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CHEMICAL SYNTHESIS OF COENZYME A ANALOGS OF A MODIFIED CYSTEAMINE MOIETY[1,2]

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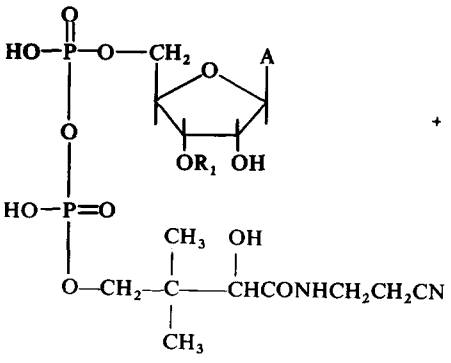
Abstract—Some coenzyme A analogs, such as α-methyl-(IVc), β-methyl-(IVd), and α-carboxy-coenzyme A (IVe), have been synthesized from the nitrile compound (Ib) and cysteamine derivatives (IIc–e) *via* the respective thiazoline intermediates (IIIc–e). Homo-coenzyme A (XII) has been synthesized by a combination of the methods of Moffatt and Khorana, for the formation of pyrophosphate, and Michelson, for 2′, 3′-cyclic phosphate fission.

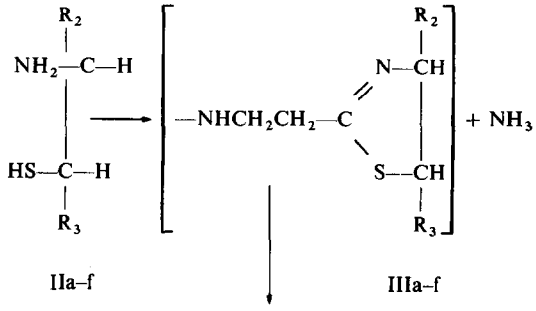
ALTHOUGH coenzyme A (CoA) participates widely in metabolic pathways, studies on the reaction mechanism of CoA-dependent reactions are limited when compared with those on other coenzymes. This should be remedied by the successful total syntheses of CoA[3–5] and our fourth one *via* the thiazoline intermediate[6] (Ia, b → IIIa, b → IVa, b in Chart 1). In addition, as the phosphotransacetylase of *Escherichia coli* B has been purified and its properties examined in detail,[7,8] CoA analogs have been synthesized in order to obtain an insight into the structural specificity of CoA for transacetylation. The cysteamine moiety of CoA was first modified in order to apply the thiazoline method[6,9] for the preparation of the desired compounds. Prior to this work, the syntheses[10] and microbiological responses[11] of panteth(e)ine analogs have been described.

In the syntheses of α-methyl-CoA (IVc) and β-methyl-CoA (IVd) by the thiazoline method, the same starting compound (nitrile) was used as in the case of CoA:[6] P^1-adenosine 3′-phosphate 5′-P^2-D-pantothenonitrile 4′-pyrophosphate (Ib). Compound Ib as a trilithium salt was allowed to react with *rac*-α-methylcysteamine (2-amino-1-propanethiol; IIc)[10,12] until completion of thiazoline ring closure which was checked by disappearance of IR absorption characteristic of the nitrile group. According to the standardized method, the thiazoline intermediate (IIIc) was hydrolyzed to the crude α-methyl-CoA (IVc). The separation and purification described in the Experimental afforded analytically pure α-methyl-CoA (P^1-adenosine 3′-phosphate [5′-P^2-N-D-pantothenoyl-*rac*-2-amino-1-propanethiol 4′-pyrophosphate; IVc) in 32·3% yield. The elution pattern of IVc is shown in Fig. 1. Use of *rac*-β-methylcysteamine (1-amino-2-propanethiol; IId)[10,13] afforded β-methyl-CoA (P^1-adenosine 3′-phosphate 5′-P^2-N-D-pantothenoyl-*rac*-1-amino-2-propanethiol 4′-pyrophosphate; IVd) in 37·2% yield. The presence of 3′-phosphate in both compounds was confirmed by paper chromatography of the alkaline hydrolysate showing a spot of adenosine 3′,5′-diphosphate. Chart 1 shows the reaction sequences.

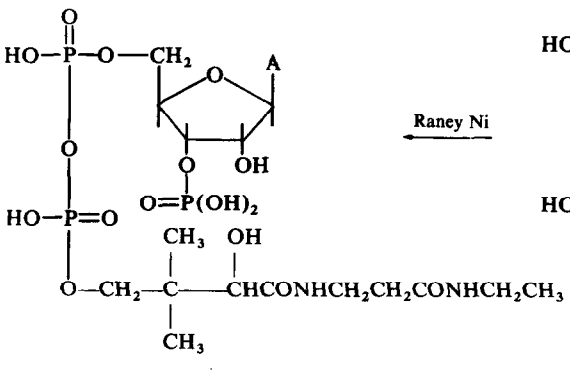
If L-cysteine is used in place of cysteamine, N-D-pantothenoyl-L-cysteine may be prepared from D-pantothenonitrile,[14] and N-D-pantothenoyl-L-cysteine 4′-phosphate from D-pantothenonitrile 4′-phosphate.[15] Consequently, Ib was allowed to react



Ia, b

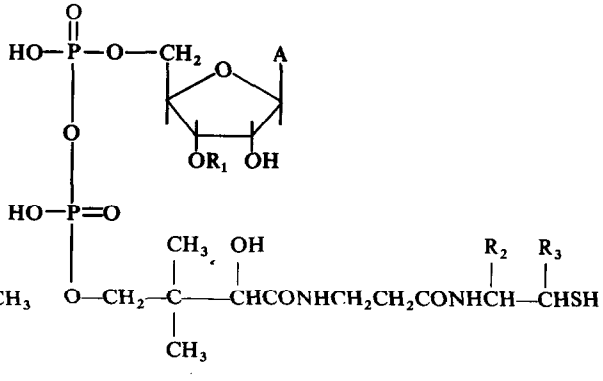


IIa–f IIIa–f



V (Desulfo-CoA)

Raney Ni



IVa (3′-Dephospho-CoA)
b (CoA)
c (α-Methyl-CoA)
d (β-Methyl-CoA)
e (α-Carboxy-CoA)
f (3′-Dephospho-α-carboxy-CoA)

	R_1	R_2	R_3
a	H	H	H
b	$-P(OH)_2$ ‖ O	H	H
c	$-P(OH)_2$ ‖ O	$-CH_3$	H
d	$-P(OH)_2$ ‖ O	H	$-CH_3$
e	$-P(OH)_2$ ‖ O	$-COOH$	H
f	H	$-COOH$	H

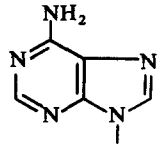
A= 

CHART 1

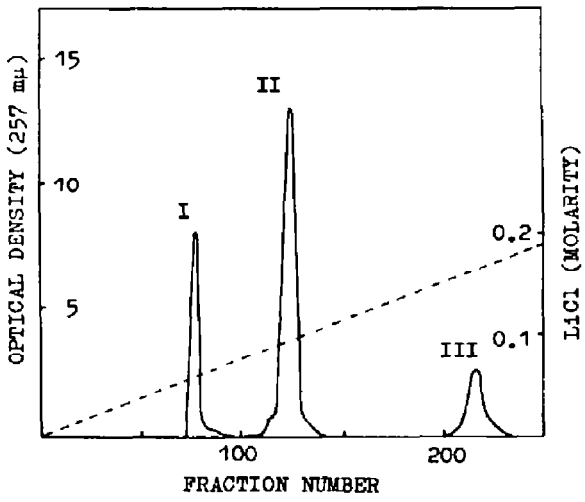


FIG. 1 Chromatography of the reaction products in the preparation of α-methyl-CoA on a DEAE-cellulose (Cl^-) column (2 × 30 cm).
Elution was carried out using a linear salt gradient. The reservoir contained 0·3M LiCl in 0·003N HCl (1 l.) and mixing vessel contained 0·003N HCl (1 l.). Fractions of 5 ml were collected at a flow rate of 0·5 ml per min. Peak I (169 OD units at 257 mμ, 16·1% based on Ib), adenosine 3',5'-diphosphate; Peak II (350 OD units, 33·5%), α-methyl-CoA (SH form); Peak III (100 OD units, 9·5%), α-methyl-CoA (disulfide form). The elution pattern of β-methyl-CoA was almost the same as one shown here.

with L-cysteine monosodium salt (IIe). The subsequent treatment for thiazoline ring opening and purification was the same as described for IVc and IVd. Analytically pure α-carboxy-CoA (P^1-adenosine 3'-phosphate 5'-P^2-N-D-pantothenoyl-L-cysteine 4'-pyrophosphate; IVe) was obtained in 28·9% yield. By using P^1-adenosine 5'-P^2-D-pantothenonitrile 4'-pyrophosphate (Ia), 3'-dephospho-α-carboxy-CoA (P^1-adenosine 5'-P^2-N-D-pantothenoyl-L-cysteine 4'-pyrophosphate; IVf) was obtained in 25·6% yield. The elution pattern of IVe is shown in Fig. 2. The paper chromatograms of acidic hydrolysates of these two compounds revealed the presence of N-D-pantothenoyl-L-cysteine 4'-phosphate.

In addition to the chemical approach, the biosynthesis of CoA[16–19] from D-pantothenic acid in rat liver was examined. Compounds IVe and IVf were very useful as possible substrates in the final stage[18,19] of the biosynthesis.

The CoA analog having homocysteamine in place of cysteamine has been named homo-CoA in accordance with Felder, *et al.*[20] who reported the synthesis of homopantethine. In a previous paper,[10] we reported an extended application of the thiazoline method to the synthesis of homopantetheine in a good yield. As hydrolytic fission of the six-membered intermediate had necessitated a longer reaction time than in the case of thiazoline, and because the pyrophosphate bond is susceptible to hydrolysis by prolonged heating at acidic pH, a combination of the methods, used by Moffatt and Khorana for the formation of pyrophosphate[3] and Michelson for 2',3'-cyclic phosphate fission,[4] were employed as shown in Chart 3.

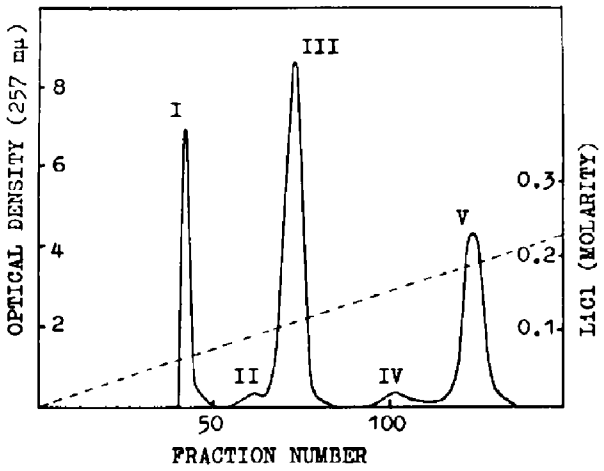


FIG. 2 Chromatography of the reaction products in the preparation of α-carboxy-CoA on a DEAE-cellulose (Cl^-) column (2 × 30 cm).

Elution was carried out using a linear salt gradient. The reservoir contained 0·45M LiCl in 0·003N HCl (1·5 l.) and mixing vessel contained 0·003N HCl (1·5 l.). Fractions of 10 ml were collected at a flow rate of 0·5 ml per minute. Peak I (117 OD units at 257 mμ, 7·1% based on Ib), adenosine 3′,5′-diphosphate; Peaks II and IV, unidentified; Peak III (605 OD units, 36·2%), α-carboxy-CoA (SH form); Peak V (312 OD units, 18·7%), α-carboxy-CoA (disulfide form).

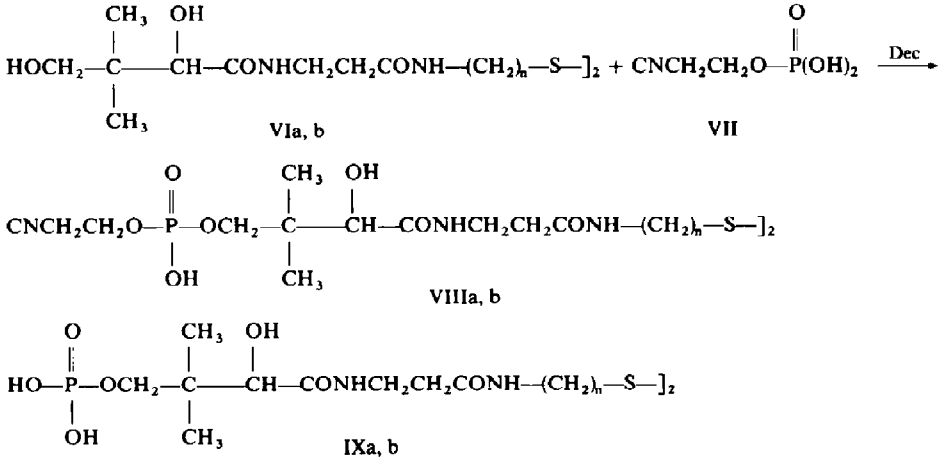


CHART 2

Homopantethine 4′,4″-diphosphate (disulfide form of N-D-pantothenoyl-homocysteamine 4′-phosphate; IXb) was synthesized by phosphorylation of homopantethine[10] with cyanoethyl phosphate.[21] A preliminary test showed that phosphorylation of pantethine (VIa) with cyanoethyl phosphate (VII) followed by mild alkaline hydrolysis gives pantethine 4′,4″-diphosphate (IXa).[15] Therefore, the pantoyl

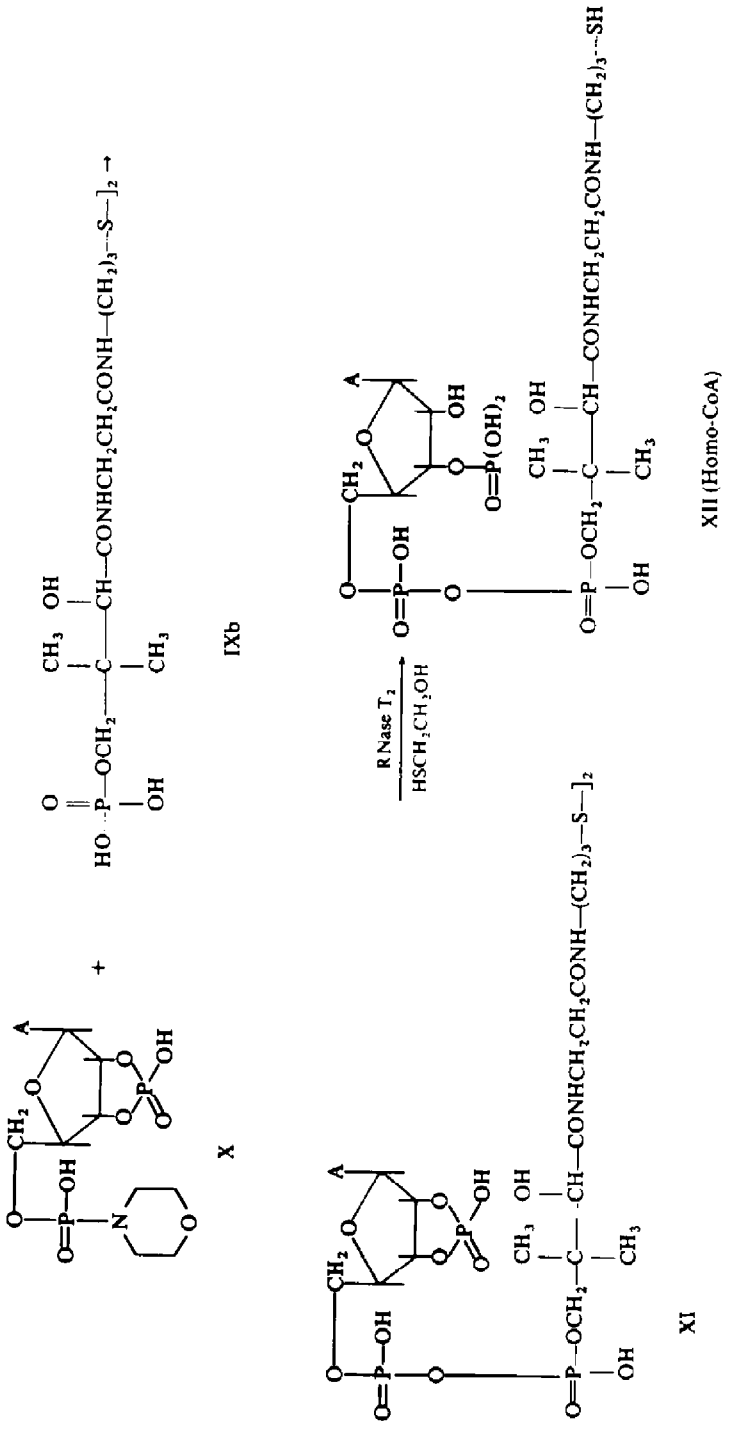


CHART 3

derivative is phosphorylated only at the primary OH group at 4'. Consequently, homopantethine (VIb) was converted to the desired diphosphate (IXb) as shown in Chart 2. The synthesis of homo-CoA (XII) was effected by condensation of homopantethine 4',4''-diphosphate (IXb) with adenosine 2',3'-cyclic phosphate 5'-phosphoromorpholidate (X) followed by selective hydrolysis of the 2',3'-cyclic phosphate and reduction as shown in Chart 3. Analytically pure homo-CoA (XII) was obtained as a trilithium salt in 8·7% yield. The elution pattern of XII is shown in Fig. 3.

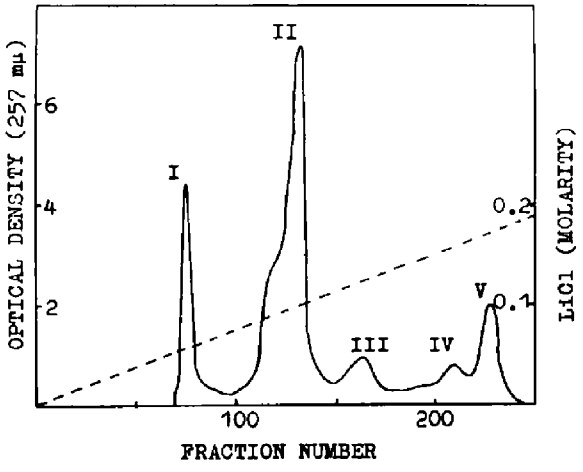


FIG. 3 Chromatography of the reaction products in the preparation of homo-CoA on a DEAE-cellulose column Peak I, adenosine 3',5'-diphosphate; Peak II, homo-CoA (SH form); Peaks III and IV, unidentified; Peak V, homo-CoA (disulfide form)

Chase, *et al.*[22] prepared desulfo-CoA (V) and examined its effects as a CoA-antagonist in various enzymic reactions. According to them, desulfo-CoA has a very strong affinity for the phosphotransacetylase from *Clostridium kluyveri*. As we propose studying the kinetics of transacetylation with the phosphotransacetylase from *E. coli* B[7] using various CoA analogs, desulfo-CoA was prepared for comparison with the data obtained by Chase *et al.*

The assay system specific for CoA according to Stadtman, *et al.*[23] has been standardized using the phosphotransacetylase partially purified from *E. coli* B.[7] The experimental data showed the relationship between the structure of CoA in the cysteamine moiety and the transacetylation by the phosphotransacetylase as shown in Table 1.* The activities of CoA analogs have been published on 3'-dephospho-CoA and iso-CoA,[3] oxy-CoA,[24] and seleno-CoA,[25] in addition to desulfo-CoA[22] using the phosphotransacetylase from *Cl. kluyveri*. All the results indicate that only α-methyl-CoA has a slight CoA activity. In order to compare the CoA analogs as CoA-antagonists, we are now carrying out kinetic studies using the phosphotransacetylase purified from *E. coli* B. The result of this work will be published elsewhere in the near future.

* Assays were performed by T. Suzuki, K. Kameda and Y. Abiko.

TABLE 1. ACTIVITY OF CoA ANALOGS IN THE PHOSPHOTRANSACETYLASE SYSTEM FROM *E. coli* B

	Unit assayed as CoA	
α-CH_3-CoA (IVc) (38 mμmoles)	0·63	0·55
β-CH_3-CoA (IVd) (38 mμmoles)	0·0	0·0
α-COOH-CoA (IVe) (38 mμmoles)	0·0	0·0
Homo-CoA (XII) (36·8 mμmoles)	0·0	0·0
Desulfo-CoA (V) (35·5 mμmoles)	0·0	0·0

CoA analogs were assayed for CoA activity by the phosphotransacetylase method. 1 mμmole of each CoA analog is equivalent to 0·315 unit of CoA.

EXPERIMENTAL

Paper chromatography was carried out by the ascending technique on Toyo Roshi No. 50 paper. The solvent systems used were: solvent A, EtOH—0·5N NH_4OAc (pH 3·8) (5:2); solvent B, EtOH–1N NH_4OAc (pH 7·5) (5:2); solvent C, BuOH–HOAc–H_2O (5:2:3); solvent D, PrOH-conc NH_4OH–H_2O (6:3:1); solvent E, saturated $(NH_4)_2SO_4$—0·1M NH_4OAc (pH 6)-isoPrOH (79:19:2). Paper electrophoresis was carried out on Toyo Roshi No. 50 paper (65 × 5 cm) impregnated with the solvents described below at 800 volt for 2 hr by the technique of Markham and Smith:[26] solvent I, 0·05M triethylammonium bicarbonate (pH 7·5); solvent II, 0·05M NH_4OAc buffer (pH 3·5).

Adenosine derivatives were located on chromatograms with UV lamp. P-containing compounds were located with Hanes and Isherwood spray[27] followed by UV irradiation, and sulfhydryl compounds with ammonia spray after nitroprusside spray and disulfide compounds with nitroprusside spray after KCN spray. The R_f values and relative electrophoretic mobilities of different compounds are given in Table 2. P was determined by the method of Boltz and Mellon.[28] Adenosine was determined by UV absorption in 0·01N HCl using $\varepsilon_{257} = 15{,}000$ as standard value. All evaporations were carried out *in vacuo* below 35°.

General method for the synthesis of CoA *analogs via thiazoline intermediates.* To a soln of the Li salt of Ib[6] (0·07–0·1 mmole) in MeOH (ca. 5 ml) IIc, IId,[29] or monosodium salt of IIe (5–6 equivts to Ib) in MeOH soln was added. The mixture was refluxed in N_2 media for 6–9 hr and concentrated to dryness to give the crude thiazoline. Its IR spectrum has no absorption band of C≡N at 2250 cm^{-1}.

An aqueous soln of the crude thiazoline was adjusted to pH 4·7–5·0 with 0·1N HCl and heated in N_2 media at 55–60° for 2–3·5 hr. After treatment with 2-mercaptoethanol, the mixture was passed through a column of Dowex 50 (H^+) (2 ml). The effluent was adjusted to pH 4·5 with 0·1N LiOH and concentrated to dryness. The residue was dissolved in MeOH (1 ml), and acetone (20 ml) was added to precipitate the crude Li salt of the CoA analog. It was treated again with 50% aqueous 2-mercaptoethanol. The soln was adjusted to pH 6·0 and applied to a column of DEAE-cellulose (Cl^-). The elution was carried out as shown in Figs 1 and 2. The soln containing the desired CoA analog (SH form) was adjusted to pH 4·5 with 0·1N LiOH and evaporated to dryness. The residue was mixed with MeOH (1 ml), and acetone (20 ml) was added to give a white powder, which was purified by repeated precipitation as above until the supernatant was free of chloride ion and dried over P_2O_5 *in vacuo* to yield the Li salt of CoA analog. The fractions containing the SS-form were worked up similarly and treatment of the resulting Li salt with 50% aqueous 2-mercaptoethanol overnight gave an additional crop of the SH-form.

Alkaline hydrolysis (1N NaOH, 100°, 20 min) of the CoA analogs yielded adenosine 3',5'-diphosphate and acid hydrolysis (1N HCl, 100°, 5 min) of IVe yielded pantothenoylcysteine 4'-phosphate.[15] The hydrolysed products were detected by paper chromatography.

In a similar manner, IVf was prepared from Ia.[6] The properties of the products were given below.

(i) α-Methyl-CoA (IVc) was obtained in 32·3% yield: IR ν_{max}^{KBr} cm^{-1}: 3340 (OH, NH), 1660–1646 (amide I), 1538 (amide II) 1245 (PO_2^-), 1125, 1087 (P—O—C, PO_2^-, C–O), 948 (P—O—P). (Found: C, 26·68; H, 5·17; N, 8·96; P, 9·82; adenosine:phosphorus = 1:3·00. $C_{22}H_{35}O_{16}N_7P_3SLi_3 \cdot 10H_2O$ requires: C, 26·97; H, 5·66; N, 10·01; P, 9·49%; adenosine:phosphorus = 1:3·00).

(ii) β-Methyl-CoA (IVd) was obtained in 37·2% yield; IR ν_{max}^{KBr} cm^{-1}: 3370, 1660–1645, 1540, 1240, 1125, 1080, 950. (Found: C, 28·20; H, 4·98; N, 10·87; P, 9·86; adenosine:P = 1:3·02. $C_{22}H_{35}O_{16}N_7P_3SLi_3 \cdot 7H_2O$ requires: C, 28·55; H, 5·34; N, 10·59; P, 10·04%; adenosine:P = 1:3·00).

TABLE 2. PAPER CHROMATOGRAPHY AND PAPER ELECTROPHORESIS OF DIFFERENT COMPOUNDS

Compound	R_f in solvent A	B	C	D	E	Electrophoretic mobility at pH 3·5
CoA (SH)	0·28	0·11				1·0[a]
α-Methyl-CoA (SH)	0·30	0·12				0·92[a]
β-Methyl-CoA (SH)	0·30	0·12				0·92[a]
α-Carboxy-CoA (SH)	0·26	0·05				1·0[a]
α-Carboxy-dephospho-CoA (SH)	0·32	0·23				1·45[b]
Homo-CoA (SH)	0·28	0·13				0·80[a]
Desulfo-CoA	0·30	0·13				
Adenosine 3',5'-diphosphate	0·16	0·01			0·42	
Adenosine 2',5'-diphosphate	0·16	0·01			0·50	
Pantethine 4',4''-diphosphate			0·33	0·27		
Homopantethine 4',4''-diphosphate			0·35	0·32		

[a] Mobility relative to adenosine 2'(3'),5'-diphosphate.
[b] Mobility relative to adenosine 5'-phosphate.

(iii) α-Carboxy-CoA (IVe) was obtained in 28·9% yield; IR ν_{max}^{KBr} cm^{-1}: 3380, 1645, 1615 (COO^-), 1540, 1410(COO^-), 1245, 1125, 1090, 1060, 953. (Found: C, 22·84; H, 6·17; N, 8·33; P, 7·34; adenosine: P = 1:3·06. $C_{22}H_{32}O_{18}N_7P_3SLi_4 \cdot 20H_2O$ requires: C, 22·05; H, 6·07; N, 8·21; P, 7·78%; adenosine: P = 1:3·00).

(iv) 3'-Dephospho-α-carboxy-CoA (IVf) was obtained in 25·6% yield; IR ν_{max}^{KBr} cm^{-1}: 3350, 1643, 1615, 1540, 1410, 1243, 1123, 1085, 1060, 950. (Found: C, 31·80; H, 5·18; P, 7·08; adenosine: P = 1:2·18. $C_{22}H_{32}O_{15}P_2SLi_3 \cdot 5H_2O$ requires: C, 31·48; H, 5·04; P, 7·38%; adenosine: P = 1:2·00).

D-*Pantethine 4',4''-diphosphate* (IXa). A mixture of VIa (277 mg, 0·5 mmole), pyridinium salt of VII (1·2 mmoles) and DCC (248 mg, 1·2 mmoles) in dry pyridine (5 ml) was kept for 22 hr at room temp. Water (5 ml) was added, after 1 hr dicyclohexylurea was filtered off, and the filtrate was evaporated to dryness. An aqueous soln of the residue was neutralized with 0·2M $Ba(OH)_2$ and concentrated to a volume of 3 ml. Addition of EtOH (12 ml) gave a ppt which was removed by filtration, and the filtrate was evaporated to dryness. The residue was treated with 2N NaOH (6 ml) at 0° for 30 min and an aqueous slurry of Amberlite IR 120 (H^+) resin was then added to acidify the soln. The mixture was applied to the top of a column of IR 120 (H^+) resin (10 ml), the column being washed thoroughly with water. The eluate was neutralized with $Ca(OH)_2$ and evaporated to dryness. Paper chromatography showed that the residue contained IXa and traces of the mixed disulfide of pantetheine 4'-phosphate and pantetheine 2',4'-cyclic phosphate (R_f 0·42 in solvent D) and the mixed disulfide of pantetheine and pantetheine 4'-phosphate (R_f 0·53 in solvent D). The crude product was dissolved in water (3 ml) and addition of MeOH (6 ml) gave a ppt (204 mg), which was repeatedly treated with water and MeOH and dried *in vacuo* at room temperature giving the calcium salt of IXa (162 mg, 38·3% yield). $[\alpha]_D^{25}$ +11·5° (c = 2·5, H_2O). Its IR spectrum and R_f values of PPC were identical with those of an authentic sample.[15] (Found: C, 31·12; H, 5·70; N, 6·28. Calc. for $C_{22}H_{40}O_{14}N_4P_2S_2Ca_2 \cdot 3H_2O$: C, 31·27; H, 5·49; N, 6·63%).

Homopantethine 4',4''-diphosphate (IXb). A mixture of VIb[10] (1·66 g, 2·8 mmoles), pyridinium salt of VII (7·84 mmoles) and DCC (1·45 g, 7·02 mmoles) in anhyd pyridine (15 ml) was kept at room temp for 40 hr. Working-up in the same manner as described for IXa gave the crude Ca salt of IXb (635 mg), which was precipitated several times from aqueous soln with MeOH to give a pure sample; IR ν_{max}^{KBr} cm^{-1}: 3400 (OH, NH), 1650 (amide I), 1537 (amide II), 1080 (P—O—C. PO_3^-, C—O), 990 (PO_3^-). $[\alpha]_D^{23}$ + 7·9° (c = 1·0, H_2O). (Found: C, 29·50; H, 5·89; N, 6·48. $C_{24}H_{44}O_{14}N_4P_2S_2Ca_2 \cdot 8H_2O$ requires: C, 29·93; H, 6·28; N, 5·82%).

Homo-coenzyme A (XII). A mixture of the pyridinium salt of IXb (0·3 mmole) and the 4-morpholine N,N'-dicyclohexylcarboxamidinium salt of X[3] (220 mg, 0·2 mmole) was rendered anhydrous by evaporation of added pyridine, dissolved in anhyd pyridine (10 ml) and left overnight at room temp. After removal of the solvent the residue was dissolved in water (3 ml) and the pH was adjusted to pH 4·6 with NH_4OH.

Partially purified ribonuclease T_2^6 (0·6 ml, activity: 378 units) and 0·16M EDTA (0·125 ml) were added, and the mixture was incubated at 37° for 2·5 hr. 2-Mercaptoethanol (0·5 ml) and RNase T_2 (0·6 ml) were added and the soln was incubated again for 1 hr. After being adjusted to pH 6·0 with NH_4OH and treated with 2-mercaptoethanol (4 ml), the mixture was chromatographed on a DEAE-cellulose (Cl^-) column (3 × 45 cm) by a linear salt gradient using 0·003N HCl (2·25 l.) in the mixing vessel and 0·225N LiCl in 0·003N HCl (2·25 l.) in the reservoir. Fractions of each 15 ml were collected every 10 min. The elution curve is shown in Fig. 3. Peak II (1526 OD units, 50·9%) and Peak V (340 OD units, 11·3%) were SH form and disulfide form of homo-CoA, respectively. They were combined, concentrated and passed through a column of Sephadex G-15 (4 × 25 cm) to remove LiCl. Fractions containing nucleotide were pooled, concentrated and treated with 2-mercaptoethanol. The mixture was chromatographed on a DEAE-cellulose column in the same manner as described above. XII (SH form) was eluted in fraction Nos 120–143 and amounted to 526 OD units (17·6%). The fractions were pooled, adjusted to pH with 0·1N LiOH and then worked up in the usual manner to give the Li salt of XII (13·3 mg, 7·0% yield); IR ν_{max}^{KBr} cm^{-1}: 3375, 1640, 1540, 1243, 1124, 1085, 955. (Found: P, 9·85; adenosine: P = 1:3·03. $C_{22}H_{35}O_{16}N_7P_3SLi_3 \cdot 8H_2O$ requires: P, 9·84%; adenosine: P = 1:3·00). Oxidised homo-CoA was eluted in fraction Nos 236–247 (105 OD units, 3·5%), which were worked up in the same way to give the lithium salt of disulfide of XII (3·2 mg, 1·7%). The structure of XII thus obtained was confirmed by alkaline hydrolysis and acid hydrolysis which yielded adenosine 3',5'-diphosphate and homopantetheine 4'-phosphate, respectively.

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REFERENCES

[1] This report represents Part XV of *Investigations on Pantothenic Acid and Its Related Compounds—Chemical Studies* (7); for Part XIV, see Ref. 11.
[2] Preliminary report of this work was published in *Chem. Pharm. Bull. Tokyo* **14**, 681 (1966).
[3] J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.* **81**, 1265 (1959); **83**, 663 (1961).
[4] A. M. Michelson, *Biochim. Biophys. Acta* **50**, 605 (1961); **93**, 71 (1964).
[5] W. Gruber and F. Lynen, *Liebigs Ann.* **659**, 139 (1962).
[6] M. Shimizu, O. Nagase, S. Okada, Y. Hosokawa, H. Tagawa, T. Suzuki and Y. Abiko, *Chem. Pharm. Bull. Tokyo* **13**, 1142 (1965); **15**, 655 (1967).
[7] Y. Abiko, T. Suzuki and M. Shimizu, *J. Biochem. Tokyo* **61**, 10 (1967).
[8] M. Shimizu, T. Suzuki and Y. Abiko, to be published.
[9] M. Shimizu, G. Ohta, O. Nagase, S. Okada and Y. Hosokawa, *Chem. Pharm. Bull. Tokyo* **13**, 180 (1965).
[10] O. Nagase, H. Tagawa and M. Shimizu, *Ibid.* **16** (1968) in press.
[11] Y. Abiko, K. Kameda and M. Shimizu, *Ibid.* **16** (1968) in press.
[12] M. Bōse, *Ber. Dtsch. Chem. Ges* **53**, 2001 (1920).
[13] S. Gabriel and E. Leupold, *Ibid.* **31**, 2838 (1898); W. Mylius, *Ibid.* **49**, 1091 (1916).
[14] G. Ohta, O. Nagase, Y. Hosokawa, H. Tagawa and M. Shimizu, *Chem. Pharm. Bull. Tokyo* **15**, 644 (1967).
[15] O. Nagase, *Ibid.* **15**, 648 (1967).
[16] M. Shimizu and Y. Abiko, *Ibid.* **13**, 189 (1965).
[17] Y. Abiko, *J. Biochem. Tokyo* **61**, 290, 300 (1967).
[18] Y. Abiko, T. Suzuki and M. Shimizu, *Ibid.* **61**, 309 (1967).
[19] T. Suzuki, Y. Abiko and M. Shimizu, *Ibid.* **62**, 642 (1967).
[20] E. Felder, L. Fumagalli and D. Pitré, *Helv. Chim. Acta* **46**, 752 (1963).
[21] G. M. Tener, *J. Am. Chem. Soc.* **83**, 159 (1961).
[22] J. F. A. Chase, B. Middleton and P. K. Tubbs, *Biochim. Biophys. Res. Commun.* **23**, 208 (1966).
[23] E. R. Stadtman and A. Kornberg, *J. Biol. Chem.* **203**, 47 (1953).
[24] T. L. Miller, G. L. Rowley and C. J. Stewart, *J. Am. Chem. Soc.* **88**, 2299 (1966).
[25] W. H. H. Günther and H. G. Mautner, *Ibid.* **87**, 2708 (1965).
[26] R. Markham and J. D. Smith, *Biochem. J.* **53**, 552 (1952).

[27] C. S. Hanes and F. A. Isherwood, *Nature Lond.* **164**, 1107 (1949).
[28] D. F. Boltz and M. G. Mellon, *Analyt. Chem.* **19**, 873 (1947)
[29] S. Gabriel, *Ber. Dtsch. Chem. Ges* **49**, 1110 (1916).