Chem. Pharm. Bull. 15(5) 655~662 (1967)

UDC 577.15/6.074

81. Masao Shimizu, Osamu Nagase, Seizaburo Okada, Yasuhiro Hosokawa, Hiroaki Tagawa, Yasushi Abiko, and Tadao Suzuki: Investigations on Pantothenic Acid and Its Related Compounds. V.*1 Chemical Studies. (4). A Total Synthesis of Coenzyme A via Thiazoline Intermediate.

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Synthesis of coenzyme A using thiazoline (Vb) as intermediate is described. D-Pantothenonitrile (I) was treated with adenosine 2′,3′-cyclic phosphate 5′-phosphoromorpholidate (IIb) in pyridine followed by ribonuclease T_2 to yield P^1-adenosine 3′-phosphate 5′-P^2-D-pantothenonitrile 4′-pyrophosphate (IVb). Condensation of IVb with cysteamine gave thiazoline (Vb), which was then hydrolyzed to afford coenzyme A.

(Received May 14, 1966)

Since its brilliant discovery by Lipmann,[1] coenzyme A has been known to play a central role in metabolism of living cells. The chemical structure of coenzyme A was finally determined by Moffatt and Khorana's chemical synthesis.[2] Chemical studies of this series have successively described the application of the thiazoline method developed by the present authors for the syntheses of some metabolic intermediates of D-pantothenic acid, *i.e.* D-pantetheine,[3] D-pantothenoyl-L-cysteine,[4] D-pantetheine 4′-phosphate and D-pantothenoyl-L-cysteine 4′-phosphate.*1 Interest in the extension of this method led to a total synthesis of coenzyme A inclusive of 3′-dephospho-coenzyme A, the results of which are reported here.

Following the first publication by Moffatt and Khorana, two independent papers on the total synthesis of coenzyme A have so far been reported by Michelson[5] and by Gruber and Lynen.[6] These three procedures involved in principle the condensation step of D-pantetheine or its derivatives with adenosine or its phosphates, though differing in the formation of the pyrophosphate bond and in the introduction of phosphate bond in the 3′-position of adenosine moiety. On the contrary, the thiazoline method is generally characterized by the formation of aletheine moiety in the last step of synthetic route for the desired compounds, using nitrile derivatives and cysteamine. Therefore, the present investigation aimed at the preparation of adequate nitrile derivative and the application of the thiazoline method for it.

As done by the three forerunners, 3′-dephospho-coenzyme A (VIa) was chosen as the first target in the present synthesis. The key nitrile intermediate, P^1-adenosine 5′-P^2-D-pantothenonitrile 4′-pyrophosphate (IIIa), was prepared by condensation of D-pantothenonitrile 4′-phosphate*1 (I) with adenosine 5′-phosphoromorpholidate (IIa) in anhydrous pyridine at room temperature according to Moffatt and Khorana's method.[2] Chromatography on ECTEOLA-cellulose of the reaction mixture gave the elution diagram shown in Fig. 1. From Peak III (79% spectrophotometrically at 257 mμ), the

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1) F. Lipmann: J. Biol. Chem., **160**, 173 (1945); F. Lipmann, N.O. Kaplan: *Ibid.*, **162**, 743 (1946).
2) J.G. Moffat, H.G. Khorana: J. Am. Chem. Soc., **83**, 663 (1961); **81**, 1265 (1959).
3) M. Shimizu, G. Ohta, O. Nagase, S. Okada, Y. Hosokawa: This Bulletin, **13**, 180 (1965).
4) G. Ohta, O. Nagase, Y. Hosokawa, H. Tagawa, M. Shimizu: This Bulletin, **15**, 644 (1967)
5) A.M, Michelson: Biochim. Biophys. Acta, **50**, 605 (1961); **93**, 71 (1964).
6) W. Gruber, F. Lynen: Ann., **659**, 139 (1962).

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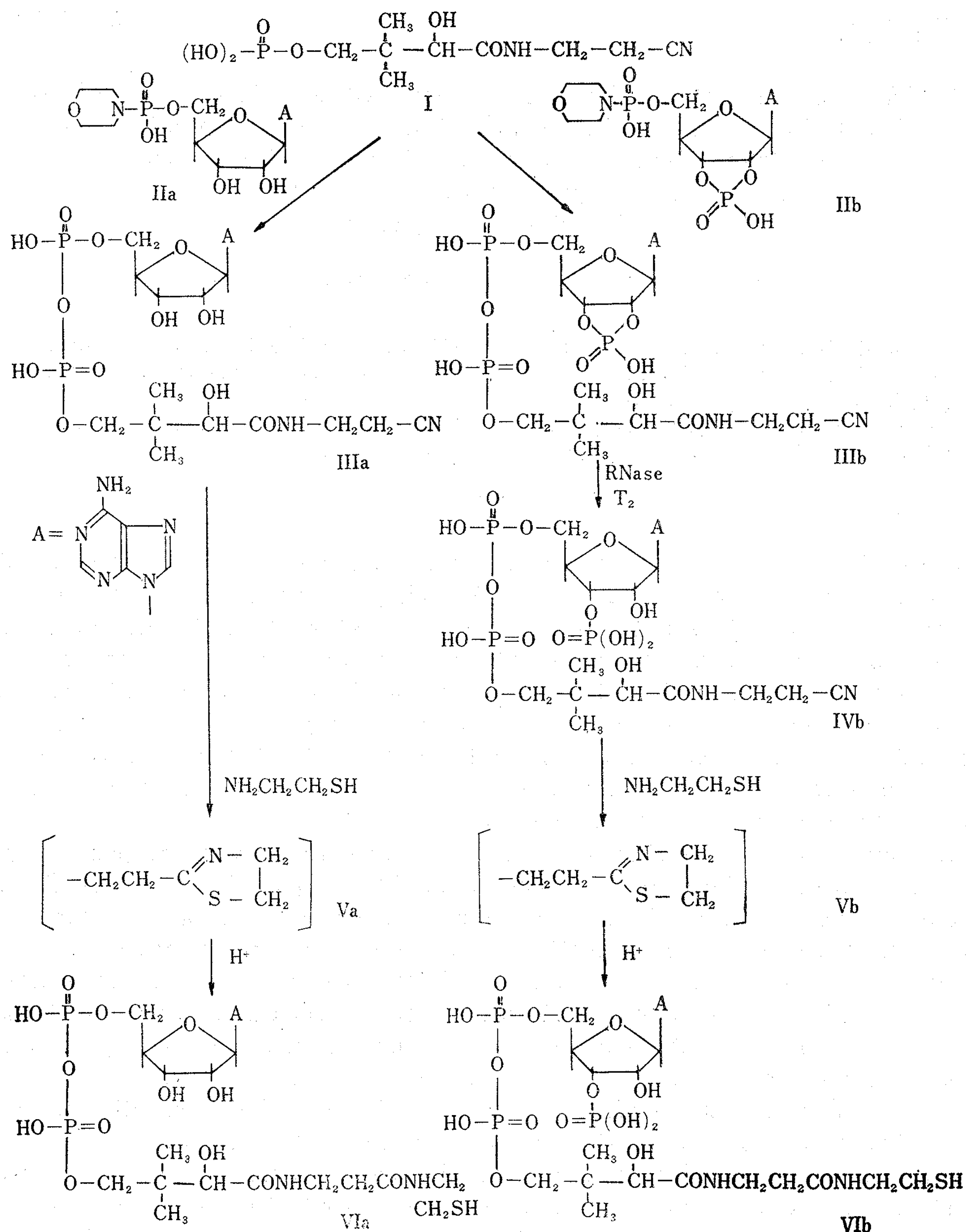

Chart 1.

desired IIIa was obtained as analytically pure dilithium salt in 52.5% yield. Refluxing of IIIa in methanolic solution with 3.3 equivalents of cysteamine afforded the thiazoline intermediate (Va), infrared spectrum of which, though not purified, did not show the absorption band of nitrile. In general, completion of thiazoline ring closure can be checked by disappearance of nitrile band in infrared spectrum and by appearance of thiazoline absorption in ultraviolet spectrum, but in this case the latter could not be used owing to overlap of thiazoline absorption with adenosine absorption. Fission of the thiazoline ring was performed by hydrolysis of crude Va in aqueous solution adjusted to pH 4.7 with hydrochloric acid at 60° for 3 hours. After purification described in the experimental part, the product obtained as dilithium salt in 63% yield was

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chromatographically and electrophoretically identical with the authentic sample of 3′-dephospho-coenzyme A prepared from D-pantetheine 4′-phosphate and IIa by the original Moffatt and Khorana's procedure.[2)]

The difficult problem in the synthesis of coenzyme A itself is the introduction of 3′-phosphate into the adenosine moiety. Acidic fission of 2′,3′-cyclic phosphate employed by Moffatt and Khorana brings about inevitable formation of 2′-phosphate besides 3′-phosphate. In the follow-up of their description, the authors found it very difficult to separate coenzyme A from iso-coenzyme A as described in the experimental part. Ribonuclease T_2[7)] from Takadiastase is well known to give

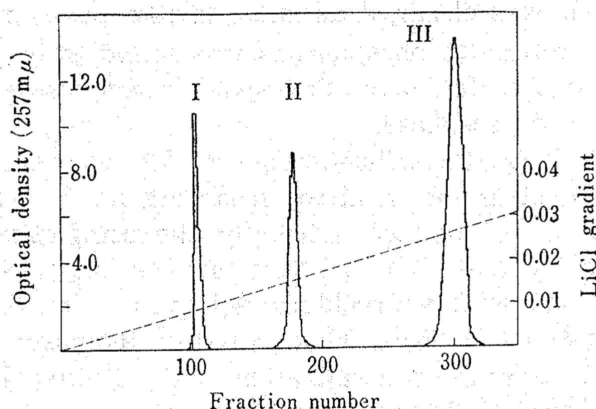

Fig. 1. Ion exchange chromatography of products formed by reaction of adenosine 5′-phosphoromorpholidate with D-pantothenonitrile 4′-phosphate.

For details see experimental text. Peak I, unidentified; Peak II, adenosine 5′-phosphate; Peak III, P^1-adenosine 5′-P^2-D-pantothenonitrile 4′-pyrophosphate.

exclusively 3′-phosphate from 2′,3′-cyclic phosphate of adenosine. Michelson[5)] utilized this property in the synthesis of coenzyme A. In the follow-up described above, the use of T_2 was found to improve both the yield and the quality of coenzyme A. In view of these experiences, the authors employed combination of Moffatt and Khorana's pyrophosphate formation method and Michelson's 2,′3′-cyclic phosphate fission method in the application of the thiazoline method for the synthesis of coenzyme A.

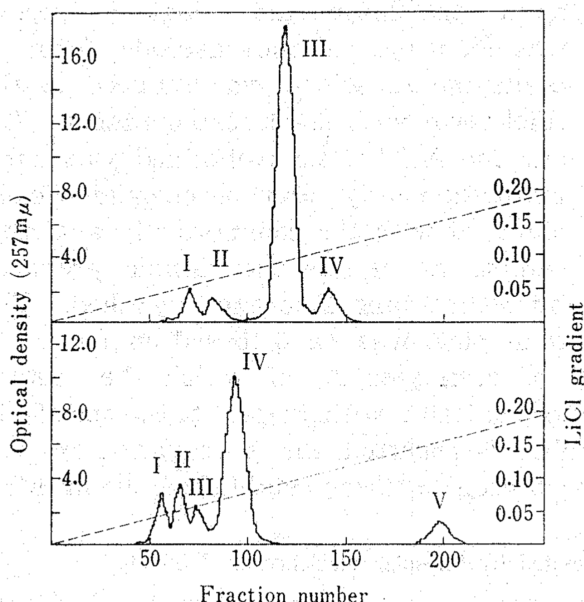

Fig. 2. Chromatography on a DEAE-cellulose column.

Top, products of the reaction of adenosine 2′,3′-cyclic phosphate 5′-phosphoromorpholidate (IIb) with D-pantothenonitrile 4′ phosphate, after RNase digestion. Peak I, adenosine 3′,5′-diphosphate; Peak II, unidentified; Peak III, P^1-adenosine 3′-phosphate 5′-P^2-D-pantothenonitrile 4′-pyrophosphate; Peak IV, unidentified. Bottom, products of the reaction of IIb with D-pantetheine 4′-phosphate, after RNase digestion. Peak I, adenosine 3′,5′-di-phosphate; Peak IV, coenzyme A (SH form); Peak V, coenzyme A (disulfide form).

In accordance with the case of 3′-dephospho-coenzyme A, the key nitrile intermediate, P^1-adenosine 3′-phosphate 5′-P^2-D-pantothenonitrile 4′-pyrophosphate (IIIb), was prepared by the following two steps. D-Pantothenonitrile 4′-phosphate (I) was allowed to react with adenosine 2′,3′-cyclic phosphate 5′-phosphoromorpholidate (IIb) in anhydrous pyridine at room temperature overnight. The crude product (IIIb) obtained by evaporation of the reaction mixture was incubated with partially purified ribonuclease T_2 at 37° for 3.5 hours in aqueous solution adjusted to pH 4.6. Chromatography on ECTEOLA-cellulose of the fission product gave the elution diagram shown in Fig. 2. From Peak III (79% spectrophotometrically at 257 mμ), the desired nitrile

7) M. Naoi-Tada, K. Sato-Asano, F. Egami: J. Biochem. (Tokyo), **46**, 757 (1959); T. Uchida, F. Egami: Progress of Ribonucleic Acid Research, **3**, 59 (1964).

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(IVb) was obtained as analytically pure trilithium salt in 61.8% yield. Its ratio of adenosine to phosphorous was found to be 1:3.06. Paper chromatography of its alkaline hydrolyzate gave the spot of adenosine 3′,5′-diphosphate, but not of the isomeric 2′,5′-diphosphate.

Next, the trilithium salt of IVb was refluxed in methanolic solution with 5 equivalents of cysteamine in nitrogen stream for 7 hours. The infrared spectrum of the reaction mixture indicated indirectly the completion of ring closure. The resultant thiazoline intermediate (Vb), without purification, was hydrolyzed in aqueous solution adjusted to pH 4.7 with hydrochloric acid at 60° for 3.5 hours. The crude coenzyme A (VIb) was obtained in 78.5% yield based on adenosine content after passage through Dowex 50 (H^+) column, neutralization with lithium hydroxide, and reduction with 2-mercaptoethanol, followed by precipitation with acetone from methanolic solution. Further purification was effected by chromatography on DEAE-cellulose column using a linear salt gradient for elution. The elution pattern is shown in Fig. 3. Peak I was not examined. Peak II (32.1%) was adenosine 3′,5′-diphosphate not mixed with 2,′5′-diphosphate. From Peak III (39.3%), analytically pure coenzyme A was isolated as its trilithium salt. Peak IV (25.9%) was identified as the disulfide form of coenzyme A, which was reduced to the thiol form with 2-mercaptoethanol. Coenzyme A (VIb) thus obtained was chromatographically and electrophoretically identical with the commercially available sample, as well as the sample prepared by Moffatt and Khorana's method. The total yield was 29.7% based on IVb.

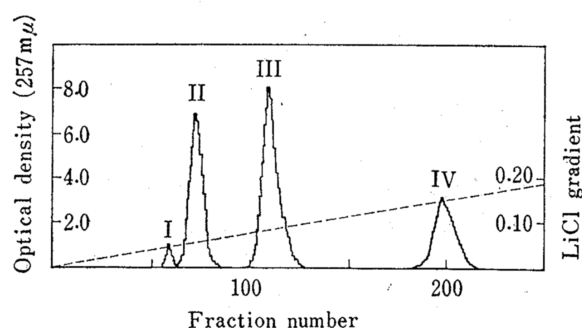

Fig. 3. Chromatography of the products in the synthesis of coenzyme A by the thiazoline method as in text.

Peak II, adenosine 3′,5′-diphosphate; Peak III, coenzyme A (SH form); Peak IV, coenzyme A (disulfide form).

There are several methods for the assay of coenzyme A, of which the method with phosphotransacetylase seems to be the most suitable with respect to the specificity for coenzyme A. While Stadtman and Kornberg[8] reported the preparation of this enzyme from *Clostridium kluiverii*, some of the present authors established the modified

Table I. Purity of CoA Preparations estimated by Phosphotransacetylase Method

Preparations	Method used	Purity based on (%)	
		adenosine	ribose
Synthetic CoA	Moffatt and Khorana's method.	82	80
Synthetic CoA	Moffatt and Khorana's method, employing hydrolysis of the cyclic phosphate by RNase T_2 in place of acid.	107	104
Synthetic CoA	Thiazoline method, including RNase T_2 digestion, crude.	80	77
Synthetic CoA	Thiazoline method, including RNase T_2 digestion, followed by further purification by DEAE-cellulose chromatography.	106	103
Commercial CoA	Isolated from yeast.[a]	86.5	80.5

a) A preparation of C. F. Boehringer & Soehne GmbH., Mannheim (Germany).
Each preparation was assayed for CoA by phosphotransacetylase method. Purities of the preparations were calculated from adenosine or ribose and CoA contents of the preparations by making 1 μ mole of CoA 316 units.[9] Adenosine and ribose were determined by spectrophotometry and iron-orcinol reaction, respectively.

8) R. E. Stadtman, A. Kornberg: J. Biol. Chem., **203**, 47 (1953).
9) F. Lipmann: J. Am. Chem. Soc., **74**, 4017 (1952).

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assay system with phosphotransacetylase from *Escherichia coliB*, the details of which will be published elsewhere in the near future. Table I shows the quality of various samples assayed by this method. Content of the crude sample by the thiazoline method was found to be nearly equal to that of the commercial sample.

In conclusion, the brief note on the thiazoline intermediate (Vb) deserves mention here. This compound corresponds to the thiazoline form of coenzyme A, which was detected by Basford, *et al.*[10] in the commercially available sample of coenzyme A using chromatographic and colorimetric techniques. Paper chromatography of the crude Vb, though not purified, was found to give a different spot from the thiol and the disulfide form of coenzyme A, but the corresponding spot was not found by paper chromatography of coenzyme A purchased here. Therefore, the presence of the thiazoline form by Basford, *et al.* remains to be determined.

Experimental

General Methods——Ascending paper chromatography was carried out on Toyo No. 50 paper using the following solvent systems : solvent I, EtOH–0.5*N* ammonium acetate buffer (pH 3.8)(5:2); solvent II, EtOH1*N* ammonium acetate (pH 7.5) (5:2); solvent III, saturated $(NH_4)_2SO_4$–0.1*M* ammonium acetate (pH 6)–*iso*-PrOH (79:19:2); solvent IV, *n*-BuOH–HOAc–H_2O (5:2:3); solvent V, *n*-PrOH–conc. NH_4OH–H_2O (6:3:1); solvent VI, EtOH–H_2O (7:3). Paper electrophoresis was carried out on Toyo No. 50 paper (65 × 5 cm.) impregnated with the solvents described below at 800 volts for 2 hr., using an apparatus similar to that described by Markham and Smith[11] : solvent A, 0.05*M* triethylammonium bicarbonate (ph 7.5); solvent B, 0.05*M* ammonium acetate buffer (pH 3.5).

Adenosine derivatives on chromatograms were detected with UV lamp. Phosphorus-containing compounds were detected with the Hanes and Isherwood spray[12] followed by ultraviolet irradiation and sulfhydryl compounds with ammonia spray after nitroprusside spray and disulfide compounds with nitroprusside spray after KCN spray. The Rf values and relative electrophoretic mobilities of various compounds are given in Table II and III, respectively. All compounds were treated with IR 120 (H^+) resin prior to chromatography.

Table II. Rf Values of Compounds

Compound	Rf	
	Solvent I	Solvent II
Adenosine 5′-phosphate	0.25	0.10
Adenosine 5′-phosphoromorpholidate		0.47
Dephospho-coenzyme A (SH)	0.38	0.38
Dephospho-coenzyme A (SS)	0.10	0.09
P^1-Adenosine 5′-P^2-pantothenonitrile 4′-pyrophosphate	0.35	0.32
Adenosine 2′(3′),5′-diphosphate	0.15	0.01
Adenosine 2′,3′-cyclic phosphate 5′-phosphoromorpholidate		0.34
P^1-Adenosine 3′-phosphate 5′-P^2-pantothenonitrile 4′-pyrophosphate	0.23	0.09
Coenzyme A (SH)	0.27	0.12
Coenzyme A (SS)	0.08	0.02

Total phosphorus was determined by the method of Boltz and Mellon.[13] Adenosine was determined by ultraviolet absorption in 0.01*N* hydrochloric acid using ε_{257} mμ = 15,000 as standard value. All evaporations were carried out ***in vacuo*** (2~3 mm.) at a temperature of 35° or below.

Preparation of RNase T_2——Ribonuclease T_2 was prepared from Takadiastase according to the method of Uchida and Egami,[7] which consisted of water-extraction of Takadiastase powder, batchwise treatments of the extracts with DEAE-cellulose, heat-treatment at 80° for 2 minutes, ammonium sulfate-fractionation,

10) R.E. Basford, F.M. Huennekens : J. Am. Chem. Soc., **77**, 3878 (1955).
11) R. Markham, J.D. Smith : Biochem. J., **53**, 552 (1952).
12) C.S. Hanes, F.A. Isherwood : Nature, **164**, 1107 (1949).
13) D.F. Boltz, M.G. Mellon : Anal. Chem., **19**, 873 (1947).

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Table III. Related Electrophoretic Mobilities of Compounds

Compound	Mobility related to adenosine 5′-phosphate	
	Solvent A	Solvent B
Adenosine 5′-phosphoromorpholidate	0.50	
Dephospho-coenzyme A	0.79	1.30
P^1-Adenosine 5′-P^2-pantothenonitrile 4′-pyrophosphate		1.30
Adenosine 2′(3′),5′-diphosphate	1.32	1.39
Adenosine 2′,3′-cyclic phosphate 5′-phosphoromorpholidate	1.05	
Coenzyme A	1.18	1.39

the first DEAE-cellulose chromatography, alcohol-fractionation and the second DEAE-cellulose chromatography. In our experiments, two final steps of the above purification steps were omitted. Activity of RNase T_2 was measured according to the method of Takahashi.[14] RNase T_2 thus obtained was found to have a specific activity of 22～28 and the ratio of the activity at pH 4.5 to that at pH 7.5 was 1.3～2.3. RNase T_2 was free of phosphomonoesterase and nucleotide pyrophosphatase activities, contamination of which must be avoided for the purpose of the experiments reported here. These activities were checked essentially by the methods of Bessey, *et al.*[15] and of Kornberg and Pricer,[16] respectively.

P^1-Adenosine 5′-P^2-D-pantothenonitrile 4′-Pyrophosphate (IIIa)——An aqueous solution of the barium salt of D-pantothenonitrile 4′-phosphate*1 (I) (374 mg., 0.83 mmole) was passed through a column of "Amberlite IR 120 (H^+)" (0.9 × 4 cm.) and the column was washed with H_2O (30 ml.). To the total eluates was added pyridine (1 ml.), the solution was evaporated to dryness *in vacuo* and the residue was dried by three evaporations with anhydrous pyridine (10 ml.), finally redissolved in pyridine (5 ml.). Separately, 4-morpholine N,N′-dicyclohexylcarboxamidinium adenosine 5′-phosphoromorpholidate[17] (IIa) (246 mg., 0.33 mmole) was dried by three evaporations with pyridine (10 ml.) and finally redissolved in pyridine (5 ml.). Both solutions were combined, evaporated twice more with pyridine (10 ml.) *in vacuo*, and the mixture was finally taken up in pyridine (7 ml.) and the solution set aside overnight at room temperature. The solvent was removed *in vacuo* and residual pyridine was removed by several evaporations with MeOH. The residue was dissolved in H_2O (50 ml.), the pH was adjusted to 4.5 with 10% NH_4OH. The solution was run onto a column of ECTEOLA-cellulose (Cl^-, 3.0 × 28 cm.) which was then washed with H_2O until no more ultraviolet absorbing material eluted. Elution was carried out using a linear salt gradient technique with 0.003*N* HCl (4 L.) in the mixing chamber and 0.07*N* LiCl in 0.003*N* HCl (4 L.) in the reservoir. Fractions of each 10 ml. were collected at a flow rate of 1.5 ml. per minute. Three well separated peaks resulted (Fig. 1). Peak I (377 optical density units at 257 mμ, 7.6% of theoretical) has not been identified; Peak II (586 optical density units, 11.8%) was adenosine 5′-phosphate; Peak III (3920 optical density units, 79%) was P^1-adenosine 5′-P^2-D-pantothenonitrile 4′-pyrophosphate (IIIa). The pooled Peak III was adjusted to pH 4.5 with 0.1*N* LiOH and evoporated to dryness *in vacuo*. The residue was dried by several evaporations with MeOH. It was dissolved in MeOH (20 ml.) and the solution was concentrated *in vacuo* to a volume of 3 ml. Consecutive addition of acetone (30 ml.) and ether (3 ml.) resulted in the separation of an oily precipitate, which was redissolved in MeOH (3 ml.) and precipitated with acetone (30 ml.) to give white powder. This was repeatedly treated with MeOH and acetone until the supernatant was free of chloride ion. The final precipitate was washed with acetone and dried *in vacuo* at room temperature giving 120 mg. (52.5%) of the tetrahydrated lithium salt of IIIa. IR ν_{max}^{KBr} cm^{-1}: 3330, 3210, 2950, 2250, 1650, 1580, 1535, 1478, 1420, 1368, 1330, 1240, 1120, 1080, 1055, 947. *Anal.* Calcd. for $C_{19}H_{27}O_{12}N_7P_2Li_2 \cdot 4H_2O$: C, 32.91; H, 5.09; N, 14.14; P, 8.94; adenosine : phosphorus=1:2. Found: C, 32.72; H, 5.45; N, 13.67; P, 8.57; adenosine : phosphorus=1:2.00.

D-3′-Dephospho-coenzyme A (VIa)——Cysteamine (29 mg., 0.376 mmole) was added to a solution of the lithium salt of P^1-adenosine 5′-P^2-D-pantothenonitrile 4′-pyrophosphate (IIIa) (65.7 mg., 0.1 mmole) in MeOH (1 ml.). The clear solution was refluxed under N_2 for 8 hr. during which evolution of NH_3 was observed. The reaction mixture was evaporated *in vacuo* to give the crude thiazoline (Va) as white powder, which in the infrared spectrum showed no band at 2250 cm^{-1} characteristic of the nitrile group. The crude thiazoline was dissolved in H_2O (3 ml.) and adjusted to pH 4.7 with 1*N* HCl. The solution was heated under N_2 at 60° for 4 hr., while the pH of the solution changed to 4.45.

14) K. Takahashi: J. Biochem., **49**, 1 (1961).
15) O.A. Bessey, O.H. Lowry, M.J. Brock: J. Biol. Chem., **164**, 321 (1946).
16) A. Kornberg, W.E. Pricer: J. Biol. Chem., **182**, 763 (1950).
17) S. Roseman, J.J. Distler, J.G. Moffatt, H.G. Khorana: J. Am. Chem. Soc., **83**, 659 (1961).

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The reaction mixture was passed through a column of "Dowex 50 (H^+)" (1 ml.), washed with H_2O (140 ml.). The eluted solution was adjusted to pH 4.5 with 0.1*N* LiOH and concentrated to a volume of roughly 0.5 ml. 2-Mercaptoethanol (0.5 ml.) was then added and the solution was left overnight. The solution was evaporated to dryness *in vacuo* and the residue was evaporated three times with H_2O. The resulting gum was dried *in vacuo* over P_2O_5 for several hours, dissolved in MeOH (5 ml.) and the solution was filtered. The filtrate was reduced *in vacuo* to a volume of roughly 2 ml. To the stirred solutioh was added acetone (15 ml.) to give a solid precipitate, which was washed with acetone and dried *in vacuo* over P_2O_5 giving the lithium salt of dephospho-coenzyme A (50.5 mg., 64.0%). IR ν_{max}^{KBr} cm^{-1}: 3330, 3240, 2940, 1650, 1608, 1533, 1477, 1420, 1367, 1330, 1240, 1122, 1080, 1055, 947. This was identical with an authentic sample (see below) in comparison of IR spectrum, paper chromatography and electropherogram. *Anal.* Calcd. for $C_{21}H_{33}O_{13}N_7P_2SLi_2 \cdot 5H_2O$: C, 31.94; H, 5.49; N, 12.42; P, 7.84; adenosine : phosphorus = 1:2. Found: C, 32.30; H, 5.10; N, 11.10; P, 7.46; adenosine : phosphorus = 1:1.89.

An authentic sample of dephospho-coenzyme A was prepared from IIa and D-pantetheine 4'-phosphate by the procedure of Moffatt and Khorana.[2] *Anal.* Calcd. for $C_{21}H_{33}O_{13}N_7P_2SLi_2 \cdot 4H_2O$: C, 32.70; H, 5.36; N, 12.71; P, 8.03; adenosine : phosphorus = 1:2. Found: C, 33.05; H, 5.11; N, 11.81; P, 7.39; adenosine: phosphorus = 1:1.92.

P¹-Adenosine 3'-Phosphate 5'-P²-D-Pantothenoitrile 4'-Pyrophosphate (IVb)——The reaction of 4-morpholine N,N'-dicyclohexylcarboxamidinium adenosine 2',3'-cyclic phosphate 5'-phosphoromorpholidate[2] (217 mg., 0.2 mmole) with pyridinium D-pantothenonitrile 4'-phosphate (from 271 mg., 0.6 mmole of the dihydrated barium salt) was carried out essentially as in the case of 3'-dephospho derivative. The solvent was removed *in vacuo* followed by several evaporations with water. The residue was dissolved in water (4 ml.) and the pH was brought to 4.6 with 2% NH_4OH. After addition of 0.16*M* EDTA (0.15 ml., $10^{-3}M$ in final concentration) and an aqueous solution (1 ml.) of 1.3 mg. of RNase T_2 (activity, 373 units per ml.), the mixture was incubated at 37° for 2.5 hr. The pH was readjusted to 4.6 with dil. HCl, a further amount (1.3 mg.) of RNase T_2 was added, and the solution was again incubated at 37° for 1 hr. The solution was then adjusted to pH 6.0 with dil. NH_4OH. The product from this reaction was chromatographed on a column of DEAE-cellulose (Cl^-, 2.8 × 30 cm.) using a linear salt gradient with 0.003*N* HCl (1.5 L.) and 0.15*N* LiCl in 0.003*N* HCl (1.5 L.), at a flow rate of 1.5 ml. per min. The elution curve is shown in Fig. 2.

Peak III (2390 optical density units, 79.7%) was adjusted to pH 4.5 with 0.1*N* LiOH and worked up in the usual way to give a white powder. Two precipitations with MeOH-acetone gave a chromatographically homogeneous white powder (101 mg., 61.8%) of the hexahydrated trilithium salt of IVb. IR ν_{max}^{KBr} cm^{-1}: 3330, 2960, 2250 (C≡N), 1690, 1652, 1610, 1540, 1478, 1420, 1370, 1330, 1240, 1120, 1080, 947. *Anal.* Calcd. for $C_{19}H_{27}O_{15}N_7P_3Li_3 \cdot 6H_2O$: C, 27.99; H, 4.82; N, 12.03; P, 11.40; adenosine : phosphorus = 1:3.00. Found: C, 27.74; H, 5.19; N, 11.56; P, 11.57; adenosine : phosphorus = 1:3.06. Alkaline hydrolysis (1*N* NaOH, 100°, 20 min.) of this sample yielded exclusively adenosine 3',5'-diphosphate.

Coenzyme A (VIb)——a) Acid hydrolysis of P¹-adenosine 2',3'-cyclic phosphate 5'-P²-D-pantetheine 4'-pyrophosphate was performed by following exactly the method described by Moffatt and Khorana.[2] Chromatography on a DEAE-cellulose column of the reaction mixture yielded 6 well-separated peaks. Fractions containing coenzyme A and iso-coenzyme A were rechromatographed on ECTEOLA-cellulose. Iso-coenzyme A and coenzyme A were eluted in two separated peaks, but the separation was not so complete. The overlapping fractions were discarded and from the remainder of both peaks the trilithium salts of iso-coenzyme A and coenzyme A were obtained separately. These samples were chromatographically and elctrophoretically homogeneous, but assay by the phosphotransacetylase method proved to be partly contaminated with each other.

b) The barium salt of D-pantetheine 4'-phosphate*[1] (318 mg. of dihydrate, 0.6 mmole) was passed through a column of "Dowex 50 (pyridinium form)" (3 ml.) and the solution was evaporated to dryness *in vacuo*. The residue was dried by three evaporations with anhydrous pyridine (10 ml.) and added to the similary dried IIb(217mg.). The mixture was dehydrated by three evaporations with anhydrous pyridine and finally dissolved in pyridine (10 ml.) and kept at room temperature overnight. The solvent was evaporated *in vacuo* and the residual pyridine was thoroughly removed by several evaporations with 10 ml. portions of H_2O. The residue was dissolved in H_2O (3 ml.) and adjusted to pH 4.6 with 10% NH_4OH. 0.16*M* EDTA (0.125 ml.) and 1.34 mg. of RNase T_2 (the same aqueous solution as used before) were added and the mixture was incubated at 37° for 2.5 hr. A furthe quantity of RNase T_2 (1.34 mg.) was added, followed by 2-mercaptoethanol (0.5 ml.) and the solution was again incubated at 37° for 1 hr. and then neutralized to pH 6 with 10% NH_4OH. 2-Mercaptoethanol (2 ml.) was added and the mixture was set aside at room temperature overnight. The reaction mixture was diluted with H_2O (50 ml.) and applied to a column of DEAE-cellulose (Cl^-, 3 × 29 cm.). The column was washed with H_2O (500 ml.) and elution was then carried out using a linear salt gradient with 0.003*N* HCl (2.25 L.) and 0.225*N* LiCl in 0.003*N* HCl (2.25 L.). Fractions of each 15 ml. were collected at a flow rate of 1.5 ml. per min. The elution pattern is shown in Fig. 2. Peak IV (1220 O.D. units, yield 40.6%) was coenzyme A, adjusted to pH 4.5 with 0.1*N* LiOH and worked up in the usual way, giving a white powder of the tetrahydrated trilithium salt of coenzyme A (51 mg., 29.7%). Peak V (250 O.D. units, 8.4%) was oxidized coenzyme A and after similar treatment,

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the resulting lithium salt was reduced with 2-mercaptoethanol to give further coenzyme A (14 mg., 8.1%). IR ν_{max}^{KBr} cm^{-1}: 3320, 2940, 1647, 1610, 1540, 1475, 1420, 1365, 1240, 1122, 1082, 947. *Anal.* Calcd. for $C_{21}H_{33}O_{16}N_7P_3SLi_3 \cdot 4H_2O$: C, 29.41; H, 4.82; N, 11.44; P, 10.82; adenosine : phosphorus = 1:3. Found: C, 29.30; H, 5.34; N, 10.53; P, 10.59; adenosine : phosphorus = 1:2.93.

c) A solution of IVb (65 mg., 0.08 mmole) and cysteamine (33.9 mg., 0.44 mmole) in MeOH (2 ml.) was refluxed under N_2 for 7 hr. during which evolution of NH_3 was observed. Evaporation of the solvent gave the crude thiazoline (Vb) as pale yellow powder which had no band of C≡N group at 2250 cm^{-1} in infrared spectrum. Paper chromatography of this crude thiazoline compound in solvent VI gave with UV light detectable 2 spots, the main spot having a Rf 0.42 and minor spot having a Rf 0.34, distinguishable from the Rf 0.48 of a synthetic sample of coenzyme A. The compound of 0.42 was thiazoline compound (Vb) and did not react with nitroprusside-KCN reagent, but the chromatogram exposed in air for 2 days reacted as a disulfide compound. The compound of Rf 0.34 corresponded to adenosine 2′(3′),5′-diphosphate.

Crude Vb was dissolved in H_2O (5 ml.) and heated at 60° for 3 hr., while the pH of the solution changed to 4.2, The reaction mixture was passed through a column of Dowex 50 (H^+) (2 ml.). The column was washed with H_2O (150 ml.) and the combined effluent was adjusted to pH 4.5 with 0.1*N* LiOH and concentrated to about 0.5 ml. 2-Mercaptoethanol (0.5 ml.) was added, and the mixture was set aside at room temperature overnight. It was concentrated to dryness *in vacuo* and the residue was evaporated three times with H_2O. Two precipitations with MeOH-acetone gave white powder of crude coenzyme A lithium salt (47 mg., 78.5% yield from IVb, based on its adenosine content). For further purification the crude lithium coenzyme A (45 mg.) was chromatographed on a column of DEAE-cellulose (Cl^-, 2.0 × 28 cm.) using a linear salt gradient with 0.003*N* HCl (0.75 L.) and 0.225*N* LiCl in 0.003*N* HCl (0.75 L.) at a drop rate of 0.7 ml. per min. The elution pattern is shown in Fig. 3. Peak II (290 O.D. units, 25.2% based on IVb) was adenosine 3′,5′-diphosphate which was identified by paper chromatography using the solvent system III. Peak III (355 O.D. units, 30.9% yield) was coenzyme A. Peak IV (236 O.D. units, 20.3% yield) was disulfide of coenzyme A. Peak III and IV were worked up as described above to give the trilithium salt of coenzyme A (13 mg, 19.8% and 6,5 mg., 9.9%, respectively). It was paper chromatographically and electrophoretically identical with the commercial coenzyme A and its infrared spectrum was agreed with that of the sample obtained above. *Anal.* Calcd. for $C_{21}H_{33}O_{16}N_7P_3SLi_3 \cdot 8H_2O$: C, 27.13; H, 5.31; N, 10.55; P, 10.00; adenosine : phosphorus = 1:3. Found: C, 26.63; H, 5.55; N, 9.30; P, 10.13; adenosine : phosphorus = 1:3.03.

Alkaline hydrolysis: a sample (1 ml.) was heated with 1*N* NaOH (0.1 ml.) in boiling water bath for 20 min. IR 120 (H^+) resin was added and the supernatant was spotted on paper and chromatographed using solvent III, IV and V. The sample obtained by the method of either b) and c) gave pantentheine 2′,4′-cyclic phosphate (Rf 0.56 in solvent IV, Rf 0.65 in solvent V) and adenosine 3′,5′-diphosphate (Rf 0.47 in solvent III). None of the isomeric 2′,5′-diphosphate (Rf 0.51 in solvent III) could be detected.

Acid hydrolysis: a sample (1 mg.) was heated with 1*N* HCl (0.1 ml.) in boiling water bath for 5 min., concentrated *in vacuo* and dissolved in H_2O. The aliquot was chromatographed on paper which showed the presence of pantetheine 4′-phosphate (Rf 0.55 in solvent IV, Rf 0.28 in solvent V) and adenine (Rf 0.65 in solvent IV, Rf 0.62 in solvent V).

The authors wish to express their deep gratitude to Dr. M. Irie, University of Tokyo, for kind helpful guidance in the preparation of ribonuclease T_2. The authors are also grateful to Dr. J. Shinoda, Chairman of the Board of Directors, and Dr. T. Ishiguro, President of this Company, for their kind encouragement and to Dr. G. Ohta for helpful advice. Thanks are due to Mr. B. Kurihara and Miss E. Kosaka for elemental analyses.

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