COENZYME A-SYNTHESIZING PROTEIN COMPLEX OF SACCHAROMYCES CEREVISIAE

Edsel T. BUCOVAZ*, Stanley J. TARNOWSKI, William C. MORRISON, Robert M. MACLEOD, John C. MORRISON, Camellia M. SOBHY, James L. RHOADES[1], James E. FRYER, Jubran M. WAKIM[2] and Walter D. WHYBREW

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Department of Biochemistry, University of Tennessee, Center for the Health Sciences, Memphis, Tennessee 38163

Summary

The coenzyme A-synthesizing protein complex (CoA-SPC) is a multienzyme complex of *Saccharomyces cerevisiae* (Bakers' yeast), which has a molecular weight in excess of 200,000 as determined by Sephadex G-200 column chromatography. This multienzyme complex, which is insoluble in the crude yeast cell lysate, has been purified 229-fold. A cellular component of the yeast cell lysate, referred to as t-Factor, with a molecular weight of 400–1000 and chloride ion are involved in the solubilization of CoA-SPC. The CoA-SPC requires L-cysteine, D-pantothenic acid and ATP as substrates. The terminal CoA-SPC-bound intermediate is dephospho-CoA, which is subsequently phosphorylated and released from the complex as CoA. The sequence of reactions for the synthesis of CoA by the CoA-SPC differs significantly from those previously proposed for other systems. It could be that the reaction sequence is unique for the yeast cell.

Introduction

The components utilized for the *in vivo* synthesis of CoA are well documented; however, the mechanism of CoA synthesis from these components appears to differ depending on the system investigated. The avenue for CoA synthesis in rat liver described by HOAGLAND and NOVELLI[1] involves a reaction between pantothenic acid and cysteine to form pantothenylcysteine. According to this research group, the pantothenylcysteine is decarboxylated to pantetheine. Pantetheine is then phosphorylated to yield 4′-phosphopantetheine. The 4′-phosphopantetheine reacts with 2 moles of ATP to form CoA.

BROWN[2] proposed a different route for the *in vivo* synthesis of CoA by certain microorganisms and the mammalian system. His proposed order of synthesis involves a reaction between pantothenic acid and ATP to form 4′-phosphopantothenic acid. The 4′-phosphopantothenic acid reacts with cysteine to form 4′-phosphopantothenylcysteine, which is decarboxylated to yield 4′-phosphopantetheine. Then the 4′-phosphopantetheine reacts with 2 moles of ATP to form CoA. Thus, variations between the pathways for CoA synthesis described by HOAGLAND and NOVELLI[1], and BROWN[2] may be due to differences in the system studied.

Thus far, the complete synthesis of CoA, regardless of the system, has not been reported to involve a multienzyme complex. In the rat liver system, however, dephospho-CoA pyrophosphorylase and dephospho-CoA kinase, two enzymes that convert 4′-phosphopantetheine to CoA via dephospho-CoA, have been reported to exist as a bifunctional enzyme complex[3]. Thus some evidence exists that two of

[1] Visiting Scientist, Department of Chemistry, Berry College, Mount Berry, GA 30149
[2] Visiting Scientist, Department of Chemistry, University of Tennessee, Martin, TN 38237

the enzymes involved in CoA synthesis, at least in rat liver, probably exist in complex form. Studies in our laboratory provide the first evidence that a multienzyme complex with the capacity to synthesize CoA is present in *Saccharomyces cerevisiae* (Bakers' yeast)[4–10]. The name coenzyme A-synthesizing protein complex (CoA-SPC) was given this multienzyme complex. This report describes a purification procedure for CoA-SPC of *S. cerevisiae* and other characteristics of this multienzyme system.

Materials and Methods

Assay for CoA-SPC Activity

Synthesis of dephospho-CoA and subsequently CoA by the CoA-SPC requires the presence of three substrates: L-cysteine, D-pantothenic acid and ATP. The course of reaction of a particular substrate was followed by utilizing the radioactively labeled form of that substrate in the reaction. Except for certain experiments described in the text, the composition of the reaction mixture was as follows: 2.50 mM disodium ATP, 0.5 ml buffer A (containing 50 mM Tris-acetate, pH 7.2, 10 mM magnesium acetate, 25 mM KCl), 0.60 mM calcium salt of D-pantothenic acid, 0.50 mM of L-cysteine, 0.05 ml of purified CoA-SPC containing 100–200 μg protein, and water to a total volume of 1 ml.

The reaction mixture was incubated at 36° for 1 h unless otherwise indicated in the text. Termination of the reaction was accomplished by adding 2 ml of 10% trichloroacetic acid (TCA) and heating the mixture at 95° for 5 min. The reaction mixture was cooled to room temperature and the precipitated protein was recovered by filtration using a Millipore filtering apparatus and Whatman 3 MM paper disc. The precipitate collected on the disc was washed 4 times with approximately 2 ml of water per wash. After washing, the disc was dried in an oven at 70°. The dried disc was transferred to a scintillation vial, and the radioactivity level measured in a Nuclear Chicago liquid scintillation counter using the scintillation liquid described by Hoskinson and Khorana[11]. A graph of protein-bound radioactivity versus the amount of enzyme added resulted in a linear increase. Reaction mixtures minus ATP and 0-time incubation were used as two types of control reactions for the assay. This method of assay of CoA-SPC activity was primarily a measurement of bound radioactively labeled intermediates as well as any CoA bound to the complex. In experiments using [^{35}S]-L-cysteine as the radioactive marker, the assay for CoA-SPC activity by this method measured only dephospho-CoA and CoA bound to the multienzyme complex. Omission of L-cysteine from the reaction mixture and using either [^{14}C-U]-ATP or [^{14}C-1]-D-pantothenic acid as the radioactive marker permitted the measurement of an intermediate believed to be 5′-ADP-4′-pantothenic acid. Specific activity for CoA-SPC was expressed as nmoles of bound radioactive substrate/h/mg protein.

The CoA-SPC-bound dephospho-CoA and the proposed intermediate 5′-ADP-4′-pantothenic acid were determined, after alkaline hydrolysis of CoA-SPC[12], by chromatography and/or by enzyme analysis[13]. CoA released by the multienzyme complex after synthesis was determined enzymically as described under "Determination of CoA."

Assay for Decarboxylase Activity

Cysteine decarboxylase activity of the CoA-SPC was determined using the reaction mixture described under "Assay for CoA-SPC Activity." For this study, [^{14}C-U]-L-cysteine or [^{14}C-1]-L-cysteine was used as the radioactive marker. In order to measure the released CO_2, the reaction vessel opened into a flask containing a 10% $Ba(OH)_2$ solution. After incubating the reaction mixture for 1 h at 36°, the reaction was terminated by adding 2 ml of 10% TCA and heating the mixture at 95° for 5 min. The protein precipitate was collected on Whatman 3 MM paper discs for radioactivity measurements as described under "Assay for CoA-SPC Activity." The CO_2 released reacted with $Ba(OH)_2$ to form $BaCO_3$. Insoluble $BaCO_3$ was recovered from the $Ba(OH)_2$ solution by filtration on a previously weighed Whatman 3 MM filter disc. The disc containing the $BaCO_3$ was washed once with 5 ml H_2O, dried, weighed and assayed for radioactivity content.

Inactivation of Dephospho-CoA Kinase Activity of CoA-SPC

The dephospho-CoA kinase activity of CoA-SPC appeared to be the most heat labile

enzyme activity of the multienzyme complex. The kinase activity was inactivated without any effect to the other catalytic functions of the complex by preincubation of the CoA-SPC at 36° for 3 min. The kinase activity of some CoA-SPC preparations was inactivated during the purification of the multienzyme complex from Bakers' yeast. As reported for the dephospho-CoA kinase activity of rat liver[3], the kinase activity of CoA-SPC was also inhibited by 0.02% deoxycholate.

Synthesis and Recovery of CoA-SPC-Bound Intermediates

The two proposed CoA-SPC-bound intermediates, 5′-ADP-4′-pantothenic acid and dephospho-CoA, were prepared as follows: the intermediate believed to be 5′-ADP-4′-pantothenic acid was synthesized using 100 ml of the reaction mixture described under "Assay for CoA-SPC Activity," except L-cysteine was omitted from the mixture. Consequently, with L-cysteine omitted from the reaction mixture, the proposed intermediate 5′-ADP-4′-phosphopantothenic acid was the terminal protein-bound reaction product. Dephospho-CoA was synthesized using the complete reaction mixture described under "Assay for CoA-SPC Activity." The CoA-SPC used in the experiments was preincubated at 36° for 3 min to inactivate dephospho-CoA kinase activity (Materials and Methods). When all substrates were present in the reaction mixture, the terminal protein-bound reaction product was dephospho-CoA.

After a 1 h incubation period, the two reaction mixtures were heated at 70° for 5 min to terminate the reaction. Following the heating step, the reaction mixtures were centrifuged for 5 min at 2,200 rpm in a Model CL International clinical centrifuge, and the supernatant liquids discarded. The protein pellets were washed two times by resuspending the pellet material in water followed by centrifugation at 2,200 rpm. The protein pellet material was resuspended for a third time in water, these mixtures were adjusted to pH 12.0 using 1 N KOH, and then heating at 70° for 1 h[12]. After incubation, the pH of the mixtures were readjusted to pH 7 by the addition of 0.5 N HCl. The mixtures were centrifuged at 2,200 rpm for 5 min and the resulting supernatant liquids were reduced to approximately one-tenth of their original volumes by lyophilization. The intemediates in the concentrated supernatant liquids were identified as described under "Paper Electrophoresis and Paper Chromatography." Dephospo-CoA was also identified by the PTA end-point method using 10.75 μg of PTA per reaction mixture[13] as described under "Determination of CoA."

Recovery of CoA

The reaction mixture was the same as described under "Assay for CoA-SPC Activity," except the quantity of the components were increased proportionally to give a total volume of 100 ml.

After incubation for 2 h at 36°, the reaction was stopped by heating the mixture at 70° for 5 min. Following the heating step, the reaction mixture was cooled in an ice bath and centrifuged at 2,200 rpm for 5 min in a Model CL International clinical centrifuge to remove the denatured protein. The resulting supernatant liquid was filtered using No. 8 dialysis tubing against a reduced pressure. The filtrate recovered was then refiltered using an Amicon Ultrafiltration membrane (UM-05) with a reported molecular weight cut-off of 500. Although CoA has a molecular weight of approximately 800, it passed through this filter. The protein-free filtrate was chromatographed on Whatman 3 MM paper using a n-butanol : acetic acid : water (4 : 1 : 1) solvent system. In this system CoA remains at the origin, but L-cysteine and D-pantothenic acid migrate down the paper. Following chromatography, the CoA was extracted from the paper with distilled water. The solution containing extracted CoA was reduced to dryness by lyophilization, dissolved in 3 ml of water and chromatographed on a column (0.9 cm × 163 cm) of Sephadex G-10. CoA was eluted from the Sephadex column immediately following the void volume. Other components such as ATP present in the material being chromatographed were eluted in later fractions. The fractions containing CoA were concentrated by lyophilization. CoA was identified by procedures described under "Paper Chromatography and Paper Electrophoresis," and by enzymatic assay as described under "Determination of CoA."

Determination of CoA

The CoA synthesized by CoA-SPC was measured by standard versions of the phosphotransacetylase (PTA) end-point method as de-

scribed by MICHAEL and BERGMEYER[13]. The end-point method using 0.43 μg PTA only determined intact CoA. Contributions by dephospho-CoA and other intermediates of CoA biosynthesis were less than 1%. In the absence or presence of CoA, dephospho-CoA was measured by the PTA end-point method by increasing the concentration of PTA from 0.43 μg to 10.75 μg per reaction flask[13]. Phosphotransacetylase (F.U.B. 2.3.1.8) from *Cl. kluyveri*, ≥1000 U/mg and the K-Li salt of acetylphosphate were obtained from P-L Biochemicals, Inc. CoA and dephospho-CoA for the preparation of standard solutions were purchased from Sigma Chemical Co. A Cary 14 spectrophotometer was used to determine the absorbance increase at 233 nm. Samples to be stored containing CoA were either lyophilized to dryness of adjusted to pH 4.5 to 6.0 with acetic acid and frozen.

Preparation of t-Factor

t-Factor was prepared from Bakers' yeast by a modification of the method previously described[7]. Bakers' yeast (454 g) was crumbled into liquid N_2 to freeze the cells. After thawing the mixture which contained lysed cells, intact cells and soluble components from both was centrifuged at 105,000 × g at 4° for 1 h. The 105,000 × g supernatant fraction of the yeast cell mixture was heated at 80° for 30 min. After cooling, the heated fraction was centrifuged at 10,000 × g to remove denatured protein. The resulting supernatant fraction was then passed through an Amicon UM-05 filter using 40 psi N_2 gas. The recovered filtrate was then passed through a column of petroleum-base activated charcoal to remove UV absorbing materials as well as certain other components. t-Factor, which does not adsorb to the charcoal, was present in the water wash. The water wash was pooled, and its volume was reduced to dryness by lyophilization. The lyophilized material was redissolved in 10 ml H_2O, and was further fractionated by chromatography on Sephadex G-10. The fractions recovered from the Sephadex G-10 column containing t-Factor were utilized for the solubilization of CoA-SPC as described under "Purification of CoA-SPC of Bakers' yeast."

Gel Filtration of CoA-SPC

Approximately 40 g of Sephadex G-200, coarse (Pharmacia), were equilibrated for 4 days in buffer A. A column (2.5 cm × 60 cm) was prepared from the material and maintained at 0–4°. The Pellet Extract containing CoA-SPC (see Table 1) was eluted using a dilute solution (0.02 mM) of ATP at a flow rate of 1 ml per min. ATP was used to elute the column because of its stabilizing action on CoA-SPC activity[5].

Molecular Weight

A Sephadex G-200 column prepared as described above was used to determine the approximate molecular weight of CoA-SPC. The total bed volume, inner gel volume, volume of gel, and void volume of the column were determined. A calibration curve[14] for the column was determined by chromatographing a mixture of glutathione, bovine serum albumin, bovine pancreas chymotrypsinogen, and bovine ribonuclease (all from Sigma Chemical).

Polyacrylamide Gel Electrophoresis

Electrophoresis in acrylamide gel was performed at 0–4° using a modification of the

Table 1
Purification of the CoA-Synthesizing protein complex

Fraction	Volume	Protein	Specific activity	Yield	Purification[a]
	ml	mg/ml	nmoles/h/mg	%	-fold
Crude Extract	175	131.00	4.4	100.00	1.0
Pellet Extract	175	4.10	185.5	131.90	42.2
Ammonium Sulfate Fraction 2[b]	20	13.40	303.0	80.50	69.0
Sephadex G-200 Fraction[c]	6	2.15	1007.7	12.88	229.0

[a] The assay procedure described under "Assay for CoA-SPC Activity" was used to assess the extent of purification of each fraction. Specific activity was obtained using 0.5 μmole [^{35}S]-L-cysteine (23,110 c.p.m.) as the radioactive marker.
[b] Ammonium sulfate used for fractionation was recrystallized 3× from 1% EDTA-deionized H_2O solution.
[c] Sephadex G-200 fractionation of CoA-SPC was accomplished by elution with deionized H_2O containing 0.02 mM ATP.

method described by DAVIS[15]. Samples containing 50 ug of protein were layered in 20% sucrose over the gels. A current of 2 ma per gel was applied for 3 h. For certain experiments, gels were fixed in 20% TCA for 4 h, and then stained with 0.25% Coomassie blue in 7% TCA containing 10% methanol for 16 h. The unbound dye was removed from the gels with 7% acetic acid containing 10% methanol. For other studies, following electrophoresis, each gel was sliced (2-mm slices). The gel slices were placed in a special scintillation liquid (containing 6 g PPO, 1000 ml toluene, 20 ml Hyamine hydroxide and 10 ml NCS) designed to detect radioactivity in gels. The gel slices were placed in the scintillation cocktail 48-h prior to counting. Still other studies required that the gels be sliced as described above, and that each slice be placed in 0.3 ml of deionized H_2O containing 0.02 mM ATP for 14 h at 0–4° to extract CoA-SPC and other materials from the gel slices.

For molecular weight determinations and CoA-SPC subunit composition studies, the SDS-polyacrylamide gel electrophoresis method of WEBER and OSBORN[16] was used.

A standard curve plotting log MW vs electrophoretic mobility of several proteins of known molecular weights (serum albumin, ovalbumin, chymotrypsinogen, myoglobin and cytochrome C) was used to obtain an approximate molecular weight of the substructures of CoA-SPC.

Paper Electrophoresis and Paper Chromatography

Paper electrophoresis was conducted using Whatman 3 MM paper at pH 4.2 in ammonium acetate buffer (15 ml of 25% NH_4OH and 20 ml acetic acid diluted to 3000 ml with deionized water) at 35 V/cm for 6 h[17]

Descending paper chromatography was carried out using Whatman 3 MM paper. Four developing systems were used. System 1 contained isobutyric acid : ammonium hydroxide : water (60 : 1 : 33); system 2 contained n-butanol : acetic acid : water (3 : 2 : 3); system 3 contained n-butanol : acetic acid : water (5 : 2 : 4); and system 4 contained isobutyric acid : 0.5 N ammonium hydroxide : 0.1 M EDTA (100 : 60 : 1.6).

Determination of Protein

Protein concentration was determined by the method of LOWRY *et al.*[18]

Identification of Reaction Products

Components known or suspected to be present in a particular reaction mixture were used as identification markers in electrophoretic and chromatographic studies. The markers used were: L-cysteine, D-pantothenic acid, Pi, PPi, 5′-ATP, 5′-ADP, 5′-AMP, 3′,5′-ADP, CoA(SH), dephospho-CoA, 4′-phosphopantothenic acid, 4′-phosphopantetheine and pantetheine. The 4′-phosphopantothenic acid, 4′-phosphopantetheine were prepared according to the method of SHIMIZA *et al.*[19]

Following paper electrophoresis and paper chromatography, the reaction components were identified by their migration patterns compared to the migration of known markers, radioactivity, UV absorption at 260 nm, phosphorus analysis[20], SH-analysis[21] and enzyme analysis for CoA[13]. The distinction between CoA and dephospho-CoA was made by chromatographic migration, using the solvent systems described under "Paper Electrophoresis and Paper Chromatography" and by enzyme analysis[13].

Authentic 5′-ADP-4′-pantothenic acid was not available; therefore, the identity of a compound believed to be 5′-ADP-4′-pantothenic acid was prepared as described under "Synthesis and Recovery of CoA-SPC-Bound Intermediates." The identity of this compound was based on chromatography, UV absorption and stoichiometry between radioactive labeling of the adenine ring and pantothenic acid moiety. Additional support for the 5′-ADP-4′-pantothenic acid structure being the intermediate was provided by its functioning as precursor for the synthesis of dephospho-CoA and CoA by the CoA-SPC.

Sources of Components

Dephospho-CoA and CoA were obtained from PL Biochemicals. Other components used in this study were obtained from Sigma Chemical Co. with the following exceptions: [^{14}C]- and [^{35}S]-L-cysteine and [^{14}C]- and [^{32}P]-ATP were purchased from Schwarz/Mann; [^{14}C-1]-D-pantothenic acid was purchased from New England Nuclear.

Experimental and Discussion

Purification of CoA-SPC of Saccharomyces cerevisiae

Bakers' yeast (Federal Yeast Corp., Baltimore) weighing 454 g was crumbled into a suitable vessel containing approximately 0.7 kg anhydrous ethyl ether. Three kilograms of compressed CO_2 were added and the mixture was stirred until the yeast was frozen. The frozen mixture was permitted to thaw for approximately 7 h at 23° to 25°. Ether was removed from the yeast by vacuum, using a water aspirator, until no detectable odor of ether remained in the vessel.

Approximately 30 ml of the yeast lysate and 5 g of KCl/454 g of yeast were stirred mechanically for 18 h at 4° to solubilize CoA-SPC (5–10). Buffer is not used in the stirring step, because experience shows that the CoA-SPC is more stable without the addition of buffer. After stirring, the yeast lysate was centrifuged at 105,000 × g for 20 min. The supernatant fraction obtained, which contained the CoA-SPC, was termed Crude Extract (see Table 1). This Crude Extract was used as the basis for establishing the extent of purification of CoA-SPC accomplished by the following procedure.

The remaining yeast cell lysate (approximately 420 g), which was in a fluid state, was centrifuged at 105,000 × g for 1 h. The resulting supernatant liquid was discarded. The pellet material was resuspended in an amount of water and t-Factor ("Preparation of t-Factor", Material and Methods) equivalent to the volume of the discarded supernatant fraction. Five grams of KCl/454 g of yeast were added, and the resuspended pellet material was stirred for 18 h at 4° to solubilize the CoA-SPC. During the course of stirring, 1 ml samples were taken at 3-h intervals, centrifuged at 105,000 × g for 20 min, and the supernatant liquid assayed for CoA-SPC activity (see Table 2). The remaining mixture was centrifuged at 105,000 × g for 20 min, and the resulting supernatant liquid (Pellet Extract, Table 1) was assayed for CoA-SPC activity and protein content. The volume of the Pellet Extract was 175 ml (Table 1). Because CoA-SPC was solubilized gradually from the yeast cell lysate during the 18-h stirring period, if t-Factor and 5 g KCl/454 g of yeast are present in the mixture, soluble proteins were removed by decantation following the initial centrifugation steps prior to stirring. Consequently, the procedure described above resulted in a 42-fold purification of the CoA-SPC.

Ammonium Sulfate Fractionation

The CoA-SPC in the Pellet Extract (Table 1) was further purified through two ammonium sulfate fractionation steps. Step one was the addition of 20 g of ammonium sulfate to 100 ml of the Pellet Extract followed by thorough mixing. After 8 h the mixture was centrifuged at 10,000 × g for 20 min. The supernatant layer (Ammonium Sulfate Fraction1) was retained and the precipitate was discarded. In step two, an additional 6.50 g of ammonium sulfate was added to 50 ml of the supernatant layer. After 7 h, the material was centrifuged at 105,000 × g

Table 2
Solubilization of CoA-SPC of the yeast cell lysate[a]

Pellet extract	CoA-SPC bound radioactivity					
	[^{35}S]-L-Cysteine		[^{14}C-1]-D-Pantothenic acid		[^{14}C-U]-ATP	
(stirring time, h)	nmoles	nmoles/h/mg	nmoles	nmoles/h/mg	nmoles	nmoles/h/mg
3	0	0	0	0	0	0
6	8.1	186	7.6	175	8.0	184
9	14.4	184	13.7	175	15.1	193
12	30.3	187	32.2	200	32.2	199
15	36.9	185	57.1	189	38.4	193
18	37.5	188	35.0	175	38.7	194

[a] At each stirring time indicated, a sample of the "Pellet Extract" was removed, centrifuged and 0.05 ml of the supernatant fraction was added to individual incubation mixtures which contained one of the three substrates as the radioactive tracer. Also present in each reaction mixture were the other two substrates and other components of the reaction mixture as described under "Materials and Methods." 0, indicates no activity. Activity is given in nmoles. Specific activity is expressed as nmoles of bound radioactive substrate/h/mg protein. The three radioactive substrates used were 0.5 μmole [^{35}S]-L-cysteine (23,110 c.p.m.), 0.6 μmole [^{14}C-1]-D-pantothenic acid (28,000 c.p.m.) and 2.5 μmoles [^{14}C-U]-ATP (116,000 c.p.m.).

for 1 h. The resulting supernatant layer, which did not contain a significant level of CoA-SPC activity, was discarded, and the pellet was redissolved in 20 ml of H_2O (Ammonium Sulfate Fraction 2, Table 1). Ammonium Sulfate Fraction 2, which contained 13.40 mg protein per ml, was chromatographed on Sephadex G-200 as described under "Materials and Methods." The fractions collected from the Sephadex G-200 column with the highest CoA-SPC activity were pooled (Sephadex G-200 Fraction, Table 1) to give 6 ml of solution containing 2.15 mg protein per ml, which represented a 229-fold purification of the CoA-SPC. The purification of CoA-SPC was monitored by measuring radioactive substrate binding to the enzyme complex rather than by measuring CoA production because of endogeneous CoA in the crude yeast lysate.

Solubilization of CoA-SPC

CoA-SPC activity could not be demonstrated in the yeast lysate prior to the stirring step (see "Purification of CoA-SPC of *S. cerevisiae*"). However, CoA-SPC was gradually solubilized by either stirring the crude yeast cell lysate or centrifuging the crude lysate at 105,000 × g for 20 min, discarding the supernatant fraction, and stirring the pellet material which had been resuspended in H_2O containing 5 g KCl/454 g of yeast and t-Factor equivalent to the volume of the discarded supernatant fraction. At 3-h intervals during the stirring procedure, samples of the resuspended pellet material were removed from the stirring flask, centrifuged at 105,000 × g for 20 min, and the resulting supernatant liquid (Pellet Extract) assayed for protein content and for CoA-SPC activity (Table 2). The three radioactively labeled substrates were tested separately as radioactive markers to monitor the levels of activity of the various enzymes of the complex in each successive pellet extract. As shown in Table 2, CoA-SPC activity was first detected in the 6-h pellet extract. Once activity was detected, a progressive increase in activity for each of the three substrates was observed during the 18 h of stirring. Also, a corresponding increase in CoA synthesis was observed. Although not shown in Table 2, some CoA-SPC preparations using other sources of commercial yeast required additional stirring time for maximum solubilization of CoA-SPC. Most preparations, however, showed a progressive decrease in CoA-SPC activity after 18 h. Following the procedure described, 18-h stirring time has resulted in the optimum level of CoA-SPC activity for most preparations.

In studies not shown, Ammonium Sulfate Fraction 2 and the Sephadex G-200 Fraction of Table 1 also showed comparable levels of activity for each of the three substrates.

Recovery and Identification of CoA

Following the procedure described under "Recovery of CoA, Materials and Methods," CoA was recovered from three separate reaction mixtures similar to those in Table 6 except the volume was increased to 100 ml and the reaction time was 2 h. The recovered CoA from reaction mixture containing [^{14}C-U]-ATP (Reaction Mixture 1, Table 6), [^{14}C-1]-D-pantothenic acid (Reaction Mixture 4), and [^{35}S]-L-cysteine (Reaction Mixture 7) as radioactive tracers were subjected to paper chromatography. In each of four chromatographic solvent systems, radioactive material was detected in the reaction mixtures with the same R_f value as authentic CoA (Table 3). Also, the radioactive component recovered from the three reaction mixtures had the same electrophoretic pattern, and gave a radioactive band with a 260-nm absorbance, identical to authentic CoA. Assay of the material in the phosphotransacetylase system[13] verified the presence of 28.5 to 30.1 mg of CoA in 100 ml of the reaction mixtures. Based on the amount of the substrates present in the reaction mixture, 38.6 mg of CoA could have been synthesized. Therefore, 74 to 78% of the theoretical yield of CoA was detected.

Molecular Weight Estimation of CoA-SPC

The molecular weight of CoA-SPC was estimated to be in excess of 200,000 based upon Sephadex G-200 column chromatography (Materials and Methods).

Polyacrylamide Gel Electrophoresis

Purified CoA-SPC was analyzed by polyacrylamide gel electrophoresis. After electrophoresis, the gels were segmented, except for control gels used for staining, and the segments were placed in 0.3 ml of 0.02 mM ATP for 14 h

Table 3
Relative chromatographic mobilities of substrates and CoA-SPC reaction products[a]

Materials tested	R_f Values			
	System 1	System 2	System 3	System 4
L-Cysteine	0.64	0.61	0.48	0.61
D-Pantothenic acid	0.69	0.84	0.79	0.72
5′-ATP	0.14	0.19	0.12	0.37
5′-ADP	0.21	0.28	0.15	0.45
5′-AMP	0.45	0.45	0.27	0.57
3′,5′-ADP	0.40	—	—	0.43
PPi	0.11	0.26	0.10	—
Pi	0.26	0.48	0.28	0.30
CoA	0.41	0.25	0.18	0.59
Dephospho-CoA	0.29	0.37	0.15	0.69
4′-Phosphopantetheine	0.49	0.59	0.51	0.64
Pantetheine	0.94	0.94	0.88	0.84
5′-ADP-4′-pantothenic acid	0.33	0.43	0.30	0.63
4′-Phosphopantothenic acid	0.50	0.52	0.49	0.53
Reaction Mixture 1[b]	0.41	0.26	0.18	0.59
Reaction Mixture 4	0.41	0.25	0.18	0.59
Reaction Mixture 7	0.40	0.24	0.18	0.60

[a] Conditions were as follows: descending chromatography; approximately 50 cm solvent movement on Whatman 3 MM paper at room temperature. Other conditions were as described under "Materials and Methods."
[b] The reaction mixtures chromatographed correspond to those in Table 6.

at 0–4° to extract the CoA-SPC. Control gels were stained with Coomassie blue as described under "Materials and Methods." The purified fraction of CoA-SPC displayed one major protein band and several minor bands (Fig. 1). Assay for CoA-SPC activity present in the gel extracts indicated that all detectable enzyme activity attributed to CoA-SPC was associated with the major protein band.

Polyacrylamide gel electrophoresis was also used to analyze radioactively labeled CoA-SPC of a reaction mixture following chromatography on Sephadex G-200. The CoA-SPC was radioactively labeled using the procedure described under "Assay for CoA-SPC Activity."

After incubation for 1 h at 36°, the reaction was stopped by cooling the mixture in an ice-NaCl bath and chromatographing its contents on a Sephadex G-200 column (Materials and Methods) as shown in Figure 2. Fractions 71 through 91, which contain proteins with a molecular weight in excess of 200,000 and have bound radioactivity, were pooled and analyzed by polyacrylamide gel electrophoresis before and after dialysis against 1% SDS as shown in Figures 3 and 4, respectively. The distribution of protein-bound radioactivity prior to and after dialysis against SDS was determined after slicing the gel into 2-mm segments. A duplicate gel representing each condition was stained with Coomassie blue and scanned with a densitometer to locate the major protein bands.

As shown in Figure 3, three bands of protein bound radioactivity were present after polyacrylamide gel electrophoresis of the pooled Sephadex G-200 fractions prior to dialysis against SDS. The first band of radioactivity was extracted from the initial (0–2 mm) gel segment, indicating that some of the protein did not penetrate the gel. The second band of radioactivity was extracted from the 10 to 20 mm gel segments, and corresponded with the migration of the major protein band. A significant portion of the radioactivity in this band should be bound to CoA-SPC. The third major radioactive peak was found in gel segments 64 through 74 mm. Dialysis of the extract from the third radioactivity band indicated that all of the radioactivity was bound to protein, or to some other material with a molecular weight large enough that it was not removed by dialysis.

A second sample of the Sephadex G-200

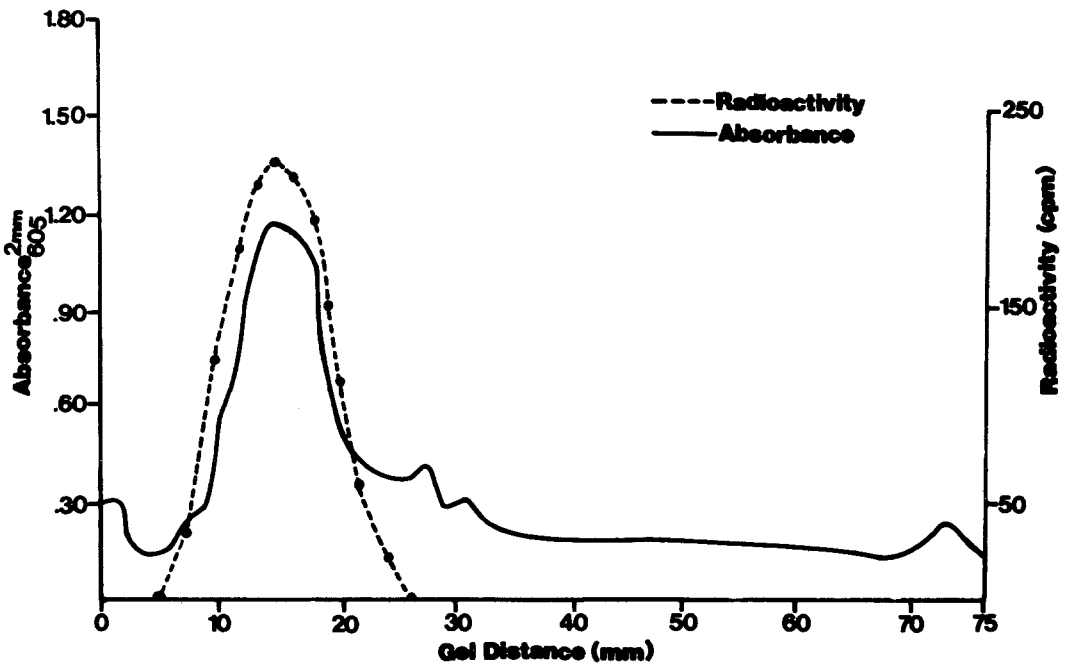

Fig. 1. Polyacrylamide gel electrophoresis pattern of Sephadex G-200 fractionated CoA-SPC (see Table 1). Following electrophoresis, the gels were segmented, except control gels used for staining. CoA-SPC was extracted by placing the gel segments in 0.3 ml of deionized water containing 0.02 mM ATP for 14 h at 0–4°. The gel extract was assayed for CoA-SPC activity, "Material and Methods," using 0.5 μmole [^{35}S]-L-cysteine (23,000 c.p.m.) as the radioactive tracer.

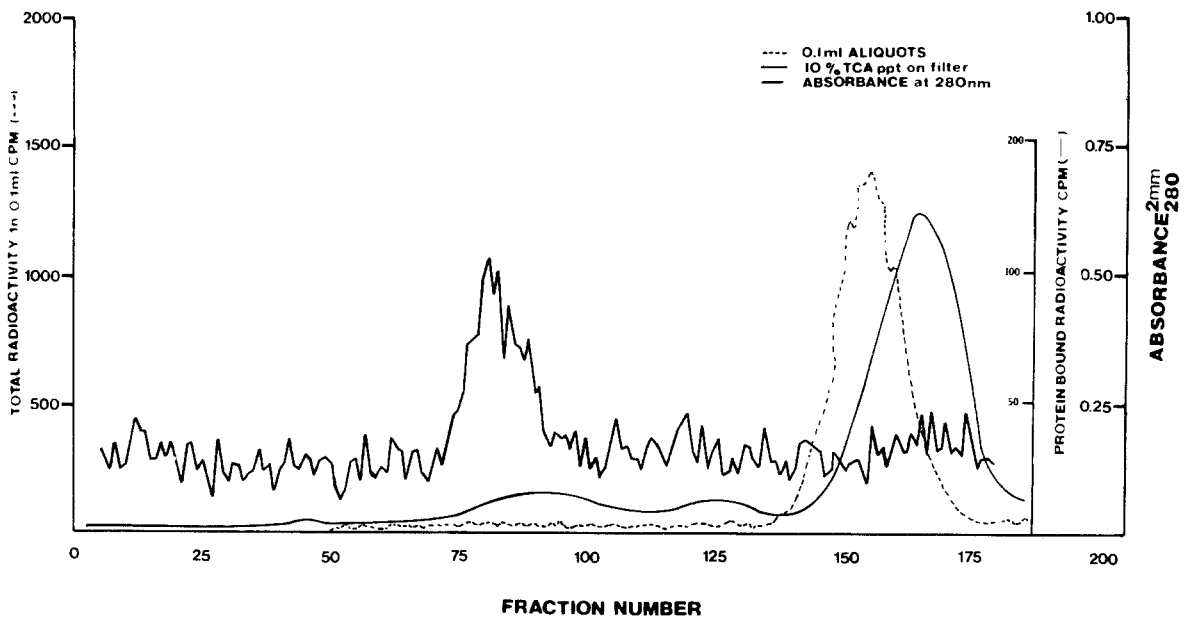

Fig. 2. Sephadex G-200 elution profile of a reaction mixture containing purified CoA-SPC. Purified CoA-SPC (see Table 1) was radioactively labeled using 0.5 μmole [^{35}S]-L cysteine (23,000 c.p.m.) per ml of reaction mixture as the marker, Following incubation for 1 h at 36°, five ml of the reaction mixture were fractionated on a column of Sephadex G-200.

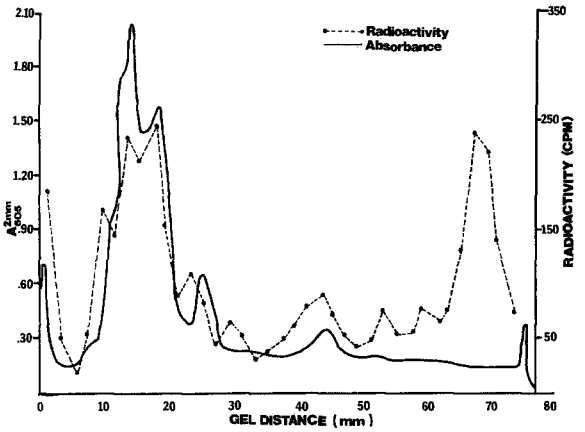

Fig. 3. Polyacrylamide gel electrophoresis pattern of the CoA-SPC reaction product. Peak fractions 71 through 91 from Sephadex G-200 chromatography (Fig. 2) were pooled, concentrated by lyophilization and electrophoresed, as described under "Polyacrylamide Gel Electrophoresis, Materials and Methods."

Fraction (see Fig. 2) containing radioactively labeled CoA-SPC was dialyzed for 14 h against 1% SDS. After polyacrylamide gel electrophoresis of the SDS treated fraction, the gels were segmented, except for control gels used for staining, and the contents of the gel segments were extracted either in the scintillation cocktail or in 0.02 mM ATP as previously described. As shown in Figure 4, scanning of the stained gels revealed one major band of protein after SDS treatment, which correlated with the band of radioactivity extracted from corresponding gel segments (2-mm segments). Molecular weight estimation of the protein following the SDS method of WEBER and OSBORN[16] indicated that protein in this band was in the molecular weight range of cytochrome C (13,000) or less.

On the basis of the SDS-polyacrylamide gel electrophoresis study, the number of polypeptide chains comprising the CoA-SPC cannot be

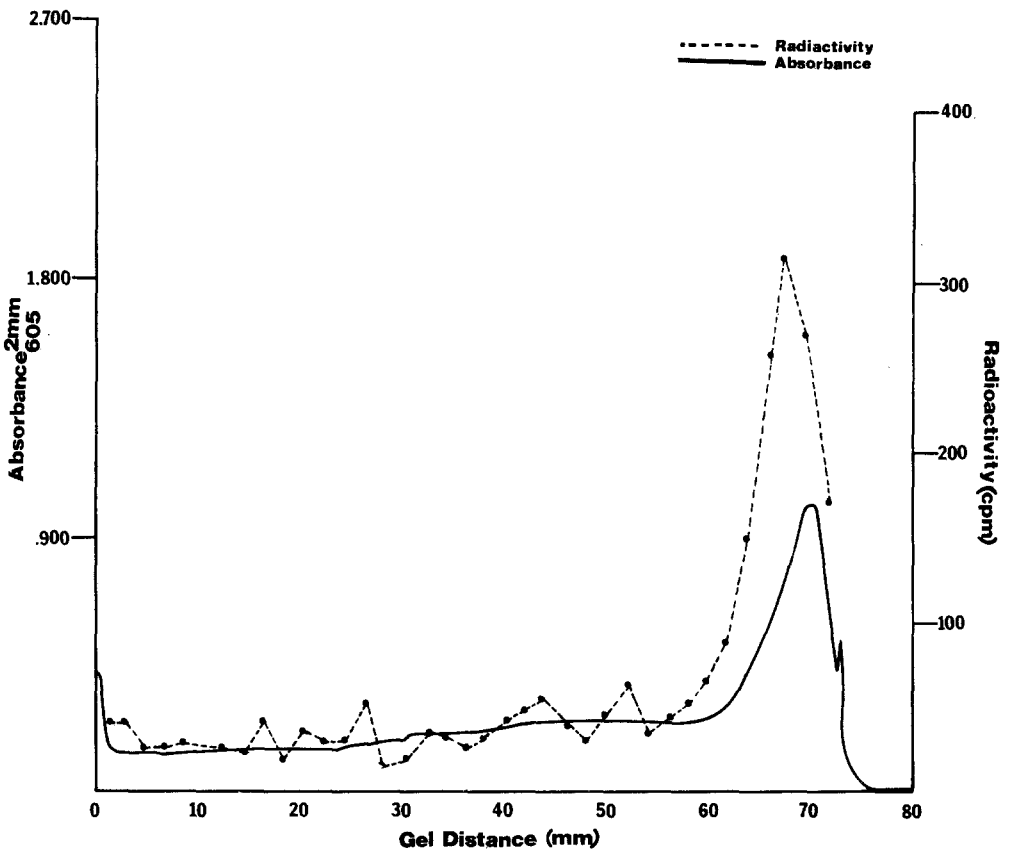

Fig. 4. SDS-Polyacrylamide gel electrophoresis pattern of the CoA-SPC-bound reaction products. The CoA-SPC had previously been radioactively labeled with [^{35}S]-L-cysteine as described under "Assay for CoA-SPC Activity." The radioactively labeled CoA-SPC of fractions 71 through 91 from Sephadex G-200 chromatography (Fig. 2) was treated as in Figure 3 except for the presence of SDS[16].

reliably estimated. However, it does indicate that CoA-SPC can be separated into several smaller subunits by treatment with 1% SDS. Also based on dialysis studies of the gel extracts, it would appear that essentially all of the radioactivity after SDS treatment is bound to one or more of the polypeptide chains in gel segments 64 through 74 mm.

Effect of temperature on CoA-SPC activity

As shown in Figure 5 at 36°, the temperature routinely used in the assay, near maximal activity was obtained with minimal CoA-SPC inactivation. In other temperature studies, purified CoA-SPC was preincubated at 36° for 3 h. At 5-min intervals, aliquots were taken and assayed for CoA-SPC activity as described under "Assay for CoA-SPC Activity." A progressive decrease in CoA-SPC activity was observed with each successive aliquot taken from the incubation flask (Fig. 6a). No CoA-SPC activity was observed after 30 min of preincubation. The addition of 0.02 mM ATP to the sample prior to preincubation protected the CoA-SPC against inactivation (Fig. 6b). In the presence of ATP, the CoA-SPC retained its initial activity level even after preincubation at 36° for 3 h. Neither L-cysteine nor D-pantothenic acid alone or combined could replace ATP in this capacity.

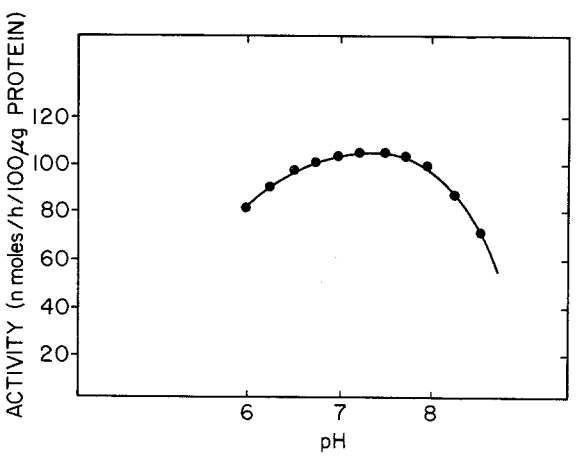

Fig. 5. Effect of temperature on CoA-SPC catalytic activity. Assay conditions were as described under "Assay for CoA-SPC Activity." The source of enzyme was the Sephadex G-200 purified fraction (Table 1), and 0.5 μmole [^{35}S]-L-cysteine (22,800 c.p.m.) was used as the radioactive marker. In separate experiments (not shown) identical patterns were observed when [^{14}C-1]-D-pantothenic acid or [^{14}C-U]-ATP instead of [^{35}S]-L-cysteine was used as the radioactive marker.

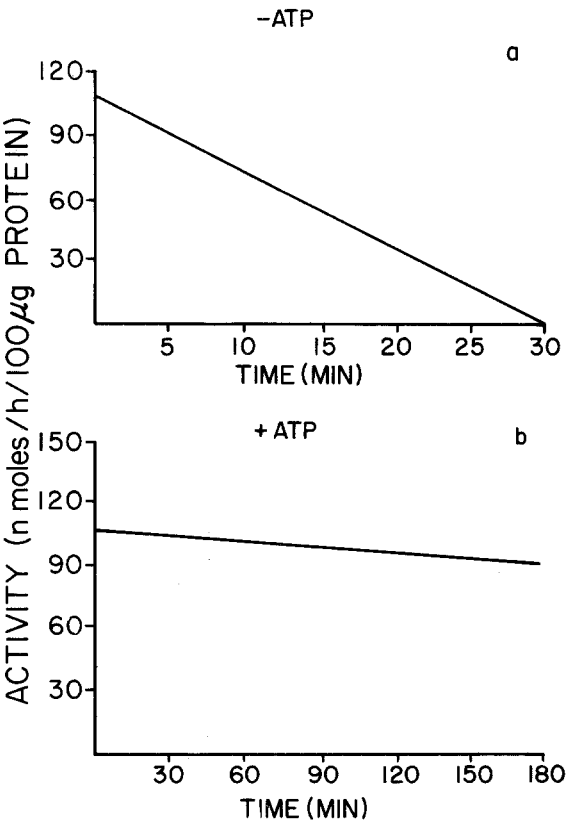

Fig. 6. Comparison of the stability of CoA-SPC activity to preincubation in the absence and presence of ATP. Preincubation was conducted on a CoA-SPC: deionized H_2O solution (1:1 v/v). (a) Represents the pattern of CoA-SPC during preincubation at 36° in the absence of added ATP. (b) Represents the pattern of CoA-SPC activity during preincubation at 36° in the presence of 0.02 mM ATP. At 5-min intervals, a 0.10 ml aliquot was removed from each of the flasks containing CoA-SPC and assayed for catalytic activity, using 0.5 μmole [^{35}S]-L-cysteine (23,110 c.p.m.) as the radioactive marker.

Effect of pH on CoA-SPC Activity

The dependence of CoA-SPC activity on pH is illustrated in Figure 7. The pattern was identical when each of the substrates was used as the radioactive marker. Normally, reaction mixtures were pH 7.2 which is near the middle of the optimum pH range for CoA-SPC activity.

Characteristics of dephospho-CoA and CoA Synthesis by CoA-SPC

The catalytic activity of CoA-SPC and the assembly of dephospho-CoA and CoA were studied by utilizing the radioactively labeled forms of L-cysteine, D-pantothenic acid, and ATP in the following studies.

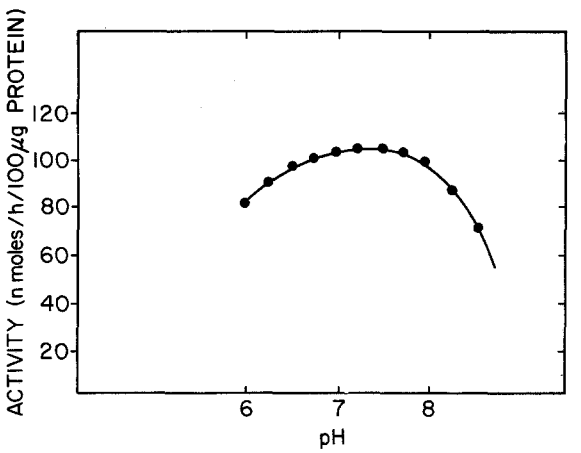

Fig. 7. Effect of pH on CoA-SPC activity. Assay conditions were as described under "Assay for CoA-SPC Activity," except for the variations in pH. The source of the enzyme was the Sephadex G-200 purified fraction (see Table 1), and the radioactive marker was 0.5 μmole [^{35}S]-L-cysteine (22,800 c.p.m.). Repeated experiments in which either [^{14}C-1]-D-pantothenic acid or [^{14}C-U]-ATP was the radioactive marker gave identical results.

Role of ATP in the Synthesis of CoA-SPC-Bound Intermediates

Either [^{32}P]- or [^{14}C]-ATP was used as the radioactive marker in the experiments shown in Table 4. [α-^{32}P]-ATP was used in Experiment 1, and [$\beta\gamma$-^{32}P]-ATP was used in Experiment 2 as the radioactive marker. Radioactively labeled CoA-SPC was present in both reaction mixtures whether or not the CoA-SPC used in the experiments had dephospho-CoA kinase activity. However, when [γ-^{32}P]-ATP was used as the radioactive marker (Experiments 3 and 4), there was a significant difference in the results depending on the condition of CoA-SPC used in the experiment. CoA-SPC with dephospho-CoA kinase activity was used in Experiment 3 and CoA-SPC without dephospho-CoA kinase activity was added to the reaction mixture of Experiment 4. As shown in Experiments 3 and 4, only when CoA-SPC had dephospho-CoA kinase activity (Experiment 3) was CoA-SPC radioactivity labeled with [^{32}P]. On the other hand, when either [^{14}C-8]-ATP or [^{14}C-U]-ATP was the radioactive tracer (Experiments 5 and 6), CoA-SPC was radioactively labeled regardless of the presence or absence of dephospho-CoA kinase activity. The data shown for Experiments 1, 2, 4 support the contention that the bond between the β- and the γ-P is hydrolyzed, releasing the γ-P group and the α- and β-P groups remaining bound to the CoA-SPC. Whereas, Experiment 3 shows CoA-SPC-bound radioactivity when CoA-SPC with dephospho-CoA kinase activity is used in the reaction mixture. Experiments 5 and 6 indicate the adenine ring of ATP also remains attached to the CoA-SPC. Thus it would appear that ATP is converted to ADP and the γ-P group was released during the course of dephospho-CoA synthesis. In an experiment not shown in Table 4, whenever L-cysteine was omitted from reaction mixtures in which CoA-SPC with inactivated dephospho-CoA kinase activity was used,

Table 4
Reaction characteristics of ATP with CoA-SPC[a]

Experiment no.	Radioactive component	System	nmoles/h/mg[b]
1	[α-^{32}P]-ATP	Complete	988
2	[$\beta\gamma$-^{32}P]-ATP	Complete	649
3[c]	[γ-^{32}P]-ATP	Complete	171
4[d]	[γ-^{32}P]-ATP	Complete	0
5	[^{14}C-8]-ATP	Complete	969
6	[^{14}C-U]-ATP	Complete	1002

[a] The reaction mixtures were the same as described under "Assay for CoA-SPC Activity," except the radioactive marker is ATP.

[b] Specific activity represents net specific activity. Identical reaction mixtures stopped at 0-time incubation were used as controls for the assay. Specific radioactivity was determined using 2.5 μmoles of the following: [α^{32}P]-ATP (117,000 c.p.m.), [$\beta\gamma$-^{32}P]-ATP (117,300 c.p.m.), [γ-^{32}P]-ATP (117,450 c.p.m.), [^{14}C-8]-ATP (115,400 c.p.m.) and [^{14}C-U]-ATP (116,000 c.p.m.) as radioactive markers.

[c] CoA-SPC with active dephospho-CoA kinase activity.

[d] CoA-SPC with heat inactivated dephospho-CoA kinase activity (Materials and Methods).

the γ-P group of ATP was also released, but the α-, β-P groups remained intact. Only when the CoA-SPC had dephospho-CoA kinase activity, which catalyzed the phosphorylation of dephospho-CoA, was the γ-P group involved. This suggested that some of the γ-P group of ATP was involved in the phosphorylation of dephospho-CoA to form CoA. The level of bound radioactivity in Experiment 3 apparently represented CoA-SPC-bound CoA.

Role of D-Pantothenic acid and L-cysteine in the formation of CoA bound intermediates

Table 5 shows the results of a study designed to determine the involvement of D-pantothenic acid and L-cysteine in the formation of CoA-SPC bound intermediates. [^{35}S]-L-Cysteine was the radioactive marker used in the reaction mixtures of Experiments 1 and 2. As shown, CoA-SPC-bound radioactivity was not present when D-pantothenic acid was omitted from the reaction mixture (Experiment 1). When D-pantothenic acid was added (Experiment 2), CoA-SPC was radioactively labeled. In Experiments 3 and 4, [^{14}C-1]-D-pantothenic acid was the radioactive marker instead of [^{35}S]-L-cysteine. The absence or presence of L-cysteine in the reaction mixture has little or no influence on the formation of radioactively labeled CoA-SPC. The data in Table 5 indicate D-pantothenic acid and ATP must be components of the reaction mixture for CoA-SPC to be radioactively labeled by [^{35}S]-L-cysteine. In contrast, [^{14}C-1]-D-pantothenic acid in the presence of ATP functioned independently of L-cysteine in the radioactive labeling of CoA-SPC.

Sequence of substrate interaction with CoA-SPC

The experimental data shown in Table 6 provided additional information regarding the function of ATP, D-pantothenic acid and L-cysteine as substrates for CoA-SPC. Experiments 1–3, utilize [^{14}C-U]-ATP as the radioactive marker. The presence or absence of L-cysteine in the reaction mixture (Experiments 1,2) had little or no influence on the extent of radioactivity bound to CoA-SPC. The omission of D-pantothenic acid (Experiment 3), however, resulted in the absence of bound radioactivity (also see Experiments 1,2, Table 4). From other studies (see Table 4) it was known that the γ-P group of ATP is hydrolyzed at the time of the reaction of D-pantothenic acid with ATP; therefore, the bound radioactive component would be ADP and not ATP.

The radioactive marker in Experiments 4–6 of Table 6 was [^{14}C-1]-D-pantothenic acid. In this case, the addition or omission of L-cysteine from the reaction mixtures (Experiments 4,5) did not influence the level of radioactivity bound to CoA-SPC. The omission of ATP, however, (Experiment 6) resulted in the absence of CoA-SPC-bound radioactivity.

The radioactive marker in Experiments 7–9 of Table 6 is [^{35}S]-L-cysteine. Experiment 7 shows that the presence of ATP and D-pantothenic acid are required for [^{35}S]-L-cysteine to bind to the CoA-SPC. The omission of either D-pantothenic acid (Experiment 8) or ATP (Experiment 9) resulted in the absence of CoA-SPC-bound radioactivity. Therefore, it would appear that ATP and D-pantothenic acid interacted to form 5′-ADP-4′-pantothenic acid.

Table 5
Reaction characteristics of cysteine and pantothenic acid with CoA-SPC

Experiment no.	Reaction mixture[a] Radioactive component	Omission	nmoles/h/mg[b] protein
1	[^{35}S]-L-Cysteine	D-Pantothenic acid	0
2	[^{35}S]-L-Cysteine		1011
3	[^{14}C-1]-D-Pantothenic acid	L-Cysteine	994
4	[^{14}C-1]-D-Pantothenic acid		999

[a] D-Pantothenic acid was omitted from Experiment 1, and L-cysteine was omitted from Experiment 3, otherwise the procedure followed was the same as described under "Assay for CoA-SPC Activity."

[b] Specific activity represents net specific activity. Reaction mixtures minus ATP were used as controls for the assay. Specific activity was determined using 0.5 μmole [^{35}S]-L-cysteine (23,110 c.p.m.) and 0.6 μmole [^{14}C-1]-D-pantothenic acid (28,000 c.p.m.) as radioactive markers.

Table 6
CoA-SPC requirements for each substrate

Experiment no.	Radioactive label	Assay system[a]	nmoles/h/mg[b] protein
1	[^{14}C-U]-ATP	Complete	983
2	[^{14}C-U]-ATP	-Cysteine	979
3	[^{14}C-U]-ATP	-Pantothenic Acid	0
4	[^{14}C-1]-D-Pantothenic Acid	Complete	987
5	[^{14}C-1]-D-Pantothenic Acid	-Cysteine	939
6	[^{14}C-1]-D-Pantothenic Acid	-ATP	0
7	[^{35}S]-L-Cysteine	Complete	997
8	[^{35}S]-L-Cysteine	-Pantothenic Acid	0
9	[^{35}S]-L-Cysteine	-ATP	0

[a] Conditions for this study were the same as described under "Assay for CoA-SPC Activity," except the reaction mixtures of certain experiments were modified as described above.
[b] Specific activity represents net specific activity. Reactions stopped at 0-time incubation were used as controls. Specific activity was determined using 2.5 μmoles [^{14}C-U]-ATP (116,000 c.p.m.), 0.6 μmole [^{14}C-1]-D-pantothenic acid (28,000 c.p.m.) and 0.5 μmole [^{35}S]-L-cysteine (23,110 c.p.m.) as radioactive markers.

The 5′-ADP-4′-pantothenic acid then reacted with [^{35}S]-L-cysteine to synthesize either dephospho-CoA or 5′-ADP-4′-pantothenyl-cysteine. Since CoA was shown to be the reaction product and dephospho-CoA was the immediate precursor, it would appear that the intermediate formed in the absence of L-cysteine was most likely 5′-ADP-4′-pantothenic acid. Additional support for this proposal was provided by the data presented in Table 4 which indicate that the γ-P group of ATP was released during the course of the reaction between ATP and pantothenic acid. Based on the information accumulated, the intermediate formed in the presence of L-cysteine with the proposed 5′-ADP-4′-pantothenic acid appeared to be either 5′-ADP-4′-pantothenylcysteine or dephospho-CoA. In order to elucidate the answer to this question, the following study was conducted.

Cysteine decarboxylase activity
Evidence for the decarboxylation of L-cysteine is shown in Table 7. In this series of experiments, [^{14}C-U]-L-cysteine and [^{14}C-1]-L-cysteine were used as the radioactive tracers.

All CO_2 evolved during the incubation period was collected as $BaCO_3$ as described under "Assay for Decarboxylase Activity." For experiments in which [^{14}C-U]-L-cysteine was the radioactive substrate, radioactivity was recovered in both the protein precipitate, which contained the CoA-SPC (see Assay for CoA-SPC-Bound Intermediates), and the [^{14}C]-CO_2 evolved was recovered as [^{14}C]-$BaCO_3$. Omission of either D-pantothenic acid or ATP from the reaction mixtures resulted in the absence of [^{14}C]-$BaCO_3$ being formed. Similar experiments in which [^{14}C-1]-L-cysteine was used as the radioactive marker in place of [^{14}C-U]-L-cysteine, radioactivity was present only as [^{14}C]-$BaCO_3$; no radioactivity was detected in the protein precipitate. Again, no radioactivity was detected in either the protein precipitate or as [^{14}C]-$BaCO_3$ when ATP or pantothenic acid was omitted from the reaction mixture. This supported the proposal that L-cysteine was decarboxylated by CoA-SPC only when all three substrates are present in the reaction mixture. This suggested the proposed intermediate 5′-ADP-4′-pantothenic acid was synthesized prior to the reaction, which resulted in the decarboxylation of L-cysteine.

Also shown in Table 7 are experiments in which [^{35}S]-L-cysteine and [^{35}S]-cysteamine were used as radioactive markers. These experiments were included to show that decarboxylation of L-cysteine did not take place prior to its observed binding to CoA-SPC. L-Cysteine apparently was decarboxylated during or immediately following its involvement in the synthesis of dephospho-CoA. Therefore, [^{35}S]-L-cysteine, not [^{35}S]-cysteamine, proved to be the substrate for CoA-SPC.

Table 7
Cysteine decarboxylase activity of CoA-SPC

Reaction mixture[a]		Specific activity[b]	
Radioactive tracer	Component omitted	nmoles/h/mg protein	nmoles/h $[^{14}C]$-CO_2 recovered
$[^{14}C$-U]-L-Cysteine		623	401
$[^{14}C$-U]-L-Cysteine	D-Pantothenic Acid	0	0
$[^{14}C$-U]-L-Cysteine	ATP	0	0
$[^{14}C$-1]-L-Cysteine		0	973
$[^{14}C$-1]-L-Cysteine	D-Pantothenic Acid	0	0
$[^{14}C$-1]-L-Cysteine	ATP	0	0
$[^{35}S]$-L-Cysteine		1002	0
$[^{35}S]$-Cysteamine		0	0

[a] Conditions for the measurement of cysteine decarboxylase activity are given under "Decarboxylation," in "Materials and Methods." Reaction mixtures were modified as indicated.

[b] Specific activity represents net specific activity. Reactions stopped at 0-time incubation were used as controls for the assay. Specific activity was determined using 0.5 μmole $[^{14}C$-U]-L-cysteine (23,500 c.p.m.), 0.5 μmole $[^{14}C$-1]-L-cysteine (23,200 c.p.m.), 0.5 μmole $[^{35}S]$-L-cysteine (23,110 c.p.m.), and 0.5 μmole $[^{35}S]$-cysteamine (24,000 c.p.m.) as radioactive markers. Good stoichiometry was obtained between CoA-SPC-bound radioactivity and $[^{14}C]$-CO_2 recovered.

Dephospho-CoA kinase activity

The dephospho-CoA kinase activity of CoA-SPC appeared to be the most heat sensitive of the known enzymic activities of this multienzyme complex. The dephospho-CoA kinase activity was inactivated within a few days when stored at −20°. Preincubation of CoA-SPC at 36° for 3 min or the addition of CoA-SPC to a reaction mixture containing 0.02% deoxycholate inactivated the dephospho-CoA kinase activity of the multienzyme complex without any apparent effect on the other enzyme activities. Concentrations of deoxycholate of 0.01% caused approximately 50% inactivation of the kinase activity. The kinase activity, however, was shown to be stable for more than a year when the CoA-SPC was stored dry at room temperature or in solution at −70°.

The presence or absence of dephospho-CoA kinase activity of CoA-SPC was monitored as described in the following experiments. Figure 8 shows the time course of $[^{35}S]$-L-cysteine binding to CoA-SPC with dephospho-CoA kinase activity (– – –), after preincubation for 3 min at 36° (–·–) and in the presence of 0.01% deoxycholate (—). Three different patterns of $[^{35}S]$-binding were observed. At 2 min intervals, a 1 ml sample of the reaction mixture was

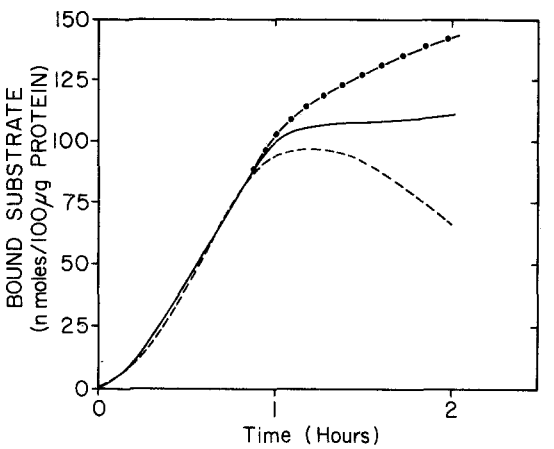

Fig. 8. Time course patterns of CoA-SPC-bound radioactivity as a method of assessing dephospho-CoA kinase activity. Separate reaction mixtures of 100 ml each were set up according to the procedure described under "Assay for CoA-SPC Activity." The only difference in these three reaction mixtures was in the CoA-SPC added. As shown, (– – –) represents CoA-SPC with dephospho-CoA kinase activity; (–·–) represents CoA-SPC after preincubation for 3 min at 36° to destroy dephospho-CoA kinase activity and (—) represents CoA-SPC exposed to 0.01% deoxycholate. At 2-min intervals, a 1 ml sample of each was removed and assayed according to the procedure described under "Assay for CoA-SPC Activity." CoA-SPC with fully active dephospho-CoA kinase activity showed a characteristic decrease in the level of CoA-SPC-bound radioactivity during the second h of incubation.

Table 8
Inhibitory effects of certain compounds on the catalytic activity of CoA-SPC

Compounds added[a]	Percent inhibition[b]		
	[^{14}C-1]-D-Pantothenic Acid	[^{14}C-U]-ATP	[^{35}S]-L-Cysteine
Pantetheine	95	95	90
4′-P-Pantetheine	90	90	95
CoA	90	90	90
Dephospho-CoA	90	90	85
DL-Pantoyl taurine	65	65	60
D-Pantothenyl alcohol	73	70	70
Cysteamine	0	0	50
S-Carbamyl-L-cysteine	0	0	95
S-Allyl-L-cysteine	0	0	75
S-Carboxymethyl-L-cysteine	0	0	65
S-Ethyl-L-cysteine	0	0	75
S-Methyl-L-cysteine	0	0	75
N-Acetyl-L-cysteine	0	0	65
L-Pantothenic acid	0	0	0
D-Cysteine	0	0	0
Dithiothreitol	0	0	0
Mercaptoethanol	0	0	0

[a] The concentration of compounds added to the reaction mixtures was 0.20 mM. Otherwise, the reaction mixtures were the same as described under "Assay for CoA-SPC Activity."
[b] Percent inhibition is based on the level of binding of each of the three radioactive substrates in the presence and absence of a particular added compound. Radioactive substrates used were: 0.6 μmole [^{14}C-1]-D-pantothenic acid (28,000 c.p.m.), 2.5 μmoles [^{14}C-U]-ATP (116,000 c.p.m.) and 0.5 μmole [^{35}S]-L-cysteine (23,000 c.p.m.).

Table 9
Measurements of dephospho-CoA and CoA in reaction mixtures containing CoA-SPC with different levels of dephospho-CoA kinase activity

CoA-SPC	Protein extract dephospho-CoA	Filtrate CoA
	μg/ml	μg/ml
Authentic CoA (180 μg/ml)	165	163
Authentic Dephospho-CoA (193 μg/ml)	149	2
Untreated	62	299
Preincubated	150	0
Deoxycholate treated	91	140

The remainder of the reaction mixtures used to obtain the time course patterns shown in Figure 8 were used for this study. After the 120-min incubation period, the reaction mixtures were heated to 70° for 5 min to inactivate CoA-SPC. The denatured protein and liquid portion were separated by filtration using Whatman 3 MM paper discs. The protein recovered was treated with alkali as described under "Synthesis and Recovery of CoA-SPC-Bound Intermediates." The filtrates were processed as described under "Recovery of CoA." CoA and dephospho-CoA content of the protein extracts and filtrates were determined by the PTA method described under "Determination of CoA." The deoxycholate concentration used was 0.01%. Authentic CoA (90% pure), and authentic dephospho-CoA (90% pure) were used as controls for the PTA assay. The dephospho-CoA contained approximately 1% CoA.

removed from each of the 3 flasks, and the samples were processed as described under "Assay for CoA-SPC Activity." After the 120 min incubation period, the remaining 40 ml of each reaction mixture was treated at 70° for 5 min to stop the reaction and the protein precipitates and filtrates were separated by filtration using Whatman 3 MM filter paper discs. The protein precipitates collected on the filter discs were exposed to alkali as described under "Synthesis and Recovery of CoA-SPC-Bound Intermediates." The filtrates were treated according to the procedure outlined under "Recovery of CoA."

As shown in Table 9, the greatest amount of CoA and the least amount of dephospho-CoA were found in the reaction mixture containing untreated CoA-SPC. No CoA was detected when CoA-SPC, which had been preincubated for 3 min at 36° to destroy dephospho-CoA kinase activity, was used in the reaction. However, the highest level of dephospho-CoA was present in the alkaline hydrolysate of this reaction mixture. Deoxycholate (0.01%) treated CoA-SPC resulted in an intermediate level of both CoA and dephospho-CoA synthesis.

Additional studies concerned with dephospho-CoA kinase activity of CoA-SPC are shown in Table 10. Table 10 shows the results of experiments in which CoA-SPC with and without dephospho-CoA kinase activity are added to reaction mixtures containing [γ^{32}P]-ATP as the radioactive marker. The assay procedure is the same as described under "Assay for CoA-SPC Activity," except the volume of each reaction mixture was increased to 100 ml. As shown in Table 10, the reaction mixture in which CoA-SPC without dephospho-CoA kinase activity was used as the enzyme source indicated no radioactivity was bound to CoA-SPC and no CoA could be detected. In contrast, in the reaction mixture containing CoA-SPC with dephospho-CoA kinase activity, some radioactivity was bound to CoA-SPC, but most of the radioactivity was incorporated into the CoA recovered. The radioactively labeled CoA recovered from the reaction mixture was treated with potato acid phosphatase (Eq 3.1.3.2) according to the method of BREMER, *et al.*[22] Descending paper chromatography in developing solvent 4 ("Materials and Methods") after acid phosphatase treatment showed the

Table 10
Phosphorylation of dephospho-CoA in the presence of CoA-SPC with and without dephospho-CoA kinase activity

	Protein Precipitate		Filtrate	
CoA-SPC	[^{32}P]-Bound Radioactivity	CoA	[^{32}P]-CoA	CoA
	nmoles/ml	μg/ml	nmoles/ml	μg/ml
Untreated	20.1	15.4	301.0	231.5
Preincubated	0	0	0	0

The composition of the reaction mixtures were the same as described under "Assay for CoA-SPC Activity". The volume was increased to 100 ml and either untreated CoA-SPC or CoA-SPC preincubated at 36° for 3 min to inactivate dephospho-CoA kinase activity was used as the source of catalytic activity.

Each reaction mixture contained 2.5 μmoles [γ-^{32}P]-ATP (117,450 c.p.m.) per ml as the radioactive tracer. [^{32}P]-bound radioactivity was measured as described under "Assay for CoA-SPC Activity." [^{32}P]-CoA activity was measured by spotting 0.1 ml of the recovered CoA on paper discs and counting as previously described[11]. The CoA-SPC-bound CoA was released by alkaline treatment of the protein precipitate[12]. CoA present in the filtrate was recovered as described under "Recovery of CoA." CoA content of the protein extracts and filtrates were determined by the PTA method described under "Determination of CoA."

appearance of [^{32}P]-orthophosphate, and the disappearance of [^{32}P]-labeled CoA.

It would appear, based on the data presented, that the most plausible hypothesis for the interaction of the three substrates with CoA-SPC in the course of CoA synthesis is as follows (Fig. 9): ATP and D-pantothenic acid bind to CoA-SPC. The initial reaction is between the β-phosphorus group of ATP and the 4′-hydroxyl group of pantothenic acid resulting in the formation of CoA-SPC-bound 5′-ADP-4′-pantothenic acid. The α-amino group of L-cysteine then reacts with the carboxyl group of the pantothenic acid moiety of 5′-ADP-4′-pantothenic acid. At that time, cysteine is decarboxylated forming CoA-SPC-bound dephospho CoA (Fig. 9), which is subsequently phosphorylated and released from the complex as CoA (4-10).

Other evidence which tends to support this hypothesis as being correct is presented under "Identification of CoA-SPC-Bound Intermediates."

Identification of CoA-SPC-bound intermediates

Reaction mixtures identical to those listed in

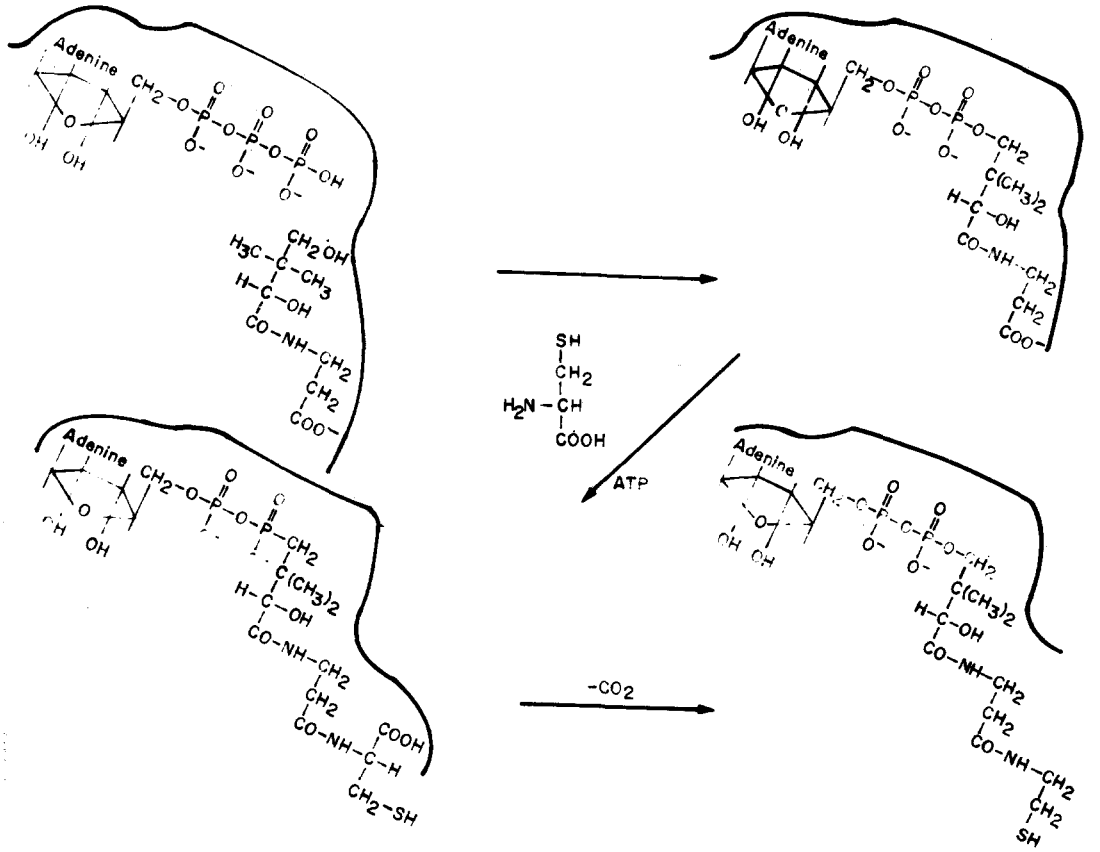

Fig. 9. Proposed reaction sequence for the syntheses of dephospho-CoA by the CoA-SPC.

Experiments 1, 2, 4, 5 and 7 of Table 6 were used to study CoA-SPC-bound intermediates, except the volume of each was increased to 100 ml. Following incubation, the reaction mixtures were treated with alkali and processed as described under "Synthesis and Recovery of CoA-SPC-Bound Intermediates," in "Materials and Methods."

The alkaline treated extracts were chromatographed using the same four developing systems used to identify CoA (see Table 3 for R_f values of reference compounds).

The extracts from Experiments 2 and 5, which contained [^{14}C-U]-ATP and [^{14}C-1]-D-pantothenic acid, respectively, showed a radioactive area on the chromatograms in each of the four developing solvent systems, R_f 0.33, 0.44, 0.35 and 0.63, respectively, believed to be 5′-ADP-4′-pantothenic acid (see Table 3). The 5′-ADP-4′-pantothenic acid would be an expected intermediate according to the hypothesis presented (Fig. 9) for CoA-SPC synthesis of CoA.

Chromatography of the alkaline treated extracts from Experiments 1, 4 and 7 showed radioactive areas on the chromatograms developed in each of the four systems with R_f values identical to those of authentic dephospho-CoA and CoA.

Reaction mixtures of Experiments 4 and 7 also showed low levels of radioactivity, which corresponded to the R_f of authentic 4′-phosphopantetheine and pantetheine. These compounds might be expected if some of the dephospho-CoA and CoA was hydrolyzed by the alkaline treatment. Experiment 1, which utilized [^{14}C-U]-ATP as the radioactive tracer, contained additional radioactive components in the areas of the chromatograms corresponding to the R_f values for authentic 5′-AMP, 5′-ADP and 3′,5′-ADP. These three nucleotides could have been present if a portion of the synthesized

dephospho-CoA and CoA underwent hydrolysis.

Effect of reaction products and related compounds on CoA-SPC activity

Compounds with structures related either to the proposed reaction products or L-cysteine or D-pantothenic acid were tested for their inhibitory effect on CoA-SPC activity. As shown in Table 8, pantetheine, 4′-phosphopantetheine, CoA, dephospho-CoA, DL-pantoyl-taurine and D-pantothenyl alcohol inhibited the formation of the proposed intermediate 5′-ADP-4′-pantothenic acid. Furthermore, as shown in Table 8, the reaction with L-cysteine was inhibited, as would be expected, because the proposed intermediate 5′-ADP-4′-pantothenic acid would not have been available to react with the α-amino group of L-cysteine.

Compounds structurally related to L-cysteine, such as cysteamine, S-carbamyl-L-cysteine, S-allyl-L-cysteine, S-carboxymethyl-L-cysteine, S-ethyl-L-cysteine, S-methyl-L-cysteine and N-acetyl-L-cysteine, specifically inhibited the reaction between cysteine and 5′-ADP-4′-pantothenic acid, but did not interfere with the formation of 5′-ADP-4′-pantothenic acid. Neither L-pantothenic acid nor D-cysteine interfered with the formation of dephospho-CoA or CoA, indicating that only D-pantothenic acid and L-cysteine along with ATP served as substrates for the synthesis of dephospho-CoA and CoA by the CoA-SPC. Also, dithiothreitol and mercaptoethanol did not inhibit the catalytic activity of CoA-SPC.

Evidence for the multienzyme complex of CoA-SPC

The multienzyme complex nature of CoA-SPC was supported throughout this study. For example, as shown in Table 2, a progressive and corresponding increase in catalytic activity for each of the three substrates was observed as CoA-SPC was solubilized. Also, Sephadex G-200 chromatography and polyacrylamide gel electrophoresis studies indicated only one major band of catalytic activity regardless of the substrate used as the radioactive marker. Furthermore, extraction of CoA-SPC from polyacrylamide gels showed that catalytic activity was associated with only one major protein band (Fig. 1). In addition, the optimum temperature (Fig. 5) and optimum pH (Fig. 7) were the same regardless of the catalytic activity being measured. Therefore, all accumulated evidence indicated that CoA-SPC is a homogeneous multienzyme complex.

Summary

A multienzyme complex contained in *S. cerevisiae*, named coenzyme A-synthesizing protein complex (CoA-SPC), utilized the substrates ATP, D-pantothenic acid and L-cysteine for the synthesis of CoA. The CoA-SPC was initially insoluble in the crude yeast cell lysate and could be separated from most of the soluble cell components by centrifugation. Mechanical stirring of either the crude yeast cell lysate or the insoluble fraction, which was obtained by centrifugation of the yeast cell lysate at $105{,}000 \times g$ for 20 min, facilitated the gradual solubilization of the CoA-SPC. The CoA-SPC was purified 229-fold. Polyacrylamide gel electrophoresis of the purified CoA-SPC indicated one major protein band and several minor bands. The major protein band contained all of the CoA-SPC activity. The molecular weight of CoA-SPC, according to chromatography on Sephadex G-200, had been determined to be in excess of 200,000. Although proteins of rather large single polypeptide chains exist, most proteins with molecular weights in excess of 50,000 are composed of subunits. SDS-polyacrylamide gel electrophoresis of CoA-SPC indicated that this multienzyme complex was probably composed of several subunits which range in molecular weights between 15,000 and 25,000.

L-Cysteine, D-pantothenic acid and ATP are the substrates for CoA-SPC; D-cysteine and L-pantothenic acid did not function in this capacity. The proposed mechanism of synthesis of CoA by this multienzyme complex is shown in Figure 9. The initial reaction was believed to be between the β-phosphorus group of ATP and the 4′-hydroxyl group of pantothenic acid resulting in the formation of the proposed intermediate CoA-SPC-bound 5′-ADP-4′-pantothenic acid. During the course of this reaction, the γ-P of ATP was released into the reaction medium. Cysteine then reacted through its α-amino group with the carboxyl group of the pantothenic acid moiety of 5′-ADP-4′-pantothenic acid. At the time of reaction,

cysteine was decarboxylated forming CoA-SPC-bound dephospho-CoA. Dephospho-CoA was subsequently phosphorylated and released as CoA. The data in Table 8, which showed the inhibition of CoA-SPC activity by proposed reaction products and compounds with structures related to the reaction products or L-cysteine or D-pantothenic acid, also supported the proposed reaction sequence.

Alkali treatment of reaction mixtures containing CoA-SPC that had inactivated dephospho-CoA kinase activity yielded only dephospho-CoA and a compound believed to be 5′-ADP-4′-pantothenic acid. Dephospho-CoA was shown to be the terminal CoA-SPC-bound intermediate, and 5′-ADP-4′-pantothenic acid appeared to be the other intermediate. Based on the accumulated information, it would appear that phosphorylation of CoA-SPC-bound dephospho-CoA by the dephospho-CoA kinase reaction to form CoA may contribute to its release from this multienzyme complex.

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