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136. Morio Ikehara: Studies on Coenzyme Analogs. VI. The Synthesis of P¹-5'-Adenosine P²-(2-Mercaptoethyl) Pyrophosphate and its Activity as a Coenzyme-A Analog.

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Coenzyme-A was first discovered by Lipman[1] in 1945 and extensively studied by many investigators for elucidation of its important catalytic action in numerous biological reactions.

On the other hand, its chemical synthesis was planned by Baddiley, *et al.*[2] and finally achieved by Khorana,[3] who tested the activity of synthetic coenzyme-A and established its structure correctly.

From the standpoint of chemical structure, it bears a thiol group, which is acetylated by acyl-AMP[4],[*2] to form acyl-coenzyme-A. The latter compound transfers its acyl group to other substrates, which are biochemical intermediates of carbohydrate, fatty acid, peptide, and steroid metabolism.

Several fragments of coenzyme-A, such as pantothenic acid[5] (II) or cysteamine (IIIa), were examined for their biological activity. Pantothenic acid was proved to be a growth factor for *Lactobacillus vulgaricus* as the form of pantothein. Acetylated cysteamine (IIIb) is a strong acylating agent and acetylates hydroxylamine spontaneously in aqueous solution

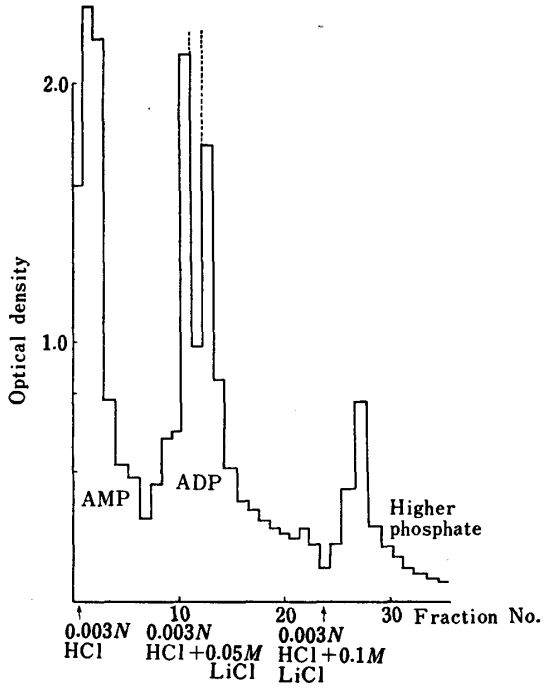

Fig. 1. Ion-exchange Chromatogram of ADP synthesis

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*2 Following abbreviations are used: AMP, adenosine 5'-phosphate; ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide.

1) F. Lipman: Science, **120**, 855(1945).
2) Coenzyme A series, J. Baddiley, *et al.*: J. Chem. Soc., **1951**, 246, et seq.
3) J. G. Moffatt, H. G. Khorana: J. Am. Chem. Soc., **81**, 1265(1959).
4) P. Berg: J. Biol. Chem., **222**, 991, 1015(1956).
5) G. D. Novelli: Physiol. Revs., **33**, 525(1953).

at room temperature.[6]

To testify the relationship between the structure of coenzyme-A and acylating activity in the enzyme system in pigeon liver extract, a compound having the structure in which pantothenyl group is absent from 3′-dephospho-coenzyme-A was first taken up for attempted synthesis.

2-Benzylthioethylamine (V) was prepared from N-(2-benzylthioethyl)phthalimide[7] by treatment with hydrazine hydrate and then phosphorylated with dibenzyl phosphite[8] to dibenzyl N-(benzylthioethyl)phosphoramidate (VI) in a fairly good yield. The latter was debenzylated to monobenzyl derivative (VII) by treatment with sodium iodide in 2-methoxy-

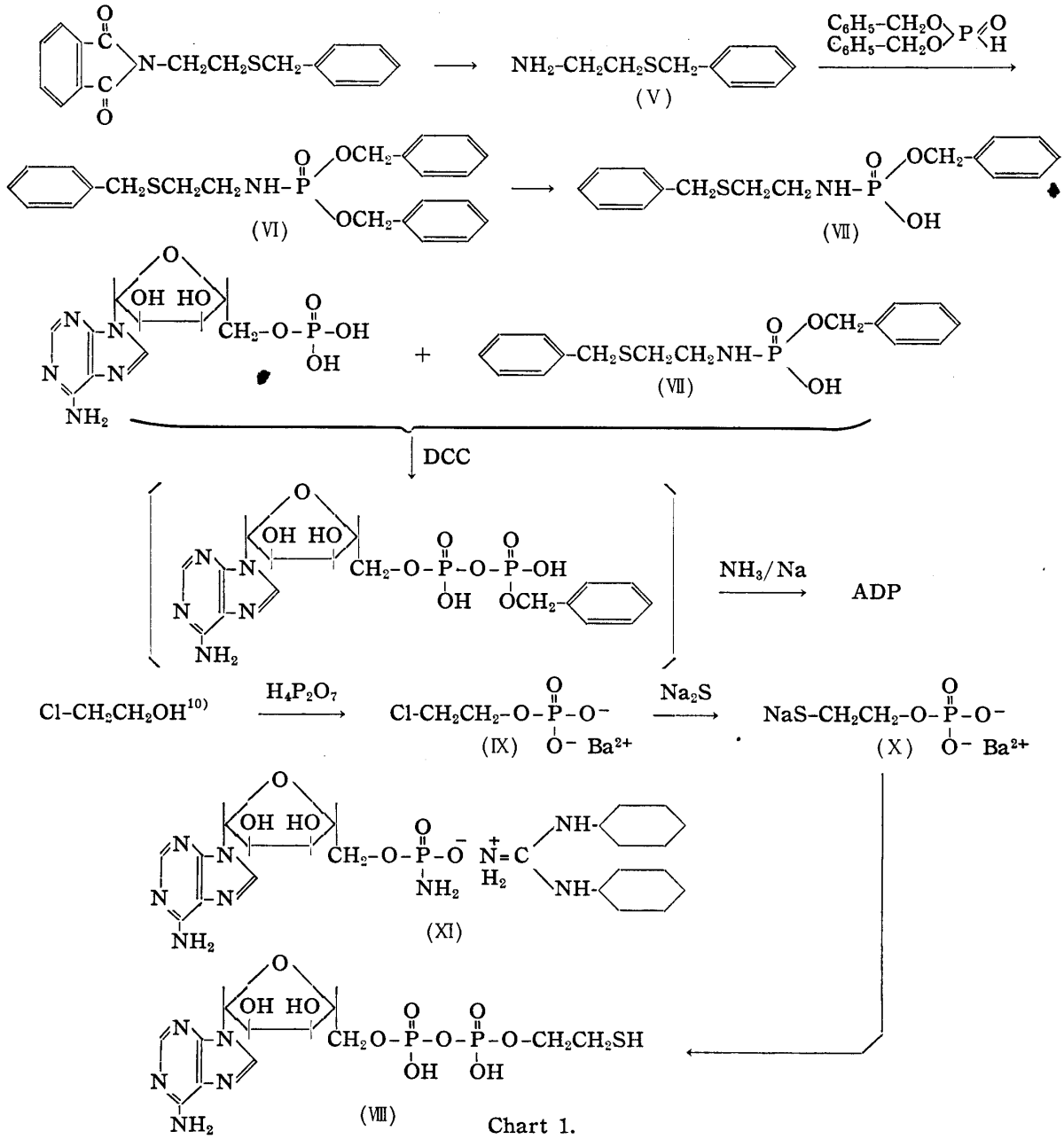

Chart 1.

6) J. Baddiley, E. M. Thain : J. Chem. Soc., **1951**, 3425.
7) M. Michells : Ber., **25**, 3050(1892).
8) F. A. Atherton, H. T. Openshaw, A. R. Todd : J. Chem. Soc., **1945**, 382.

ethanol. Pyridinium salt of AMP[9] was then reacted with (VII) in the presence of DCC in acetonitrile and DMF for 4 days at room temperature.

Contrary to expectations, ADP and some higher organic phosphates not containing sulfur were separated by ion-exchange column chromatography (Fig. 1). The mechanism of this reaction may be explained tentatively by the assumption of an intermediate given in brackets in Chart 1. Further investigation of this type of reaction will be reported later.

Then attention was turned to the synthesis of P^1-5′-adenosine P^2-(2-mercaptoethyl) pyrophosphate (VIII), in which imino group of (IV) was substituted with oxygen atom.

2-Chloroethyl phosphate (IX) was prepared by the procedure of Cherbliez,[10] and was converted to 2-mercaptoethyl phosphate (X) by reaction with sodium sulfide. (X) was then reacted with adenosine 5′-phosphoramidate dicyclohexylguanidinium salt (XI)[11] in pyridine for 120 hours. Ion-exchange chromatography (Fig. 2) of this reaction mixture gave AMP,

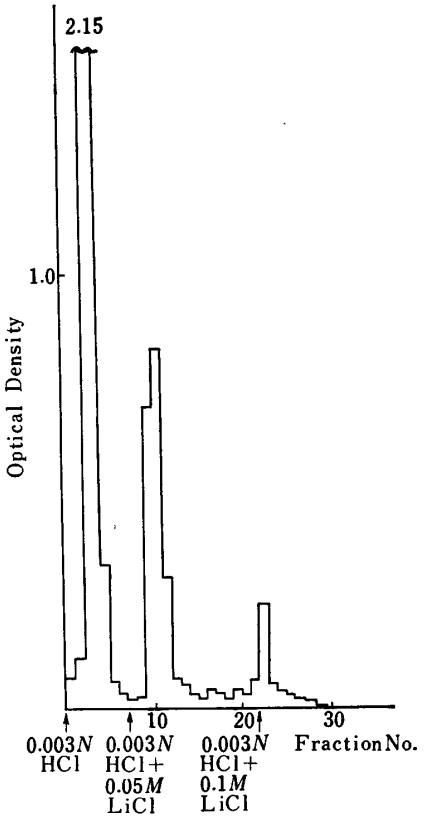

Fig. 2. Ion-exchange Chromatogram of P^1-5′-Adenosine P^2-(2-Mercaptoethyl) Pyrophosphate Synthesis

P^1-5′-adenosine P^2-(2-mercaptoethyl) pyrophosphate (VIII), and another unidentified higher phosphate, which did not have a sulfhydryl group. Lithium salt of (VIII) was converted to barium salt in order to obtain a non-hygroscopic material. Elementary analytical data corresponded to the molecular formula for barium salt of adenosine 2-thioethylpyrophosphate with 5 moles of water and its purity calculated on the basis of ultraviolet absorption of adenine at 260 mμ (ε 10840) was 83%. Paper chromatography in several solvent systems showed (see Experimental) migratory behavior of this substance to be intermediate of AMP and ATP by the cochromatography technique. The violet color reaction with Grote's reagent[12] gave proof for the presence of thiol group.

9) J. Baddiley, A. M. Michelson, A. R. Todd : *Ibid.*, **1947**, 648; N. S. Korby, G. W. Kenner, A. R. Todd: *Ibid.*, **1952**, 3669.
10) E. Cherbliez : Helv. Chim. Acta, **41**, 1693(1958).
11) R. W. Chambers, J. G. Moffatt : J. Am. Chem. Soc., **80**, 3752(1958).
12) I. Grote : J. Biol. Chem., **93**, 25(1931).

When (VIII) was hydrolyzed in 1*N* hydrochloric acid at 100° for 7 minutes, inorganic phosphate, AMP, and thioethanol phosphate were detected of the paper chromatogram. Further extensive hydrolysis with 2*N* hydrochloric acid, at 100° for 30 minutes showed some increase in organic phosphates and adenine, in addition to above fragments. From these evidences and other chemical nature of this compound, as well as from the ion-exchange chromatographic pattern (Fig. 2), the structure of P^1–5′-adenosine P^2–2-thioethylpyrophosphate was unambiguously established.

Biological activity of this compound as the coenzyme of acylating reaction in the enzyme systems in pigeon liver was carried out by the method of Lipmann and Kaplan.[13] The results are summarized in Fig. 3, in which, relative to standard coenzyme-A, entire lack of acetylation activity of this compound is shown.

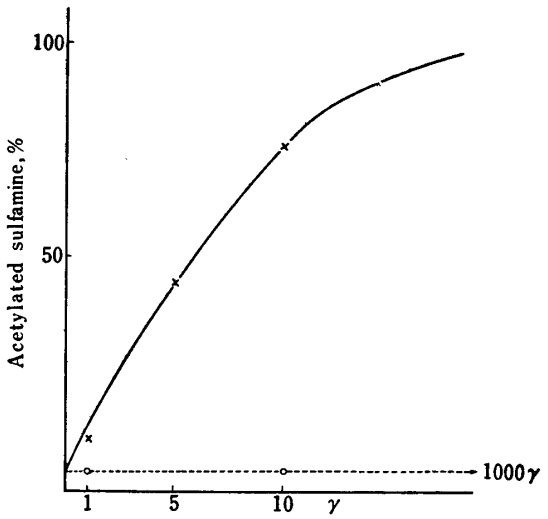

Fig. 3. Assay of P^1–5′-Adenosine P^2–(2-Mercaptoethyl) Pyrophosphate by Lipmann-Kaplan Method

—x— Coenzyme-A
····o···· P^1–5′-Adenosine P^2–(2-mercaptoethyl) pyrophosphate

These findings suggest that lack of pantothenic moiety in 3′-dephospho-coenzyme-A definitely diminishes the acetylating activity in the enzyme systems of pigeon liver. Further investigation on the rôle of pantothenyl moiety in coenzyme-A will be deferred to a later date.

Experimental

2-Benzylthioethylamine (V)—To a solution of 7.0 g. of N-(2-benzylthioethyl)phthalimide dissolved in 20 cc. of anhyd. EtOH, 1.18 g. of hydrazine hydrate was added. After 15 min. of heating on a water bath, the whole solution solidified. Into this, 5 cc. of 2*N* HCl was added and heated for further 15 min. Precipitate was removed by filtration and EtOH was evaporated *in vacuo*. Resulting syrup solidified after several days. Recrystallization from petr. ether gave colorless needles, m.p. 68~69°; yield, 2.5 g.

Dibenzyl N-(2-Benzylthioethyl)phosphoramidate (VI)—A solution of 1.3 g. of dibenzyl phosphite and 2.0 g. of 2-benzylthioethylamine (V) dissolved in 25 cc. of CCl_4 at −10° during 5 min. was kept standing overnight at room temp., washed with 10 cc. of 10% HCl, 10% NH_4OH, and H_2O, and then dried over Mg_2SO_4. Evaporation of CCl_4 afforded a vitreous residue (yield, 1.85 g.), which contained N, S, and P by quantitative test.

Benzyl Hydrogen N-(2-Benzylthioethyl) Phosphoramidate (VII)—To a solution of 1.8 g. of crude (VI) dissolved in 10 cc. of 2-methoxyethanol, 0.5 g. (3 equiv.) of LiCl was added. After heating for 5 min. at 100°, a precipitate appeared and heating was continued for further 2 hr. The mixture was cooled and the precipitate was collected (0.65 g.). The filtrate was diluted with water and extracted with three 10-cc. portions of Et_2O. Aqueous layer was acidified with *N* H_2SO_4 and the precipitate was collected on a filter (0.5 g.). This material gave positive test for N, S, and P. *Anal.* Calcd. for $C_{16}H_{19}O_3NLiPS$:

13) "Method of Biochemical Analysis," II, 201. Interscience Publishers, Inc., New York.

C, 56.16; H, 5.25; N, 4.08. Found: C, 55.52; H, 5.65; N, 3.84.

Attempted Synthesis of P¹-5′-Adenosine P²-N-(2-Benzylthioethyl) Pyrophosphoramidate—390 mg. (1 m. mole) of AMP was evaporated with a small amount of pyridine and dried over P_2O_5 for 8 hr. at 2 mm. Hg. To this pyridinium salt, 22 cc. of DMF was added, followed by the addition of 250 mg. of free (Ⅶ) (freed from Li by passing through Amberlite IR-120 and evaporated *in vacuo*) and 0.6 g. of DCC. The solution in a stoppered flask was set aside for 4 days at room temp. Separated dicyclohexylurea was collected, 2 cc. of water was added, and extracted several times with Et_2O. Yellow glass thus obtained was treated with Na (ca. 0.1 g.) in liquid NH_3 (10 cc.) until blue color remained. NH_3 was allowed to evaporate at room temp. and pH was adjusted to 7.0 (total optical density at this stage was 12950) and applied on the top of a column (0.8×7 cm) of Amberlite IRA-400 (Cl′ form, 100~200 mesh) at a flow rate of 1 cc./min. After washing with 400 cc. of H_2O, the column was eluted with 0.003*N* HCl (80 cc.), 0.003*N* HCl + 0.05*M* LiCl (1300 cc.), 0.003*N* HCl + 0.1*M* LiCl (700 cc.). Results are shown in Fig. 1. The second peak was collected, neutralized with 1*N* LiOH, and evaporated below 30° *in vacuo*. The residue was dried completely over P_2O_5 and extracted with MeOH (anhyd.) to remove LiCl. Resulting Li salt was chromatographed on a filter paper (Toyo Roshi No. 51A; solvent I) with authentic ADP and both samples gave UV absorbing spot at Rf 0.33. This material did not contain S and was identical with ADP in many respects (yield, 190 mg., 28%).

2-Mercaptoethyl Phosphate (X)—H_2S gas was bubbled through fused Na_2S (1.25 g., 15 m. mole) on a water bath. After saturation, equal volume of EtOH was added and resaturated with H_2S. The solution thus obtained was mixed with EtOH-H_2O solution containing 2.5 g. of Ba chloroethylphosphate.[9] The reaction mixture was shaken vigorously for 2 hr. Yellow color appeared both in supernatant and precipitate, which changed to a heavy white mass. After standing overnight at room temp., the precipitate was collected by filtration (yield, 2.1 g.). *Anal.* Calcd. for $C_2H_4O_4BaNaPS \cdot 2H_2O$: C, 6.83; H, 2.28. Found: C, 7.01, 6.42; H, 2.67, 2.50.

P¹-5′-Adenosine P²-(2-Mercaptoethyl) Pyrophosphate (VIII)—A solution of 100 mg. (0.176 m. mole) of dicyclohexylguanidium salt[10] of AMP-amidate dissolved in a solution of 2-mercaptoethyl phosphate (freed from 233 mg. of the salt obtained as above by passing through Amberlite IR-120 column) in 10 cc. of pyridine in a tightly stoppered reaction vessel was kept standing at room temp. for 10 hr. in a desiccator. The reaction mixture was then evaporated *in vacuo*, adjusted to pH 7, and applied on top of a column (0.8×7 cm.) of Amberlite IRA-400 (Cl′ form, 100~200 mesh). After washing with H_2O, the column was eluted with 0.003*N* HCl (300 cc.), 0.003*N* HCl + 0.05*M* LiCl (1200 cc.), and 0.003*N* HCl + 0.1*M* LiCl (1500 cc.), and the pattern is shown in Fig. 2. Each peak was separately collected, neutralized with 1*N* LiOH, and evaporated below 30° *in vacuo*. Paper chromatography of each fraction showed that the first peak is unreacted AMP and the second, S-containing diphosphate. The second fraction was dried thoroughly over P_2O_5 at 2 mm. Hg for 10 hr. and extracted with anhyd. MeOH. The residue was a white hygroscopic powder which was redissolved in H_2O, adjusted to pH 8, and equivalent quantity of $(AcO)_2Ba$ (1*M* solution) was added. The precipitated Ba salt was collected by centrifugation and washed with EtOH-H_2O, EtOH, EtOH-Et_2O, and finally with Et_2O. Dried material weighed 45 mg. (32.9% $Ba_{1.5} \cdot 5H_2O$), which agreed with the amount calculated from ion exchange pattern on the basis of UV absorption (ε_{260} 10840). *Anal.* Calcd. for $C_{12}H_{16}O_{10}N_5Ba_{1.5}P_2S \cdot 5H_2O$: C, 18.50; H, 3.34; N, 8.98; P, 7.96. Found: C, 18.06; H, 4.01; N, 8.52; P, 8.23.
Labile phosphate (1*N* HCl, 100°, 7 min.) gave AMP and 2-mercaptoethyl phosphate. Grote's reagent gave violet color in the free SH form.

Table I.

	Rf value					
	Pi	*	AMP	ATP	2-Mercaptoethyl phosphate	Adenine
Solvent I	0.55	0.17	0.33	0.09		
II	0.77	0.30		0.11		
III	0.36	0.43		0.60		
HCl hydrolysis (Solvent I)						
1*N*, 100°, 7 min.	0.47		0.40		0.22	
2*N*, 100°, 30 min.	0.45		0.38		0.21	0.95

* P¹-5′-Adenosine P²-(2-mercaptoethyl) pyrophosphate

Paper Chromatography—Results are listed in Table I. All samples were cochromatographed with authentic adenine, AMP, or ATP. Solvent I: iso-PrOH-1% $(NH_4)_2SO_4$=2:1; II: iso-PrOH (75 cc.)-H_2O (25 cc.)-CCl_3COOH (5 g.)-NH_4OH (0.25 cc.); III: iso-PrOH-NH_4OH-H_2O=7:1:2.

Biological Assay—Slightly modified Lipman-Kaplan method[12] was used. 1) Preparation of pigeon liver extract: Me_2CO-dried powder of fresh pigeon liver (28 g.) was extracted with 0.02*M* $KHCO_3$ (28 cc.) for 20 min., centrifuged, and the supernatant was kept in dry ice. This was aged at 28° for

4 hr. to destroy coenzyme-A involved, centrifuged (10000 r.p.m.), and kept in dry ice. Acetylation activity of this enzyme solution was tested with standard coenzyme-A (purchased from Sigma Chemical Co., 75% pure) and this enzyme solution proved to have sufficient activity and did not contain undestroyed coenzyme-A.

ii) Assay : a) Incubation : A mixture of 1, 5, and 10 γ/0.3 cc. of coenzyme-A or 1, 10, 100, 1000 γ/0.3 cc. of (Ⅷ), 0.1 cc. of 1M $NaHCO_3$, 0.1 cc. of 0.1M cysteine hydrochloride, 0.3 cc. of the reaction mixture,[*3] and 0.25 cc. of enzyme solution were incubated for 2 hr. at 37°. 4.0 cc. of 5% CCl_3COOH was gradually added, centrifuged, and 2 cc. each of the supernatant was used for analysis.

iii) Photometry : 2.0 cc. of mixture described in (ii) was added with 3.0 cc. of H_2O and 0.5 cc. of 0.1% $NaNO_2$, and kept for 3 min., 0.5 cc. of 0.5% $(NH_4)_2SO_4$ and, after 2 min., 0.5 cc. of 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride was added and the amount was colorimetrically estimated (Filter S-55 of Hitachi photoelectric colorimeter).

iv) Results : Fig. 3 shows the result in which activity of (Ⅷ) is given by dotted line and that of standard coenzyme-A is shown by solid line representing percentage of acetylated sulfanilamide.

The author thanks Mr. Michio Ui for his interest in this research and help in carrying out biochemical assay. Elementary analysis was carried out by Mr. Kusuo Narita of Elementary Analysis Laboratory of this Faculty, to whom the author's thanks are due.

Summary

P^1–5′–Adenosine P^2–(2–mercaptoethyl) pyrophosphate was prepared by the reaction of adenosine 5′–phosphoramidate dicyclohexylguanidinium salt and 2–mercaptoethyl phosphate. This compound has no activity for acetylation reaction in the pigeon liver enzyme systems.

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[*3] The reaction mixture : $5\times10^{-3}M$ sulfanilamide 10.0 cc., 1M AcONa 2.5 cc., $5\times10^{-2}M$ ATP Na salt 8.0 cc., $5\times10^{-1}M$ sodium citrate 10.0 cc.