Oxytocin prepared as described above is identical with a sample of highly purified oxytocin prepared in the laboratory of du Vigneaud[36] as compared by paper or thin layer chromatography and by paper electrophoresis. Paper chromatography on Whatman No. 4 paper and thin layer chromatography were performed in butanol–acetic acid–water (4:1:5, ascending) and the chromatograms were developed with ninhydrin. Paper electrophoresis was carried out on Schleicher and Schüll 2043-B paper for 4.75 hr in pyridine–acetate buffer of pH 4 at 300 v, or for 16 hr in pyridine–acetate buffer of pH 5.6 at 80 v, or for 2 hr in glycine buffer of pH 9.25 at 300 v, and bromophenol blue–mercuric chloride reagent[49] was used for development.

The oxytocin obtained as described above was submitted to partition chromatography on Sephadex.[38] A sample of 14 mg of oxytocin was dissolved in 1 ml of the upper phase of the solvent system 1-butanol–benzene–pyridine–0.1% acetic acid (6:2:1:9) and applied to a Sephadex G-25 column (1.1 × 112 cm) which had been equilibrated with both phases. Elution with the upper phase was performed and 137 fractions (1.5 ml) were collected at a flow rate of approximately 5 ml/hr. The Folin–Lowry color values showed a main peak having a R_f of 0.27 (reported R_f for oxytocin under the same conditions 0.24[38]). The recovery of oxytocin from the central portion of this peak was 9.5 mg. Materials taken before and after partition chromatography exhibited the same behavior electrophoretically and chromatographically.

(49) E. L. Durrum, *J. Am. Chem. Soc.*, 72, 2943 (1950).

B. N-Carbobenzoxy-oxytoceine (X, 0.154 g) was finely ground and dissolved in 2 ml of freshly prepared, bromine-free 2.5 *N* hydrogen bromide in glacial acetic acid. After 30 min at room temperature 100 ml of cold ether was added. The supernatant liquor was decanted and the hydrobromide was washed five times with cold ether, each washing being followed by decantation. The residue was dried *in vacuo* over potassium hydroxide and calcium chloride. The hydrobromide was dissolved in 150 ml of air-free water and was brought to a pH of *ca.* 7 by addition of a few drops of 2 *N* ammonium hydroxide. After adjustment of the pH to *ca.* 6.8 with 1 *N* acetic acid, the solution was aerated with CO_2-free air for 5 hr. The pH of the solution was adjusted to *ca.* 4, before concentrating it in a flash evaporator to a volume of 50 ml and submitting it to countercurrent distribution as described under A. After 388 transfers, determination of the Folin–Lowry color values indicated a main peak with a partition coefficient (*K*) of approximately 0.5. The contents of tubes 110–150 were concentrated and lyophilized to give 30 mg of oxytocin which was identical with preparation A in its chromatographic (paper and thin layer) and electrophoretic behavior. A sample was hydrolyzed in 6 *N* HCl at 110° and analyzed[36] according to the method of Spackman, Stein, and Moore. The following molar ratios of amino acids and ammonia were obtained, with the value of glycine taken as 1.0: aspartic acid, 1.0; proline, 1.0; glycine, 1.0; glutamic acid, 1.0; cystine, 1.0; isoleucine, 1.0; leucine, 1.0; tyrosine, 0.8; and ammonia, 3.

Acknowledgments. The author wishes to thank Mr. V. Bardakos for helpful technical assistance and Dr. H. Mantzos for the microanalyses.

Coenzyme A Analogs. Synthesis of D-Oxypantetheine-4′ Phosphate and Oxy-Coenzyme A[1a,b]

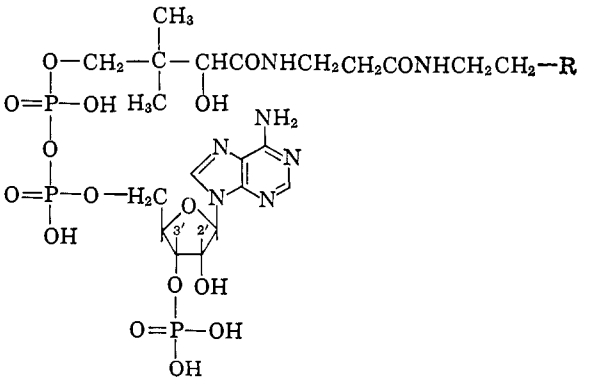
Terry L. Miller,[2] Gerald L. Rowley, and Charles J. Stewart[3]

Contribution from the Chemistry Department, San Diego State College, San Diego, California. *Received January 3, 1966*

Abstract: The syntheses of D-oxypantetheine-4′ phosphate, 3′-dephosphooxy-coenzyme A, isooxy-coenzyme A, and oxy-coenzyme A are described. Oxy-coenzyme A is shown to be a competitive inhibitor of coenzyme A in the phosphotransacetylase reaction with a $K_I = 3.5 \times 10^{-7}$ *M*.

The ubiquitous role that coenzyme A (CoA) plays in intermediary metabolism is well known.[4] It is desirable to have available a specific CoA antagonist in order to facilitate the study of cofactor binding effects on the various apoenzymes and advance one's knowledge of the reaction mechanisms of CoA-dependent reactions. The successful syntheses of CoA by Moffatt and Khorana,[5] Michelson,[6] and Gruber and Lynen[7] have provided routes for the synthesis of CoA analogs; *e.g.*, the synthesis of seleno-coenzyme A (seleno-CoA) has been reported recently by Günther and Mautner.[8]

The oxygen analog of CoA, oxy-coenzyme A (oxy-



R = SH; CoA
R = OH; oxy-CoA
R = SeH; seleno-CoA

(1) (a) This investigation was supported by Public Health Service Grant GM-07977, from the National Institute of General Medical Sciences. (b) A part of this work was reported previously: C. J. Stewart and T. L. Miller, *Biochem. Biophys. Res. Commun.*, 20, 433 (1965), and portions were submitted in partial fulfillment of the requirements for the M.S. degree by G. L. R. in June 1963, and by T. L. M. in Aug 1965.

(2) National Science Foundation Undergraduate Research Participant, 1962–1963.

(3) Author to whom inquiries concerning this paper should be addressed.

(4) L. Jaenicke and F. Lynen, *Enzymes*, 3B, 3 (1960).

(5) J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 633 (1961).

(6) A. M. Michelson, *Biochim. Biophys. Acta*, **93**, 71 (1964).

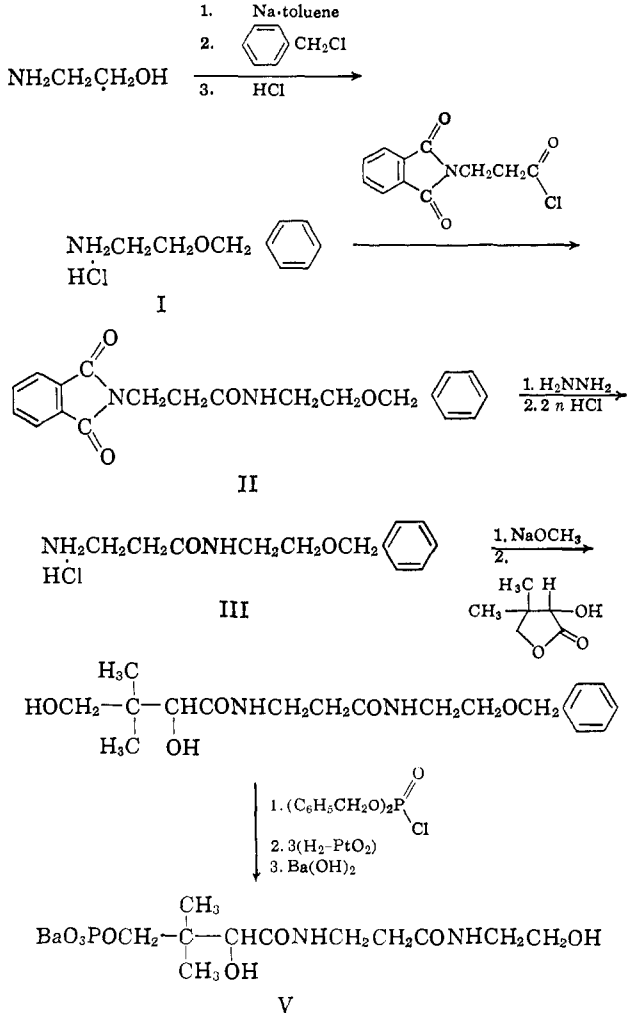
(7) W. Gruber and F. Lynen, *Ann.*, **659**, 139 (1961).

(8) W. H. H. Günther and H. G. Mautner, *J. Am. Chem. Soc.*, **87**, 2707 (1965).

CoA), is a potential CoA antagonist since oxypantetheine[9,10] is a competitive growth inhibitor of pantetheine requiring microorganisms.

This report describes the synthesis of oxy-CoA. The sequence of reactions leading to the synthesis of the key intermediate, D-oxypantetheine-4′ phosphate (V), is indicated in Scheme I. The condensation of V with

Scheme I

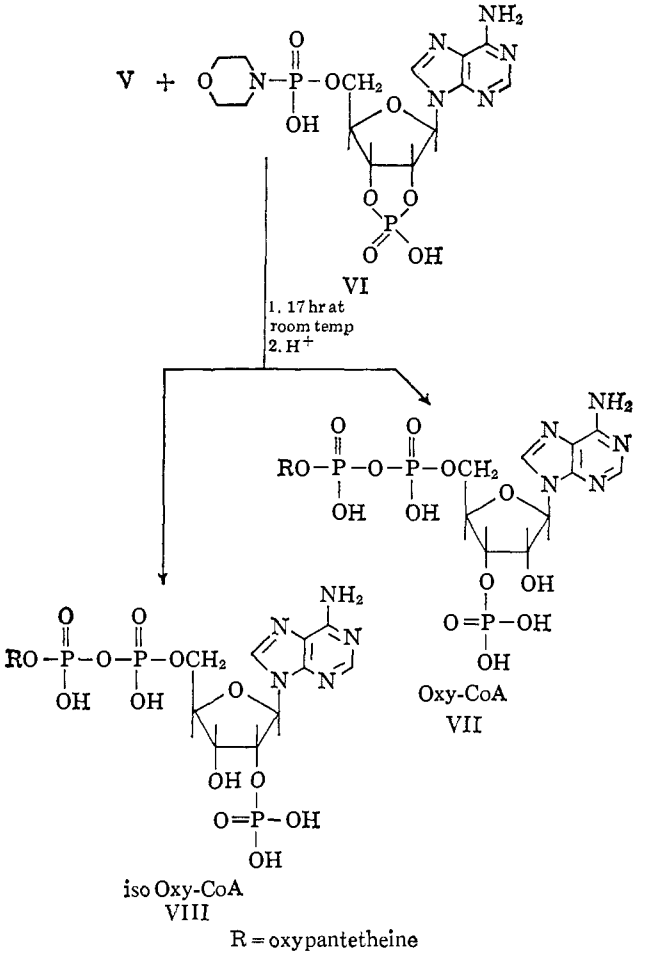


bis(4-morpholine N,N′-dicyclohexylcarboxamidinium)-adenosine-2′,3′-cyclic phosphate-5′ phosphoromorpholidate[5] and subsequent mild acid hydrolysis to yield the isomeric mixture of oxy-CoA (VII) and isooxy-CoA (VIII) is shown in Scheme II. 3′-Dephosphooxycoenzyme A was prepared by an analogous procedure, condensation of V with adenosine 5′-phosphoromorpholidate.

Synthesis of D-Oxypantetheine-4′ Phosphate (V). The synthesis of this key compound was patterned after procedures used in the synthesis of pantetheine analogs[9] and pantetheine-4′ phosphate.[5,11] Benzyl chloride was condensed with 2-aminoethanol by a modification of the procedure of Lappas and Jenkins[12] to yield 2-benzyloxyethylamine hydrochloride (I), which was

(9) C. J. Stewart, V. H. Cheldelin, and T. E. King, *J. Biol. Chem.*, 215, 319 (1955).
(10) W. S. Pierpoint, D. E. Hughes, J. Baddiley, and A. P. Mathias, *Biochem. J.*, 61, 190 (1955).
(11) J. Baddiley and E. M. Thain, *J. Chem. Soc.*, 1610 (1953).
(12) L. C. Lappas and G. L. Jenkins, *J. Am. Pharm. Assoc. Ed.*, 41, 257 (1952).

Scheme II



treated with 3-phthalimidopropionyl chloride to form N-(2-benzyloxyethyl)-3-phthalimidopropionamide (II). Treatment of II with hydrazine to remove the phthalyl group produced benzyloxyaletheine hydrochloride (III). The free base, benzyloxyaletheine, was prepared by addition of sodium methoxide to an absolute ethanol solution of III in order to precipitate sodium chloride. The over-all yield of various preparations of III, based on 2-aminoethanol, ranged from 31 to 50%.

D-Benzyloxypantetheine (IV) was produced by fusing D-pantolactone with benzyloxyaletheine. During the early stages of this work it was observed that there was considerable variation in yield of IV and that there were variable amounts of unreacted benzyloxyaletheine present in the reaction mixtures. The addition of a trace amount of sodium methoxide to the fusion mixture obviated this difficulty presumably by acting as a base catalyst. Traces of sodium methoxide, sodium chloride, and any unreacted free base of III were effectively removed by passing an aqueous solution of the fusion mixture through ion-exchange columns. By this procedure IV was obtained as a colorless, viscous, hygroscopic oil in 85% yield.

For the synthesis of D-oxypantetheine-4′ phosphate from IV, dibenzylphosphorochloridate was chosen as the preferred phosphorylating reagent. Moffatt and Khorana[5] have reported that dibenzylphosphorochloridate only attacks the 4′-hydroxyl group of pantethine to yield pantethine-4′ dibenzylphosphate. D-Benzyloxypantetheine (IV) was readily phosphorylated to produce D-benzyloxypantetheine-4′ dibenzylphosphate

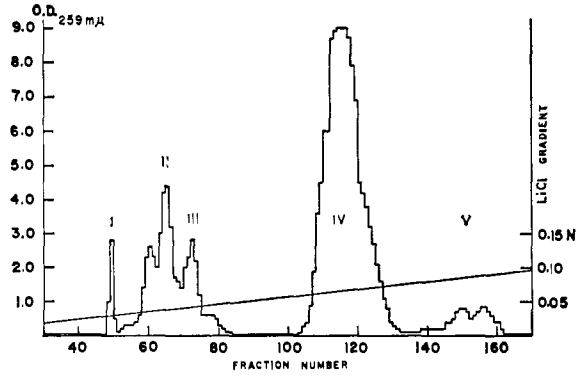


Figure 1. Products of the reaction of adenosine-2′,3′-cyclic phosphate 5′-phosphoromorpholidate with oxypantetheine-4′ phosphate. Chromatography on a DEAE-cellulose column using a linear salt gradient as shown: peak I, adenosine-5′ phosphate; peak II, adenosine-2′(3′),5′ diphosphate; peak III, unidentified; peak IV, isooxy-coenzyme A + oxy-coenzyme A; peak V, P^1,P^2-bis(2′(3′)-O-phosphoryladenosine-5′) pyrophosphate.

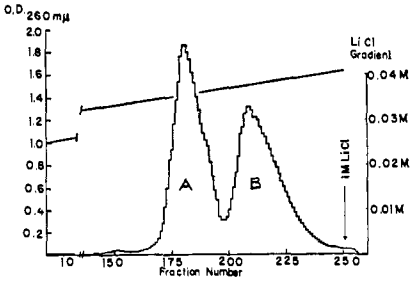


Figure 2. Separation of oxy-coenzyme A isomers on a TEAE-cellulose column using a linear gradient as shown: peak A, isooxy-coenzyme A; peak B, oxy-coenzyme A.

which was not isolated. The benzyl blocking groups were removed by catalytic hydrogenolysis. The catalyst, Pd-$BaSO_4$, was originally employed for hydrogenolysis but complete removal of the benzyl groups was not achieved. Adams catalyst, PtO_2, effected complete removal of the blocking groups to yield V in 50–73% among the various preparations. When V was subjected to paper chromatography a minor contaminant was observed as a slower moving peptide, phosphorus-containing spot (R_f 0.30 compared to 0.45 in system A). Although the contaminant was not present in sufficient quantity to permit isolation and identification, it was assumed to be 4′-phosphopantothenic acid on the basis of its chromatographic behavior. Purification of V was easily achieved by adsorption on a DEAE-cellulose column and elution with a LiCl linear gradient.

The observation that D-oxypantetheine-4′ phosphate gave a positive Biuret reaction[13] greatly facilitated the location of this compound in the DEAE-cellulose column eluates. Aliquots of each fraction were added to the reagent and only those fractions containing V produced the characteristic blue-violet color of the Biuret reaction.

The synthetic approach employed in this work has permitted retention of optical purity. D-Oxypantetheine-4′ phosphate exhibited $[\alpha]^{26}D$ +16.7°, compared to literature values ranging from +10.8°[11] to +14.6°[14] for D-pantetheine-4′ phosphate and +8.4°[8] for D-selenopantethine-4′ phosphate.

Synthesis of Oxy-Coenzyme A (VII) and 3′-Dephosphooxy-Coenzyme A. The condensation of D-oxypantetheine-4′ phosphate with adenosine-2′,3′-cyclic phosphate-5′ phosphoromorpholidate (VI) proceeded readily in anhydrous pyridine at room temperature overnight. Treatment with dilute hydrochloric acid for 1 hr at room temperature opened the 2′,3′-cyclic phosphate ring and decomposed any unreacted phosphoromorpholidate. The reaction mixture was resolved by ion-exchange chromatography on a DEAE-cellulose column. The elution pattern, shown in Figure 1, was very similar to the pattern obtained in the synthesis of CoA.[5] The mixed isomers, VII and VIII, were isolated as a single peak, peak IV (59% yield), at a LiCl gradient concentration range of 0.06–0.07 *M* in 0.003 *N* HCl. Under the same conditions the mixed CoA[5] isomers were reported to be eluted at approximately 0.075 *M* LiCl in 0.003 *N* HCl. However, the oxy-CoA isomers were not resolved on an ECTEOLA-cellulose column under conditions used for the separation[5] of CoA isomers. When the mixed oxy-CoA isomers were subjected to ECTEOLA-cellulose chromatography traces of adenosine-2′(3′),5′ diphosphate were removed and an analytically pure sample of the isomeric oxy-CoA (VII)–isooxy-CoA (VIII) mixture was obtained. The ultraviolet absorption spectrum was characteristic of an adenosine-containing compound at pH 7.0.

Separation of isomers was accomplished by chromatography on a TEAE-cellulose column as shown in Figure 2. Peaks A and B were carefully isolated and characterized by digestion of aliquots with venom phosphodiesterase. Peak A was found to be isooxy-CoA, producing only adenosine-2′,5′ diphosphate and oxypantetheine-4′ phosphate as enzymatic degradation products. Peak B was oxy-CoA, yielding only adenosine-3′,5′ diphosphate and oxypantetheine-4′ phosphate on enzymatic degradation.

3′-Dephosphooxy-coenzyme A was readily prepared by an analogous procedure. D-Oxypantetheine-4′ phosphate was condensed with 4-morpholine N,N′-dicyclohexylcarboxamidinium adenosine-5′ phosphoromorpholidate[15] overnight in pyridine. The product was separated from the reaction mixture by DEAE-cellulose column chromatography in a yield of 52%. Only adenosine-5′ phosphate and oxypantetheine-4′ phosphate were produced on venom phosphodiesterase degradation.

Phosphotransacetylase Inhibition Studies. The phosphotransacetylase enzyme[16–18] has been shown to exhibit a high specificity for CoA. The disulfide forms of CoA iso-CoA are enzymatically inactive.[5] Dephospho-CoA is *ca.* 0.5% as active as CoA.[17] Seleno-coenzyme A[8] is *neither* inhibitory nor catalytically active in this enzyme system. Oxy-coenzyme A, on the other hand, is a remarkably active competitive inhibitor of CoA.

From the Lineweaver-Burk plot[19] (Figure 3) of the

(13) E. Layne, *Methods Enzymol.*, 3, 450 (1957).
(14) J. M. Osbond, British Patent 749, 715 (1956); *Chem. Abstr.*, 51, 2853 (1957).
(15) J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, 83, 649 (1961).
(16) E. R. Stadtman, *J. Biol. Chem.*, 196, 527 (1952).
(17) H. U. Bergmeyer, G. Holz, H. Klotzsch, and G. Lang, *Biochem. Z.*, 338, 114 (1963).
(18) Systematic name, phosphate acetyltransferase, E. C. 2.3.1.8.
(19) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, 56, 658 (1934).

Table I. R_f Values of Compounds

Compound	R_f Solvent A	Solvent B	Solvent C	Solvent D
2-Benzyloxyethylamine hydrochloride	0.76			
N-(2-Benzyloxyethyl)-3-phthalimidopropionamide	0.94			
Benzyloxyaletheine hydrochloride	0.78			
Benzyloxypantetheine	0.86			
D-Oxypantetheine-4′ phosphate	0.45	0.53	0.53	
Adenosine-5′ phosphate	0.24	0.35	0.15	
Adenosine-5′ phosphoromorpholidate	...	...	0.51	
3′-Dephosphooxy-coenzyme A	0.25	0.56	0.45	
Adenosine-2′,5′ diphosphate	0.15	0.25	0.04	0.51
Adenosine-3′,5′ diphosphate	0.15	0.25	0.04	0.44
Adenosine-2′,3′-cyclic phosphate-5′ phosphoromorpholidate	...	...	0.49	...
Isooxy-coenzyme A	0.16	0.37	0.16	0.47
Oxy-coenzyme A	0.16	0.37	0.16	0.45

data obtained with oxy-CoA, the [I]/[S] ratio at 50% inhibition is approximately 0.001.

The K_I's obtained for oxy-CoA and the isomeric isooxy-CoA–oxy-CoA mixture were 3.5×10^{-7} and 6.0×10^{-7} M, respectively. These values are consistent with the observation that the isomeric mixture is approximately 50% 3′ isomer and that the 2′ isomer, isooxy-CoA, is only 0.5% as active a CoA antagonist as oxy-CoA. 3′-Dephosphooxy-CoA has only 0.1% the antagonistic activity of oxy-CoA.

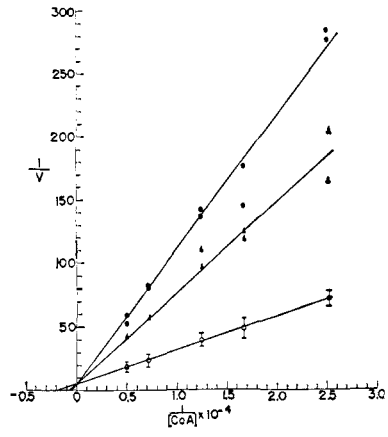


Figure 3 Double reciprocal plot showing competitive inhibition of the phosphotransacetylase reaction by oxy-CoA. The ordinate is the number of seconds required to observe an $OD_{233\ m\mu}$ change of 0.1, after addition of enzyme: Φ, average of replicate determination plus or minus the standard error of CoA alone; ▲, CoA in the presence of 1.01×10^{-6} M oxy-CoA mixed isomers; ●, CoA in the presence of 1.05×10^{-6} M oxy-CoA.

Oxy-coenzyme A to the best of our knowledge is the only known antimetabolite specific for coenzyme A in the phosphotransacetylase reaction. As such it offers the potential as a coenzyme A antagonist uniquely suited for the study of coenzyme A dependent reactions.

Experimental Section

Methods and Materials. Paper chromatography was carried out by the descending technique on Whatman No. 1 or No. 3MM paper. The solvent systems used were: solvent A, *n*-butyl alcohol–acetic acid–water (5:2:3); solvent B, *n*-propyl alcohol–concentrated ammonia–water (55:10:35); solvent C, ethyl alcohol–1 M ammonium acetate, pH 7.5 (7:3); solvent D, saturated ammonium sulfate–1 M sodium acetate, pH 6.0–isopropyl alcohol (79:19:2).

Adenine-containing compounds were located by their characteristic ability to absorb ultraviolet light. Phosphate-containing compounds were located by the method of Bandurski and Axelrod.[20] The chlorine–starch–iodine method of Rydon and Smith[21] was used to locate amino- or amide-containing compounds. Table I lists the R_f values of the various compounds.

DEAE-cellulose (Selectacel standard type) and ECTEOLA-cellulose (Selectacel Type 40), purchased from Carl Schleicher and Schuell, and TEAE-cellulose (Serva cellulose ion exchanger, Lot B 2253), purchased from Gallard Schlesinger, were used for column chromatography. Venom phosphodiesterase (*Crotalus adamanteus*) was purchased from Worthington Biochemical Corp. Phosphotransacetylase, Lot No. 06155109, was purchased from Boehringer Mannheim Corp.

2-Benzyloxyethylamine Hydrochloride (I).[12] Ethanolamine (30.5 g, 0.5 mole) and sodium (11.5 g, 0.5 g-atom) were added to 150 ml of toluene. The mixture was refluxed gently with constant stirring until disappearance of metallic sodium was complete (5 hr). Sixty-one milliliters of benzyl chloride (0.5 mole) in toluene (61 ml) was added dropwise with stirring. Following the addition of benzyl chloride the mixture was refluxed for an additional 20 min and then permitted to cool to room temperature. Sodium chloride was removed by suction filtration and washed with toluene (25 ml). The combined filtrate and washings were cooled to 5° and saturated with anhydrous hydrogen chloride gas. The temperature was carefully maintained during this period. The crude salt was isolated by suction filtration to yield 2-benzyloxyethylamine hydrochloride (64.5 g, 69%) melting at 134–138°. Recrystallization from anhydrous isopropyl alcohol (250 ml) gave 53.2 g (57%) of white platelets, mp 143–145°.[22]

Anal.[23] Calcd for $C_9H_{14}NOCl$: C, 57.60; H, 7.52; N, 7.46; Cl, 18.89. Found: C, 57.48, 57.42; H, 7.90, 7.84; N, 7.42, 7.52; Cl, 19.26, 19.12.

N-(2-Benzyloxyethyl)-3-phthalimidopropionamide (II). 2-Benzyloxyethylamine hydrochloride (18.8 g, 0.10 mole) and pyridine (16.6 g, 0.21 mole) were dissolved in N,N-dimethylformamide (100 ml) and the solution was placed in an ice bath to chill. 3-Phthalimidopropionyl chloride[24] (23.8 g, 0.10 mole), dissolved in N,N-dimethylformamide (100 ml), was added to the previously chilled solution. The resultant solution was placed in a refrigerator at 4°. After standing overnight, the solution was slowly added to 1.2 l. of water with constant stirring to precipitate the product. The product was collected by suction filtration and sucked as dry as possible. Then the damp filter cake was dissolved in methyl alcohol (150 ml) by heating and allowed to crystallize at 5°. The crude product (28.1 g, 80% yield) melted at 131–135°. Recrystallization from methyl alcohol yielded 24.8 g, 71%, of flocculent, white needles, mp 136–137°.

Anal. Calcd for $C_{20}H_{20}N_2O_4$: C, 68.16; H, 5.72; N, 7.95. Found: C, 68.35, 68.58; H, 5.63, 5.66; N, 7.92, 7.72.

Benzyloxyaletheine Hydrochloride (III). N-(2-Benzyloxyethyl)-3-phthalimidopropionamide (28.16 g, 0.08 mole) was dissolved in

(20) R. S. Bandurski and B. Axelrod, *J. Biol. Chem.*, **193**, 405 (1951).
(21) H. N. Rydon and P. W. G. Smith, *Nature*, **169**, 922 (1952).
(22) Melting points are uncorrected.
(23) Elemental analyses were performed by either West Coast Analytical Co., Inc., El Cerrito, Calif., or C. F. Geiger, Ontario, Calif.
(24) E. E. Snell and E. L. Wittle, *Methods Enzymol.*, **3**, 919 (1957).

methyl alcohol (200 ml) by heating. Hydrazine (95%, 2.72 ml, 0.084 mole) was added and the mixture was refluxed for 1.5 hr with constant stirring. The resulting mixture was brought to room temperature and the solvent removed *in vacuo*. The residue was warmed to 50° with 2 *N* hydrochloric acid (200 ml) for 10 min and allowed to cool to room temperature. Phthalylhydrazide was removed by suction filtration and the filtrate was concentrated *in vacuo*. The residue was dissolved in absolute ethyl alcohol, any insoluble material was removed by filtration, and the clear solution was evaporated to dryness *in vacuo*. The residue was dried by two more evaporations with absolute ethyl alcohol, dissolved in absolute ethyl alcohol (50 ml), and precipitated with anhydrous ether. The precipitate was collected by centrifugation and immediately dried in a moisture-free atmosphere (the product is highly hygroscopic) to yield benzyloxyaletheine hydrochloride (15.8 g, 77%) in the form of a white powder, mp 121–124°.

For purification the product was dissolved in anhydrous isopropyl alcohol (100 ml) by gentle heating and allowed to crystallize at room temperature. The crystallized product was collected by suction filtration, not allowing the product to be exposed to moisture. The product was washed with anhydrous ether and dried *in vacuo* to yield purified benzyloxyaletheine hydrochloride (12.1 g, 77%) in the form of a white, crystalline powder melting at 125–127°. An additional 2.8 g of crude product could be recovered by adding anhydrous ether to the filtrate.

For purposes of analysis, benzyloxyaletheine hydrochloride was recrystallized from anhydrous isopropyl alcohol, washed with anhydrous ether, and dried *in vacuo*, and a portion was submitted for analysis, mp 125–127°.

Anal. Calcd for $C_{12}H_{19}N_2O_2Cl$: C, 55.70; H, 7.40; N, 10.83; Cl, 13.70. Found: C, 55.97, 56.02; H, 7.63, 7.77; N, 10.92, 10.88; Cl, 13.5, 13.7.

D-Benzyloxypantetheine (IV). Benzyloxyaletheine hydrochloride (5.17 g, 0.02 mole) was dissolved in absolute ethyl alcohol (60 ml). Methanolic sodium methoxide (17.4 ml, 0.02 mole, 1.15 *N*) was added dropwise with swirling and the mixture was allowed to stand for 20 min at room temperature. Sodium chloride was removed by filtration and the solvent was removed *in vacuo* to yield an oily residue. The residue was evaporated twice with absolute ethyl alcohol to obtain dry benzyloxyaletheine. When dissolving in absolute ethyl alcohol, a small amount of insoluble material appeared which was removed by filtration. D-Pantoyl lactone (2.6 g, 0.02 mole) and 1 drop of the sodium methoxide solution as a catalyst were added to the flask and the contents of the stoppered flask melted together at 55° in a water bath. The flask was placed in a 65° oven for 12 hr. Then, the contents were dissolved in water (75 ml) and passed through a Dowex 1-X4 (hydroxide form) column. The effluent was concentrated *in vacuo* to a volume of 75 ml and passed through an Amberlite IR-120 (hydrogen ion form) column. The effluent was concentrated *in vacuo* and evaporated twice with absolute ethyl alcohol. D-Benzyloxypantetheine (6.00 g, 85%) was obtained in the form of a colorless, viscous liquid. A portion of the product was dried *in vacuo* at 80° for 2 days and submitted for analysis.

Anal. Calcd for $C_{18}H_{28}N_2O_5$: C, 61.34; H, 8.01; N, 7.95. Found: C, 61.26, 61.40; H, 8.23, 8.14; N, 7.90, 7.98.

D-Oxypantetheine-4′ Phosphate (V). D-Benzyloxypantetheine (2.93 g, 8.31 mmoles) was evaporated twice with anhydrous pyridine, then dissolved in anhydrous pyridine (50 ml), and frozen in a Dry Ice–acetone bath. A solution of dibenzylphosphorochloridate was prepared by dissolving N-chlorosuccinimide (3.33 g, 24.9 mmoles) in warm, dry benzene (50 ml) and adding dibenzyl phosphite (6.53 g, 24.9 mmoles) dropwise with swirling. After standing at room temperature for 2 hr, the phosphorochloridate solution was decanted into the frozen pyridine solution, and the resultant mixture was thawed and quickly refrozen. The mixture was placed in the deep freeze (−18°) and allowed to stand for 20 hr. Water (28 ml) was added, the flask was swirled, and after 20 min at room temperature the yellow solution was evaporated *in vacuo* with a bath temperature less than 35°. The residue was dissolved in a mixture of ethyl acetate (25 ml) and 2 *N* H_2SO_4 (25 ml), and extracted. The organic phase was extracted three times each with 2 *N* H_2SO_4 (25 ml), 10% $NaHCO_3$ (25 ml), and saturated Na_2SO_4 (25 ml), in that order. The ethyl acetate phase was dried over anhydrous sodium sulfate and the solvent evaporated *in vacuo*. The resulting syrup was dissolved in a mixture of isopropyl alcohol (40 ml) and water (10 ml), and Adams catalyst (2 g) was added. Hydrogenolysis was commenced at room temperature and atmospheric pressure and allowed to proceed until hydrogen uptake ceased (6.5 hr). The catalyst was removed by centrifugation, washed once with isopropyl alcohol–water, and recentrifuged. The wash was combined with the supernatant solution and evaporated *in vacuo* with a bath temperature less than 35° yielding a pale yellow syrup. The syrup was dissolved in water (50 ml) and adjusted to pH 7.5 with 1 *N* barium hydroxide. The precipitate was removed by centrifugation at 10,000 rpm, yielding a clear, colorless solution. The supernatant solution was evaporated *in vacuo* to yield a clear glass. The glass was dissolved in methyl alcohol (15 ml) and a small amount of turbidity was removed by centrifugation at 18,000 rpm. Acetone (200 ml) was added to precipitate the product. The white precipitate was collected by centrifugation and dried *in vacuo* over P_2O_5. The crude barium salt of D-oxypantetheine-4′ phosphate (2.34 g, 59%) was obtained in the form of a white powder.

For purposes of purification, a portion of the above barium salt (315 mg) was dissolved in water (10 ml) and applied to a 2.7 × 50 cm DEAE–cellulose (chloride form) column. After washing the column well with water it was eluted with a linear gradient using 2.0 l. of 0.5 *N* lithium chloride in the reservoir and 2.0 l. of water in the mixing vessel. Peptide-containing material, located by a positive Biuret[18] reaction, was found in fractions 48–56. Alternatively, this peak could be located by spotting aliquots on paper and spraying with phosphate reagent.[20] This peak was pooled and the solvent was removed by evaporation *in vacuo*. The white solid residue was treated repeatedly with a mixture (40 ml) of methyl alcohol–acetone (1:15) until a negative chloride test was obtained. The precipitate was dried overnight *in vacuo* over P_2O_5 to yield the dilithium salt of D-oxypantetheine-4′ phosphate (180 mg, 78%) in the form of a white powder. The product was found homogeneous on paper chromatography (see Table I). The specific rotation was observed to be $[\alpha]^{26}D$ +16.7°.

For purposes of analysis, a portion of the product (100 mg) was dissolved in methyl alcohol and a trace of turbidity removed by centrifugation at 18,000 rpm. Acetone was added to precipitate the product which was dried *in vacuo* at 100° for 6 hr.

Anal. Calcd for $C_{11}H_{21}N_2O_8PLi_2$: C, 37.30; H, 5.98; N, 7.91. Found: C, 37.59; H, 5.97; N, 8.03.

Oxy-Coenzyme A and Isooxy-Coenzyme A (VII and VIII). D-Oxypantetheine-4′ phosphate, dilithium salt, (223 mg, 0.48 mmole), was converted to the pyridinium salt by passage through a 1 × 5 cm Dowex 50W-X8 (pyridinium form) column and evaporating the effluent to dryness *in vacuo*. Final traces of water were removed by several repeated additions and evaporations *in vacuo* of anhydrous pyridine. The D-oxypantetheine-4′ phosphate in anhydrous pyridine (10 ml) was added to an anhydrous pyridine solution (3 ml) of bis(4-morpholine N,N′-dicyclohexylcarboxamidinium)adenosine-2′,3′-cyclic phosphate 5′-phosphoromorpholidate (222 mg, 0.2 mole) and the mixture was evaporated to a viscous oil *in vacuo*. After an additional evaporation with anhydrous pyridine, the mixture was dissolved in anhydrous pyridine (10 ml) and permitted to react overnight at room temperature in a tightly stoppered flask. Pyridine was subsequently removed by several evaporations *in vacuo* with water; the unreacted morpholidate decomposed and the 2′,3′-cyclic phosphate was opened by treatment with 0.1 *N* HCl (10 ml) for 1 hr at room temperature. Several evaporations *in vacuo* with methyl alcohol were used to remove the HCl. The residue was dissolved in water (25 ml), the pH was adjusted to 6.0 with dilute NH_4OH, and the solution applied to a 2.2 × 30 cm DEAE–cellulose (chloride form) column. The column was washed with water until the effluent was essentially free of ultraviolet absorbing material. Then the adsorbed compounds were eluted by application of an acidic lithium chloride linear gradient. The reservoir contained 2.0 l. of 0.15 *M* LiCl in 0.003 *N* HCl and the mixing vessel contained 2.0 l. of 0.003 *N* HCl. Fifteen-milliliter fractions were collected. The oxy-CoA (mixed isomers) was found in tubes 106–129. This peak was pooled and found to contain 2132 OD units at 259 mμ (72% of the total applied OD units). After adjusting the pH to 4.5 with 1 *N* LiOH, the peak was evaporated to dryness *in vacuo*. Lithium chloride was removed from the solid white residue by repeated extraction with 40-ml fractions of methyl alcohol–acetone (1:15). After drying over P_2O_5 *in vacuo* at room temperature overnight a yield of 89 mg (59%) of the mixed isomers of oxy-CoA was obtained.

In an attempt to separate the 2′,3′-phosphate isomers of oxy-CoA an analytically pure sample of the mixed isomers was prepared. Oxy-CoA, mixed isomers, 1940 OD units, was applied to a 2.2 × 30 cm ECTEOLA–cellulose (chloride form) column and eluted by an acidic lithium chloride linear gradient. The reservoir contained 2 l. of 0.10 *M* LiCl in 0.003 *N* HCl and the mixing vessel contained 2 l. of 0.03 *M* LiCl in 0.003 *N* HCl. Tubes 47–58 contained the

major peak, 1540 OD units (97% of the total recovered OD units). A white powder was obtained by working up this peak in the manner previously described for the DEAE–cellulose column effluent peak. After drying for 3 hr at 100° over P_2O_5 *in vacuo*, the mixed oxy-CoA isomers were obtained as the trilithium salt trihydrate in a yield of 78 mg (52%).

Venom phosphodiesterase digestion of this material produced spots identical with oxypantetheine-4′ phosphate and adenosine-2′(3′),5′ phosphate when subjected to paper chromatography.

Anal. Calcd for $C_{21}H_{33}N_7O_{17}P_3Li_3 \cdot 3H_2O$: C, 30.64; H, 4.78; N, 11.91. Found: C, 30.24; 30.51; H, 4.93, 5.14; N, 12.40.

The ultraviolet absorption spectrum, at pH 7.0, was determined on a Cary Model 14 spectrophotometer: λ_{max} 259 mμ, λ_{min} 230 mμ; absorbency ratios, 280/260 = 0.15, 250/260 = 0.81; the extinction coefficient (calculated from the spectrum of the trilithium salt trihydrate, mol wt 823) was $\epsilon_{259m\mu}$ 14.8×10^3.

Anal.[25] Calcd for $C_{21}H_{33}N_7O_{17}P_3Li_3 \cdot 3H_2O$ (adenosine:P): P, 11.3; A:P = 1:3. Found: P, 11.6; A:P = 1:3.09.

Separation of Oxy-Coenzyme A (VII) from Isooxy-Coenzyme A (VIII). The isooxy-coenzyme A–oxy-coenzyme A mixed isomers (53 mg, 825 OD units) were dissolved in 0.003 *N* HCl (25 ml) and applied to a 1.8 × 80 cm TEAE–cellulose (chloride form) column. The column was washed with 0.003 *N* HCl (150 ml) and then a linear gradient of lithium chloride in 0.003 *N* HCl was applied. The mixing vessel contained 4 l. of 0.025 *M* LiCl in 0.003 *N* HCl and the reservoir contained 4 l. of 0.055 *M* LiCl in 0.003 *N* HCl. Fifteen-milliliter fractions were collected and the optical density (260 mμ) of each fraction was determined. The effluent did not rise above 0.05 OD until fraction 164. Then two well-defined peaks with a slight overlapping area were obtained (Figure 2). Isooxycoenzyme A was present in peak A, fractions 166–193 inclusive (320 OD units). The overlapping fractions, 194–202, contained 62 OD units. Peak B, fractions 203–240, contained oxy-coenzyme A (374 OD units).

The total recovery in fractions 166 to 240 inclusive was 756 OD units (92%). Each of the peaks was characterized by venom phosphodiesterase degradation. The only detectable nucleotides after enzyme digestion and paper chromatography were adenosine-2′,5′ diphosphate from the peak A and adenosine-3′,5′ diphosphate from peak B.

3′-Dephosphooxy-Coenzyme A. 4-Morpholine N,N′-dicyclohexylcarboxamidinium adenosine-5′ phosphoromorpholidate[15] (153 mg, 0.2 mmole) was dried by three evaporations with anhydrous pyridine (5 ml each). Separately, D-oxypantetheine-4′ phosphate dilithium salt (223 mg, 0.48 mmole) was converted to the pyridinium salt and dried as described in the preparation of oxy-CoA. The D-oxypantetheine-4′ phosphate in anhydrous pyridine (10 ml) was added to the morpholidate and the mixture evaporated to an oil *in vacuo*. The residue was dissolved in anhydrous pyridine (10 ml) and permitted to react overnight at room temperature in a tightly stoppered flask. Pyridine was subsequently removed by several evaporations *in vacuo* with water; the residue was dissolved in water (25 ml) and applied to a 2.2 × 30 cm DEAE–cellulose (chloride form) column. The column was washed well with water and the adsorbed compounds were eluted by application of an acidic lithium chloride linear gradient. The reservoir contained 2.0 l. of 0.07 *N* lithium chloride in 0.003 *N* hydrochloric acid and the mixing vessel contained 2.0 l. of 0.003 *N* hydrochloric acid. Fifteen-milliliter fractions were collected. The 3′-dephosphooxy-CoA was found in fractions 75–93. Only oxypantetheine-4′ phosphate and adenosine-5′ phosphate were detected on paper chromatograms of the venom phosphodiesterase digestion mixture. 3′-Dephosphooxy-coenzyme A dilithium salt tetrahydrate (78 mg, 52% yield) was obtained as a fine, white powder after working up the peak in the manner described in the oxy-coenzyme A preparation and drying *in vacuo* over P_2O_5 at room temperature overnight.

The sample submitted for analysis was dried an additional 3 hr *in vacuo* over P_2O_5 at 100°.

Anal. Calcd for $C_{21}H_{33}N_7O_{14}P_2Li_2 \cdot 4H_2O$: C, 33.39; H, 5.47; N, 12.98. Found: C, 33.66; H, 5.88; N, 12.60.

Venom Phosphodiesterase Degradations. Degradations were accomplished by incubating 1–2 mg of the coenzyme A analogs, 100 μmoles of Tris–HCl buffer[26] (pH 9.0), and 0.2 mg of venom phosphodiesterate in a total volume of 0.4 ml for 90 min at 38°. Aliquots were directly spotted on sheets of chromatography paper and developed with the various solvent systems.

Phosphotransacetylase Inhibition. Phosphotransacetylase assays were performed by the method of Bergmeyer, *et al.*[17] The components of the system were: 235 μmoles of Tris–HCl buffer, pH 7.4; 1.5 mg of glutathione; 8 mg of acetylphosphate (lithium salt); 30 μmoles of ammonium sulfate; 0.33 μg of phosphotransacetylase; 0.134 to 0.670 μmole of CoA; inhibitor (Figure 3); and 3.0 ml total volume.

Acknowledgments. The authors are indebted to Dr. Grant R. Bartlett for aiding with the phosphorus analyses, and to Mr. Bruce Chassy for assisting in the early stages of the synthetic work.

(25) Phosphorus was determined by the Fiske–SubbaRow method as modified by G. R. Bartlett, *J. Biol. Chem.*, 234, 466 (1959).

(26) Tris = 2-amino-2-hydroxymethyl-1,3-propanediol.

Synthesis of the Carboxyl-Terminal Heptapeptide Sequence of Bovine Pancreatic Ribonuclease

James E. Shields and H. Renner

Contribution from the Department of Chemistry, Case Institute of Technology, Cleveland, Ohio 44106. *Received December 23, 1965*

Abstract: The carboxyl-terminal heptapeptide sequence of ribonuclease A, L-valyl-L-histidyl-L-phenylalanyl-L-aspartyl-L-alanyl-L-seryl-L-valine, has been synthesized. The hexapeptide was prepared by the sequential addition of carbobenzoxy derivatives of amino acids to L-valine *t*-butyl ester by the mixed carbonic anhydride procedure. The final residue was added as the *p*-nitrophenyl ester.

The synthesis of peptide sequences from bovine pancreatic ribonuclease has been reported from two laboratories, those of Hofmann[1] in the United States and of Rocchi, *et al.*,[2] in Italy. Hofmann's work has concerned itself so far with the amino terminal eicosapeptide sequence cleaved from the native enzyme by subtilisin.[3] This sequence contains one of the two

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(2) R. Rocchi, F. Marchiori, and E. Scoffone, *Gazz. Chim. Ital.*, 93, 823 (1963).