might differ from those expected if the same groups were in an aqueous environment.[4]

It is important therefore, to ascertain how the behavior of a chemical reaction is altered when it occurs at such hydrocarbon–water interfaces. The most common approach to this problem has involved the study of reactivity in the presence of detergent micelles.[5] Recently, we reported a model system[6] which permits the study of the properties of amino acid side chains when present in a hydrocarbon–water interface. It is based on the fact that N^{α} long-chain acylamino acids in aqueous solutions form micelles with the hydrocarbon chains directed away from the water, while the amino acid side chains would lie in the micellar surface at the hydrocarbon–water interface. As an initial example of the model we showed that mixed micelles of N^{α}-myristoyl-L-histidine (MirHis) and cetyltrimethylammonium bromide (CTABr) catalyze the hydrolysis of *p*-nitrophenyl esters at significantly higher rates than those reported for histidine, imidazole, or histidine-containing peptides or polymers.[6] These results indicated the potential use of such micelles to create catalytic surfaces

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(3) W. Kauzmann, *Advan. Protein Chem.*, **14**, 1 (1959).

(4) D. B. Wetlaufer, J. T. Edsall, and B. R. Hollingworth, *J. Biol. Chem.*, **233**, 1421 (1958); S. Yanari and F. A. Bovey, *ibid.*, **235**, 2818 (1960).

(5) E. F. J. Duynstee and E. Grunwald, *J. Am. Chem. Soc.*, **81**, 4540, 4547 (1959); J. L. Kurz, *J. Phys. Chem.*, **66**, 2239 (1962); D. G. Herries, W. Bishop, and F. M. Richards, *ibid.*, **68**, 1842 (1964); L. J. Winters and E. Grunwald, *J. Am. Chem. Soc.*, **87**, 4608 (1965); M. T. A. Behme, J. G. Fullington, R. Noel, and E. H. Cordes, *ibid.*, **87**, 266 (1965); F. M. Menger and C. E. Portnoy, *ibid.*, **89**, 4698 (1967).

(6) A. Ochoa-Solano, G. Romero, and C. Gitler, *Science*, **156**, 1243 (1967).