[Chem. Pharm. Bull. 15(7) 944~948 (1967)]

UDC 577.158.062

# 116. Shigeru Kurooka, Kanoo Hosoki, and Yoshio Yoshimura: Enzymatic Synthesis of 3′-$^{32}$P-Labeled Coenzyme A from 3′-Dephospho-Coenzyme A.*1

(*Research Laboratory, Dainippon Pharmaceutical Co., Ltd.**2)

It has been confirmed that 3′-$^{32}$P-labeled CoA was enzymatically prepared by incubating 3′-dephospho-CoA with $^{32}$P-labeled *p*-nitrophenyl phosphate and cell suspensions of *Proteus mirabilis*. The formed labeled CoA with a specific radioactivity of 2 μc per mg. was separated from the reaction mixture by an ECTEOLA cellulose column chromatography. The amount of the labeled CoA was determined enzymatically by phosphotransacetylase-hydroxamic acid assay, spectrophotometrically based on the molecular extinction coefficient of CoA, and radiochemically from the known specific radioactivity of $^{32}$P-labeled *p*-nitrophenyl phosphate.

(Received June 16, 1966)

If labeled Coenzyme A (CoA) is available, it will provide a convenient tool for the metabolic studies on CoA which in trace amount participates in many biochemical reactions. S. P. Sen, *et al.*[1] has reported the method for preparing $^{32}$P- and $^{35}$S-labeled CoA by paper chromatography of boiled extract of *Saccharomyces cerevisiae* grown in the medium containing $^{32}$P-labeled phosphate and $^{35}$S-labeled sulphate. Recently, Mitsugi, *et al.*[2,3] reported that adenosine 3′, 5′- or 2′, 5′-diphosphate could be enzymatically synthesized by incubating adenosine 5′-monophosphate (5′-AMP) with *p*-nitrophenyl phosphate (pNPP) and cell suspensions of *Proteus mirabilis* IFM, OM-9. This investigation prompted us to apply the reaction to 3′-dephospho-CoA (3′-deP-CoA) to prepare 3′-$^{32}$P-labeled CoA by the use of $^{32}$P-labeled *p*-nitrophenyl phosphate (pNP$^{32}$P) as a phosphate donor.

It is the purpose of the present communication to describe the method for the preparation of 3′-$^{32}$P-CoA by the above mentioned reaction starting from 3′-deP-CoA prepared from hydrolysate of CoA by 3′-nucleotidase[4] of *Bacillus subtilis* IFO 3032.

## Experimental

### Materials and Methods

**CoA**——CoA was prepared from baker's yeast by a modification of the procedure of Beinert, *et al.*[5]

**3′-deP-CoA**——About 10 mg. of CoA was dissolved in 5 to 10 ml. of water and the pH was adjusted to 7.5 with the addition of 1*M* tris buffer (pH 8.0). Ten ml. of crude 3′-nucleotidase was added to the solution and incubated in a shaking incubator at 37° for 3 hrs. The reaction mixture was adjusted with the addition of 1*N* HCl to pH 6.0 and was added to an ECTEOLA cellulose ($Cl^-$) column (3×25 cm.). The column was washed well with water until no UV absorbing materials appeared. Then, after AMP and adenosine 3′,5′-diphosphate were eluted with 0.003 *N*-HCl, 3′-deP-CoA was eluted with 0.0125*M* LiCl in 0.003*N* HCl. Fractions containing 3′-deP-CoA were collected and lyophylized. About 2 mg. of 3′-deP-CoA was obtained with contamination of CoA (1 to 2%).

**Disodium pNP$^{32}$P**[6]——Three ml. of pyridine containing 1.8 mmoles of *p*-nitrophenol (pNP) was added dropwise to 2.5 ml. of pyridine containing 3.1 mmoles of $^{32}POCl_3$ (3.78 mc)*3 in 30 ml-Erlenmyer flask. After

---

*1 This work was reported at the Kansai Branch Meeting of the Pharmaceutical Society of Japan, May 21, 1966.

*2 Ebie-kami 2, Fukushima-ku, Osaka (黒岡 繁, 細木 和, 吉村嘉男).

1) S. P. Sen, A. C. Leopold: Biochim. et Biophys. Acta, **18**, 320 (1955).
2) K. Mitsugi, E. Nakazawa, M. Takahashi, H. Yamada: Agr. Biol. Chem., **28**, 571 (1964).
3) *Idem*: *Ibid.*, **28**, 859 (1964).
4) S. Igarashi, A. Kakinuma: *Ibid.*, **28**, 131 (1964).
5) H. Beinert, R. W. Von Korff, D. E. Green: J. Biol. Chem., **200**, 385 (1953).

30 minutes stirring, 4 ml. of cold water was added and pH was adjusted to 7.0 with 20% NaOH. The reaction mixture was concentrated by a rotary-evaporator under reduced pressure to remove water and pyridine (under 50°). The dried reaction mixture was extracted with 5, 2.5, and 1.5 ml. of hot 87% ethanol and the combined solution was filtered while hot. The filtrate was left in a refrigerator for a night at 5°. The formed crystalline was washed with 87% ethanol, absolute ethanol, and absolute ether successively, and then was dried over $P_2O_5$ *in vacuo*. The first crystal of *p*-NPP was 124.5 mg. in weight and its chemical purity was 86% with contamination of inorganic phosphate (Pi). After recrystallization of the crystalline from hot 87% ethanol, radiochemically pure pNP$^{32}$P with the specific radioactivity of 4.47 μc per mg. was obtained, giving a single spot when chromatographed on paper by ascending technique in the solvent system, *n*-butanol : acetic acid : $H_2O$ (5:2:3).

**Phosphotransferase**——*Proteus mirabilis* IFM, OM-9 was grown in the synthetic medium containing 5 g. of glucose, 5 g. of Ehrlich meat extract, and 5 g. polypeptone per 1 L. of water. The cells were harvested by centrifugation after incubation at 30° for 24 hr., washed twice with 0.02% KCl and dried over $P_2O_5$ under reduced pressure. Sixty mg. of cell suspensions in 5 ml. of water was used as phosphotransferase. About 50% of 5'-AMP was phosphorylated to form adenosine 2',5'- or 3',5'-diphosphate when incubated with 50 μ moles of 5'-AMP, 5 μ moles of $CuSO_4$, 1.2 mmoles of acetate buffer (pH 4.0) and 140 μ moles of pNPP in the presence of 1 ml. of the enzyme.

**Analytical Methods**

CoA activity was measured by the phosphotransacetylase-hydroxamic acid method, using phosphotransacetylase from *Clostridium kluyveri* ATCC 8527 as described by Stadtman.[7] The radioactivity of $^{32}$P-labeled compounds was measured in a $2\pi$ gas flow counter (TEN model GCS, Kobe Kogyo Corp.). The Shimazu model QV-50 spectrophotometer was used for all spectrophotometrical measurements.

**Recovery of CoA Activity from 3'-deP-CoA**——The reaction mixture contained, in 2 ml., 0.59 μ moles of 3'-deP-CoA, 1.00 μ moles of $CuSO_4$, 0.16 mmoles of acetate buffer (pH 4.0), 28 μ moles of pNPP, and 0.10 ml. of phosphotransferase. The blank systems contained all the components except the enzyme or 3'-deP-CoA. The reaction mixtures were incubated at 37° for 15 hr. and after removal of the cells (enzyme) by centrifugation, CoA activity in the mixture was measured. As seen from Table II, the increase in CoA activity was observed with complete systems, while no increase in CoA activity was found in the blank systems, suggesting phosphorylation of 3'-deP-CoA at 3'-position by the phosphotransferase. Twelve percent of 3'-deP-CoA was converted into CoA when the firstly prepared cell suspensions were used as the enzyme, while the phosphorylating activity was lower by 1/8 when the secondary prepared cell suspensions were used, suggesting the culture conditions of the cells had a considerable influence on the enzyme activity.

**ECTEOLA Cellulose Column Chromatography of the Reaction Mixtures**——The reaction mixtures were applied to an ECTEOLA cellulose ($Cl^-$) column (1 × 10 cm.) and after washing out pNP with water, pNPP, 3'-deP-CoA, and CoA were eluted with 0.003*N* HCl, followed by step wise elution with 0.0125, 0.025 and 0.05*M* LiCl in 0.003*N* HCl. Reduced CoA and oxidized CoA were eluted with 0.025 and 0.05*M* LiCl respectively and were detected by nitroprusside reagent[9] after concentrating the eluates by lyophilization. Oxidized CoA was detected by the reagent only after cyanide cleavage of S-S bond. The flow rate of elution was approximately 1 ml. per minute and 5.5 ml. fractions were collected.

**Paper Chromatography of Reaction Mixtures**——The following five solvent systems were used to separate pNP, pNPP, Pi, 3'-deP-CoA, and CoA by ascending paper chromatography.

a) isopropanol : 28% $NH_4OH$ : $H_2O$ (7:1:2),
b) 70% ethanol,
c) saturated $(NH_4)_2SO_4$ : isopropanol : 1*M* sodium acetate (40:1:9),
d) isobutyric acid : 0.5*N* $NH_4OH$ (10:6),
e) *n*-butanol : acetic acid : $H_2O$ (5:2:3).

Table I. Rf Values of Pi, pNPP, CoA, and 3'-deP-CoA in Five Solvent Systems

| Compounds | Solvent systems | | | | |
|---|---|---|---|---|---|
| | a | b | c | d | e |
| pNP | 0.85 | 0.96 | 0.20 | 0.87 | 0.99 |
| pNPP | 0.34 | 0.80 | 0.53 | 0.60 | 0.68 |
| Pi | 0.07 | 0.43 | 0.98 | 0.27 | 0.49 |
| 3'-deP-CoA | 0.09 | 0.36 | 0.07 | 0.46 | 0.35 |
| CoA | 0.03 | 0.27 | 0.02 | 0.31 | 0.28 |

*3 Purchased from Sumitomo Genshiryoku Kogyo Co., Ltd.

6) O.A. Bessy, R.H. Love : J. Biol. Chem., **196**, 175 (1952).
7) E.R. Stadtman, G.D. Novelli, F. Lipmann : *Ibid.*, **191**, 365 (1951).

After drying, the papers were inspected under the following conditions;

1) ultraviolet light, (pNP, pNPP, 3′-deP-CoA, and CoA),
2) Hanes Isherwood's reagent,[8] (pNPP, Pi, 3′-deP-CoA, and CoA),
3) nitroprusside reagent,[9] (3′-deP-CoA, and CoA),
4) periodate-benzidine reagent,[10] (3′-deP-CoA).

Among them, solvents b), c) and e) separated pNPP, Pi, and CoA, and were found suitable for the detection of 3′-$^{32}$P-CoA by paper chromatography (Table I).

## Results and Discussion

**Formation of 3′-$^{32}$P-CoA from 3′-deP-CoA**——The reaction mixture contained, in 2 ml., 1.334 $\mu$moles of 3′-deP-CoA, 0.16 $\mu$moles of $CuSO_4$, 0.20 mmoles of acetate buffer (pH 4.0), 23 $\mu$moles of pNP$^{32}$P, and 0.20 ml. of the enzyme. The blank system contained all the components except the enzyme. The recovery of CoA activity was similar to the results obtained in the cold runs D) and E) (Table II). Changing the molar ratio of 3′-deP-CoA to pNP$^{32}$P from 1:20 to 1:1 did not have a considerable influence on the regain of CoA activity, indicating 2 to 3 moles of pNP$^{32}$P to 20 moles of 3′-deP-CoA was enough to convert 1 percent of added 3′-deP-CoA into 3′-$^{32}$P-CoA.

Table II. Recovery of CoA Activity from 3′-deP-CoA

| Reaction systems | Phospho-transferase (ml.) | 3′-deP-CoA ($\mu$moles) | pNPP or pNP$^{32}$P ($\mu$moles) | $CuSO_4$ ($\mu$moles) | Acetate buffer (pH 4.0) (mmoles) | CoA activity in the reaction mixture ($\mu$moles) | CoA activity derived from 3′-deP-CoA ($\mu$moles) |
|---|---|---|---|---|---|---|---|
| A) Complete | 0.10[a] | 0.58 | 28.0[c] | 0.16 | 0.20 | 0.095 | |
| B) Substrate blank | 0.10[a] | — | 28.0[c] | 0.16 | 0.20 | 0.002 | 0.081(12.20%) |
| C) Enzyme blank | — | 0.58 | 28.0[c] | 0.16 | 0.20 | 0.012[e] | |
| D) Complete | 0.20[b] | 1.334 | 28.0[c] | 0.16 | 0.20 | 0.035 | |
| E) Enzyme blank | — | 1.334 | 28.0[c] | 0.16 | 0.20 | 0.012[e] | 0.023( 1.72%) |
| F) Complete | 0.20[b] | 1.334 | 23.0[d] | 0.16 | 0.20 | 0.035 | |
| G) Enzyme blank | — | 1.334 | 23.0[d] | 0.16 | 0.20 | 0.009[e] | 0.026( 1.96%) |
| H) Complete | 0.20[b] | 1.334 | 1.3[d] | 0.16 | 0.20 | 0.024 | 0.015( 1.13%) |

*a*) firstly prepared enzyme *b*) secondaly prepared enzyme *c*) pNPP *d*) pNP$^{32}$P
*e*) This CoA activity is due to contaminated CoA in 3′-deP-CoA preparation.

**Paper Chromatography of Reaction Mixtures**——Solvents used were c) and e). The dried chromatograms were cut into 1 cm. wide strips after locating each spot on the dried papers, and each strip was counted for radioactivity. The radio chromatogram of the reaction mixture A) revealed three radioactive peaks each coincided with the spots of Pi, pNPP, and CoA, while no radioactivity at the CoA spot was observed with the enzyme blank system C), indicating no formation of 3′-$^{32}$P-CoA in the latter system. As seen from the distribution of radioactivity in Fig. 1, the molar ratio of pNPP to 3′-deP-CoA 1:1 was enough to convert about 1% of $^{32}$P of pNP$^{32}$P into $^{32}$P of 3′-$^{32}$P-CoA, while that of 23:1 was 0.2~0.4%, suggesting preferable use of pNP$^{32}$P as a substrate.

**ECTEOLA Cellulose Column Chromatography of Reaction Mixtures F) and G)**——The reaction mixtures were chromatographed on an ECTEOLA cellulose ($Cl^-$). From each

8) C. S. Hanes, F. A. Isherwood: Nature, **164**, 1107 (1949).
9) G. Toennies, J. J. Kolb: Anal. Chem., **23**, 823 (1951).
10) M. Viscontini, P Karrer: Helv. Chim. Acta, **38**, 642 (1955).

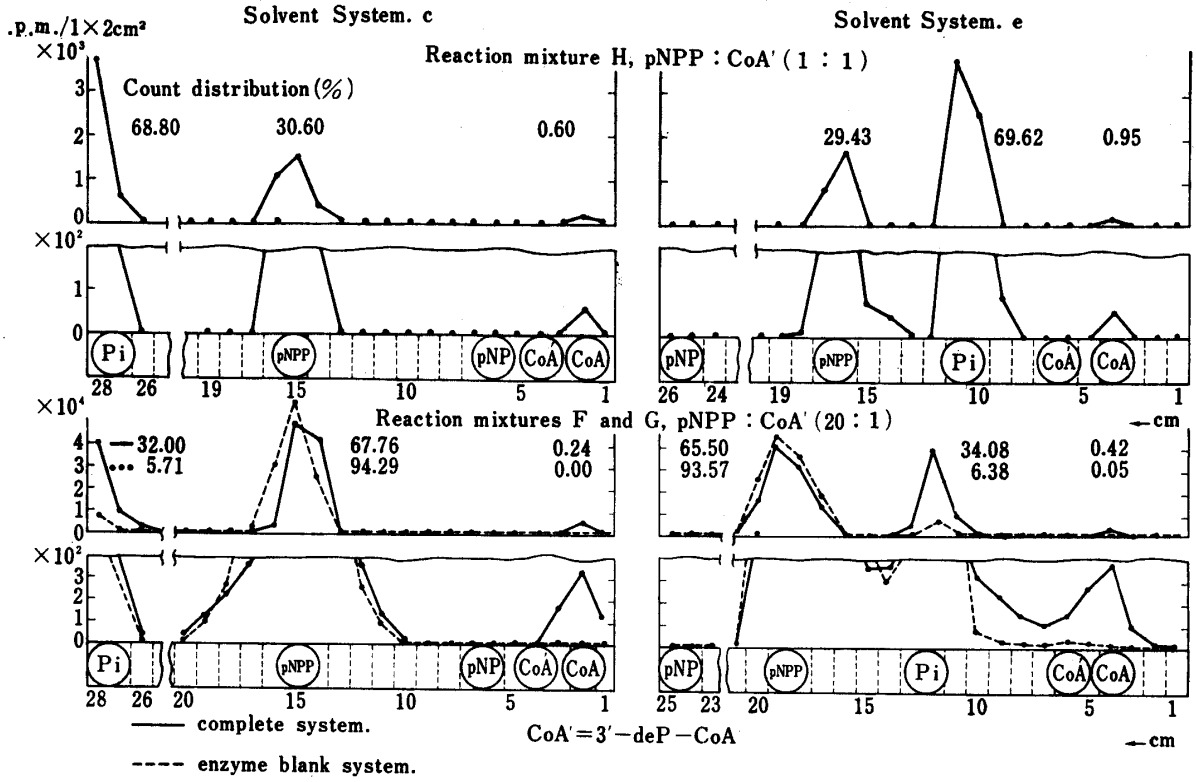


Fig. 1. Paper Chromatograms and Radiograms of Reaction Mixtures, H, F and G

fraction of 0.1 ml. the sample was taken and dried on the planchets under an infrared lamp and the radioactivity was measured. Fig. 2 shows radio column chromatograms and ultraviolet absorbancy at 260 m$\mu$ of reaction mixtures F) and G). Radioactivity and ultraviolet absorbancy due to formed 3'-$^{32}$P-CoA could be recognized at the last two peaks, reduced and oxidized CoA, in the system F), while no radioactivity was detected at the same peaks in the system G), agreeing with the results in the paper radiochromatograms (Fig. 1). The large peaks with ultraviolet absorbancy and radioactivity eluted between peaks, pNP and pNP$^{32}$P, and between peaks, pNP$^{32}$P and 3'-deP-CoA, were composed of $^{32}$Pi and pNP, and pNP$^{32}$P respectively which were identified on paper radiochromatography and paper electrophoresis (0.05 *M* ammonium formate buffer, pH 3.5, 3000 volt, 40 mA, Toyo Filter Paper No. 51, 35×10 cm., 25 minutes). These pNP and pNP$^{32}$P can be explained by the sharp rise in salt concentration of eluting solution, causing elution of tailing pNP and pNP$^{32}$P in concentrated state.

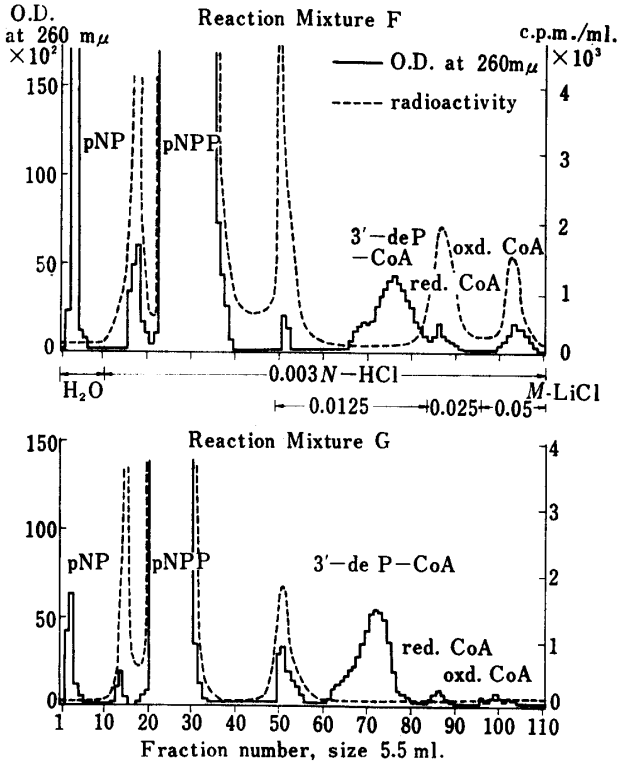


Fig. 2. ECTEOLA Cellulose Column Chromatograms and Radiograms of Reaction Mixtures, F and G

**Specific Radioactivity of Formed 3′-$^{32}$P-CoA**——CoA fractions with radioactivity and ultraviolet absorbancy at 260 mμ were pooled and 3′-$^{32}$P-CoA in them were estimated radiochemically using pNP$^{32}$P (1 μmole=1.92×10$^6$ c.p.m.) as standard and spectrophotometrically, using CoA molecular extinction coefficient, 14,600 at 260 mμ[11] and molecular weight 767.6. As seen from Table Ⅲ, the amount of formed 3′-$^{32}$P-CoA obtained from phosphotransacetylase-hydroxamic acid assay was agreed with that estimated from ultraviolet absorbancy and specific radioactivity. The formed labeled CoA was found to have a specific radioactivity of 2 μc per mg.

Table Ⅲ. CoA Content Derived from 3′-Dephospho-CoA

| Assay methods | CoA content in 1 ml. of reaction mixtures, F and G systems: fraction number | complete system F (μg.) | enzyme blank system G (μg.) | derived from 3′-deP-CoA (μg.) | (μmoles) |
|---|---|---|---|---|---|
| Phosphotransacetylase-hydroxamic acid assay, using Sigma CoA as standard | | 13.9 | 3.6 | 10.3 | 0.013 |
| UV absorbancy, CoA molecular extinction coefficient, 14600 at 260 mμ | 85～90 | 14.9 | 8.3 | 12.3 | 0.016 |
| | 102～105 | 10.4 | 4.7 | | |
| Radioactivity, based on pNP$^{32}$P (1 μmole=1.92×10$^6$ c.p.m.) | 85～90 | 22400 c.p.m. | 0 c.p.m. | 34600 c.p.m. | 0.018 |
| | 102～105 | 11200 | 0 | | |

The formation of 3′-$^{32}$P-CoA could be explained by the radiochromatograms and CoA activity by the phosphotransacetylase-hydroxamic acid assay. However, the result that the value of CoA estimated from radioactivity is higher than those from the other two methods, suggests that by phosphotransferase, 3′-deP-CoA is phosphorylated preferentially at 3′-position giving rise to CoA, but small amount of iso-CoA (2′-phospho-3′-dephospho-CoA) with no CoA activity,[12] might be formed by phosphotransferase just as 5′-AMP was phosphorylated by the enzyme to form both adenosine 2′,5′- and 3′,5′-diphosphates. It is of interest to study the structural specificity of the enzyme, because phosphorylation of 3′-deP-CoA by the enzyme was far below that of 5′-AMP which was phosphorylated about 50%.

The authors are greatly indebted to the Institute of Food Microbiology, Chiba University and the Institute for Fermentation, Osaka, for their kind gifts of *Proteus mirabilis* IFM, OM-9, and *Bacillus subtilis* 3032, to Mr. Y. Minaki for his advice in the radiochemical analysis, and to Mr. S. Nishimura for his technical assistance in this experiment. Thanks are also given to Dr. S. Ose, director, and Dr. H. Takamatsu, vice-director, of this laboratory, for their encouragement throughout this work.

11) National Academy of Sciences—National Research Council Publication 719, Washington D.C. (1960).
12) J.G. Moffatt, H.G. Khorana : J. Am. Chem. Soc., **83**, 663 (1961).