and evaporated to dryness. Guanosine diphosphate mannose (92 mg.) was isolated by precipitation from methyl alcohol with acetone and ether in the standard way. It was now pure when tested paper-chromatographically and electrophoretically. Spectrophotometric determination of its guanosine content showed it to be a hexahydrate, the yield thus being 63%.

Anal. Calcd. for $C_{16}H_{23}N_5O_{16}P_2Li_2 \cdot 6H_2O$: P, 8.50; P:guanosine:mannose = 2:1:1. Found: P, 8.00; P:guanosine:mannose = 1.88:1.0:1.02.

After drying at 100°, it was obtained as the trihydrate. Calcd. for $C_{16}H_{23}N_5O_{16}P_2Li_2 \cdot 3H_2O$: C, 28.64; H, 4.36; N, 10.44. Found: C, 28.45; H, 4.89; N, 9.77.

Another sample, which was directly isolated by chromatography on Dowex-1 (Cl^-) at *p*H 2.7 and which contained a minor unidentified impurity, was assayed using a partially purified pyrophosphorylase from yeast.[27] Within the limits of the assay the product was completely active. In addition, the synthetic compound served as a substrate for the enzymatic synthesis of L-fucose and colitose.[36]

Acknowledgments.—The work at British Columbia Research Council has been supported by grants from the Life Insurance Medical Research Fund and the National Research Council of Canada. The Rackham Arthritis Research Unit is supported by a grant from the Horace H. Rackham School of Graduate Studies of the University of Michigan. This investigation was supported in part by grants from the National Institutes of Health (A-512), the Michigan Chapter, Arthritis and Rheumatism Foundation, and from the American Cancer Society.

(36) E. C. Heath, *Fed. Proc.*, **19**, 85 (1960).

[CONTRIBUTION FROM THE CHEMISTRY DIVISION OF THE BRITISH COLUMBIA RESEARCH COUNCIL, VANCOUVER, CANADA]

Nucleoside Polyphosphates. XII.[1] The Total Synthesis of Coenzyme A[2]

BY J. G. MOFFATT AND H. G. KHORANA

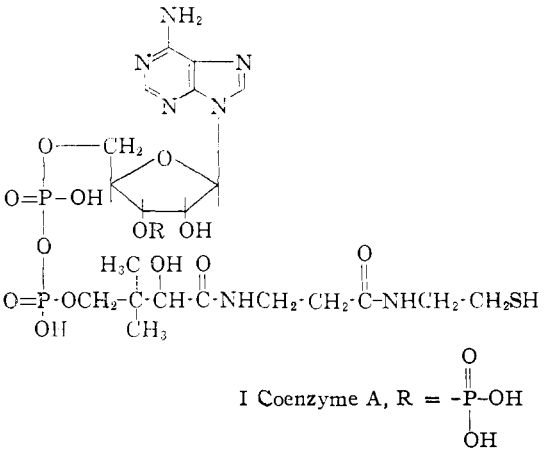
RECEIVED MAY 23, 1960

An improved procedure for the preparation of D-pantetheine-4′ phosphate by direct phosphorylation of D-pantetheine is described. DL-Pantetheine-4′ phosphate was prepared *via* 2′-O,S-di-benzyl-DL-pantetheine by modifications of synthetic procedures previously developed by Baddiley and Thain.[14] In attempts to develop alternative routes to the optically active D-pantetheine-4′ phosphate, several new derivatives of pantethenic acid and related compounds were prepared. The reaction of DL-pantetheine-4′ phosphate with adenosine-5′ phosphoromorpholidate in anhydrous pyridine at room temperature gave 3′-dephospho Coenzyme A (I; R = H) containing the racemic pantetheine moiety in 63% yield. The key intermediate used in the synthesis of Coenzyme A (I) itself was adenosine-2′,3′ cyclic phosphate-5′-phosphoromorpholidate (III), and it was prepared in 91–98% yield by the reaction of adenosine-2′(3′),5′-diphosphate with dicyclohexylcarbodiimide and morpholine. The reaction of III with D-pantetheine-4′ phosphate followed by acidic treatment and reduction with 2-mercaptoethanol gave a mixture of Coenzyme A (I) and *iso*-Coenzyme A (XXV) in an isolated yield of 65%. Rechromatography of the mixture on an ECTEOLA cellulose column gave pure Coenzyme A which in its chemical and enzymic properties was identical with the naturally occurring compound. Chromatographic purification of commercial Coenzyme A is described.

Introduction

In the accompanying papers,[1,3] a general and satisfactory method for the preparation of nucleoside-5′ phosphoromorpholidates and the use of these intermediates in the synthesis of a variety of nucleotide coenzymes has been reported. The progress made in this field encouraged us to undertake the problem of the synthesis of Coenzyme A, which embodies, from the synthetic standpoint, easily the most complex of the chemical structures represented by the nucleotide coenzymes group. The present communication contains a detailed report of our experiments leading to the successful conclusion of this synthetic problem. A brief announcement has been made previously.[4]

Coenzyme A was discovered by Lipmann[5] in 1945 as a co-factor required for the biological acetylation of amines. A further discovery, which focussed attention sharply on the chemistry of its function, was the isolation of "active acetate" and its identification as S-acetyl-Coenzyme A by Lynen[6] and co-workers in 1951. Intensive biochemical research which ensued has, during the past decade, established for Coenzyme A a central position as a mediator of biosynthetic reactions.[7] A number of groups of workers contributed towards the elucidation of its chemical structure[7] and these efforts culminated in the formula I which uniquely ex-



I Coenzyme A, R = $-\overset{\overset{O}{\|}}{P}-OH$ (OH)

(1) Paper XI, S. Roseman, J. J. Distler, J. G. Moffatt and H. G. Khorana, THIS JOURNAL, **83**, 659 (1961).

(2) This work has been supported by grants from the Life Insurance Medical Research Fund, New York, and the National Research Council of Canada. Ottawa.

(3) J. G. Moffatt and H. G. Khorana, THIS JOURNAL, **83**, 649 (1961).

(4) J. G. Moffatt and H. G. Khorana, *ibid.*, **81**, 1265 (1959).

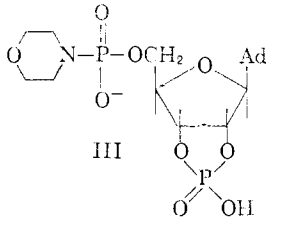
(5) F. Lipmann, *J. Biol. Chem.*, **160**, 173 (1945).

(6) F. Lynen, E. Reichert and L. Rueff, *Ann.*, **574**, 1 (1951).

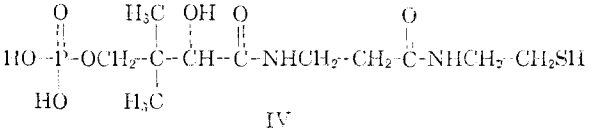
(7) The historical developments associated with Coenzyme A, its structure and function, have been reviewed in a large number of excellent presentations and writings. Only a few are cited here: (a) F. Lipmann, *Harvey Lectures, Ser.*, **44**, 99 (1948–1949); Les Prix Nobel, Stockholm, 1954, p. 151; (b) F. Lynen, *Harvey Lectures, Ser.*, **48**, 210 (1952–1953); (c) J. Baddiley, *Advances in Enzymology*, **16**, 1 (1955); (d) F. M. Strong, "Topics in Microbial Chemistry," John Wiley and Sons, Inc., New York, N. Y., 1958, p. 44.

plained all the data. This structure has been generally accepted for some years, even though the substance has until recently not been obtained in chemically pure form.[8]

Many alternative approaches to the synthesis of Coenzyme A can readily be conceived. All these must converge to the central problems of (a) establishing a pyrophosphate bridge between the two major components, pantetheine and the nucleoside, and (b) introducing, either prior or subsequent to step (a), a phosphomonoester group in the 3′-position of the adenosine moiety.[9] General considerations of the chemistry of phosphate esters at different levels of esterification, of the behavior of phosphomonoesters bearing vicinal hydroxyl groups[10] and, in particular, of the properties of Coenzyme A[7c] led us to conclude that the most promising approach lay in the activation of the 5′-phosphomonoester group of an adenine nucleotide, which already carried the 'extra'-phosphate group in the form of a diester as shown in structure III. The cyclic diester in III was then not expected to interfere in the pyrophosphate forming reaction[1] with the phosphomonoester group of the pantetheine phosphate (IV). The major problems in this approach, thus, were (1) the synthesis of D-pantetheine-4′ phosphate (IV)[11]; (2) the synthesis of III; (3) the formation of the pyrophosphate bond, followed by ring-opening of the 2′,3′-cyclic phosphate group. In the first phase of the work on the formation of the pyrophosphate bond, adenosine-5′ phosphoromorpholidate (V)[1] was used in place of III and the resulting product was P′-



III

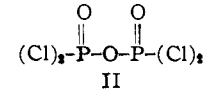


IV

adenosine-5′,P²-pantetheine-4′ pyrophosphate (I; R = H) which is frequently called 3′-dephospho-

(8) In retrospect, it is a great tribute to the large body of ingenious analytical, chemical and enzymic work that permitted the derivation of the correct structure during the years (1948–1953) when preparations of the substance were only 10–60% pure.

(9) A different approach would be the successive phosphorylations of the appropriate hydroxyl functions of the two main organic fragments of Coenzyme A using a pyrophosphorylating agent such as II [M. Becke-Goerhing and J. Sambeth, *Angew. Chem.*, **69**, 640 (1957)].



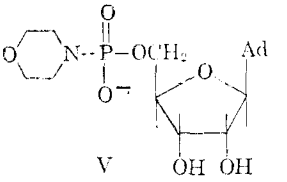
II

This approach was investigated by Lynen and co-workers, F. Lynen, *Proc. 4th Intern. Congr. Biochem.*, Vol. II, 1958, p. 230.

(10) H. G. Khorana, G. M. Tener, J. G. Moffatt and R. S. Wright, THIS JOURNAL, **79**, 430 (1959).

(11) In *Chemical Abstracts* the primary and secondary hydroxyl groups in the pantoyl fragment of pantetheine are numbered 4′ and 2′, respectively.

Coenzyme A and has been established to be the immediate biological precursor of Coenzyme A.[12]



V

Syntheses of D- and DL-Pantetheine-4′ Phosphate (IV).—Most of the previous work on the synthesis of pantetheine phosphates is due to Baddiley and Thain.[7c,13] The direct phosphorylation of D-pantethine (VI), the oxidized form of pantetheine, with dibenzylphosphorochloridate followed by simultaneous removal of the benzyl groups and reduction of the disulfide bond with, sodium in liquid ammonia was reported to give, after a multi-step work-up, D-pantetheine-4′ phosphate[14,15] in 22% yield. In our experiments on the direct phosphorylation of D-pantetheine by the above method followed by the liquid ammonia step, we invariably obtained two major phosphorus- and sulfhydryl-containing products, one of which evidently was the desired D-pantetheine-4′ phosphate. The latter could not, however, be effectively separated from the contaminant, which was encountered in varying amounts (sometimes in amounts equal to that of the 4′-phosphate) by any of the chromatographic procedures tried at this stage of the work. Attention was therefore turned to alternative routes to the synthesis of D-pantetheine-4′ phosphate using appropriately blocked intermediates. While these studies, which are described below, led to more satisfactory means for the synthesis of the racemic pantetheine-4′ phosphate, they failed to provide an alternative procedure for the preparation of the optically pure compound. Recourse was therefore had to a closer study of the products obtained on direct phosphorylation of D-pantethine.

This reinvestigation led to the identification of the side product discussed above as pantetheine-2′,4′ cyclic phosphate (VIII), a compound which has previously been synthesized by Baddiley and Thain.[16] Thus, it could be separated from pantetheine-4′ phosphate by paper-electrophoresis at *p*H 7.5, it having roughly half the mobility of the latter, whereas at *p*H 3.5 the two compounds had the same mobility. The inference from this result that the substance lacked a secondary phosphoryl dissociation was supported by its inertness toward prostate phosphomonoesterase, which under comparable conditions completely dephosphorylated D-pantetheine-4′ phosphate. Subsequently, the

(12) G. D. Novelli, *Federation Proceedings*, **12**, 675 (1953).

(13) The extensive literature on pantetheine itself up to 1952 has been reviewed by E. E. Snell and G. M. Brown, "Advances in Enzymology," Vol. XIV, Interscience Publishers, Inc., New York, N. Y., 1953, p. 49.

(14) J. Baddiley and E. M. Thain, *J. Chem. Soc.*, 1610 (1953).

(15) The hindered 2′-hydroxyl group in pantethine is inert to the phosphorylating agent and the product obtained by direct phosphorylation of pantethine was found to be identical in chromatographic properties with a sample of the racemic pantetheine-4′ phosphate synthesized by an unequivocal route, which is discussed below.

(16) J. Baddiley and E. M. Thain, *J. Chem. Soc.*, 903 (1953).

CHART I

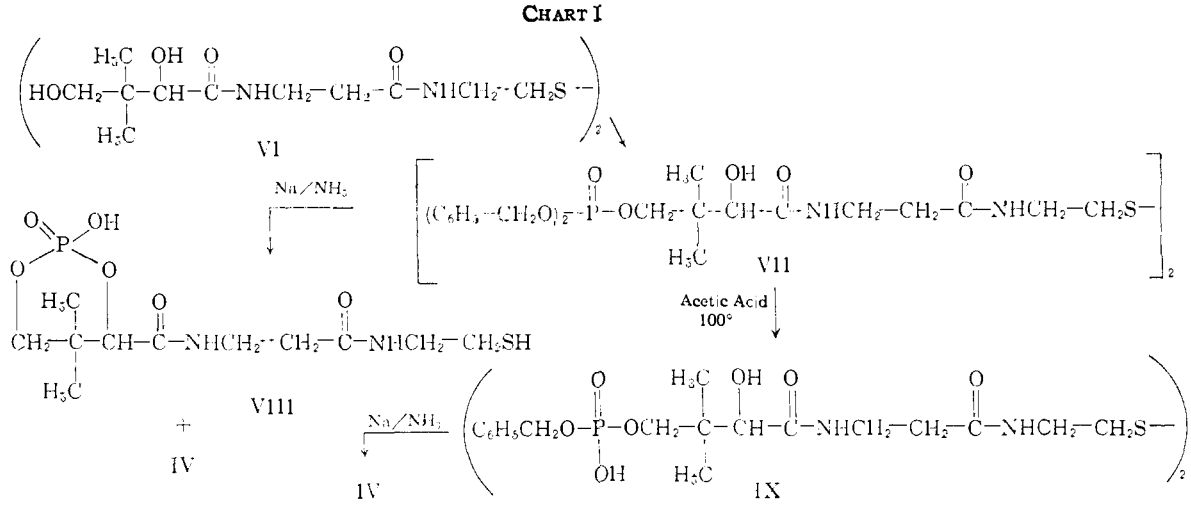
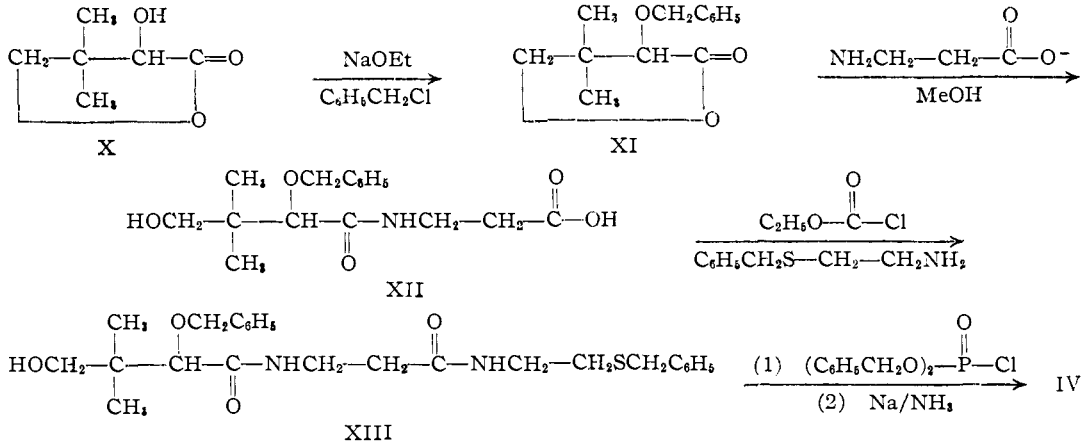


CHART II



cyclic phosphate was isolated in analytically pure form by chromatography.

The formation of the cyclic phosphate presumably occurred as a transesterification reaction of the tertiary phosphate (VII), the initial product of phosphorylation, during treatment with sodium in liquid ammonia. Many examples of this general type of reaction are known[10] and, more specifically, the experience of Baddiley and Thain[16] during the condensation of pantoyl lactone-2-diphenyl phosphate with N-β-alanyl-2-mercaptoethylamine provides a close analogy.

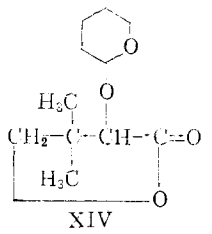
The intramolecular transesterification reactions are known to occur far more rapidly at the tertiary phosphate ester level than at the diester level[10] and in order to minimize it in the present case, VII was first treated with 50% acetic acid at 100° for 1 hr.[17] in order to convert it to, presumably, IX. Subsequent treatment with sodium in liquid ammonia gave a product which was essentially pure D-pantetheine-4′ phosphate. Traces of the cyclic diester (VIII) which were present probably arose during isolation of VII after phosphorylation in pyridine, although care was taken to use as mild conditions as possible. The trace contamination was, however, not considered serious in the use of the product in the pyrophosphate synthesis since diesters of phosphoric acid have previously been found not to enter into this type of condensation reactions.[1] Pantetheine-4′ phosphate (IV) prepared by the modified method was obtained in yields of 40–44% and had $[\alpha]^{24}D$ + 11.7° to + 12.8° as calculated for the anhydrous barium salt.[18]

DL-Pantetheine-4′ phosphate, which was used in much of the early work, especially in the synthesis of 3′-dephospho-Coenzyme A, was prepared by the route (Chart II) previously developed by Baddiley and Thain[14] but with substantial modifications at different steps resulting in higher over-all yields (Experimental section). Thus DL-2′-O,S-dibenzyl pantetheine (XIII) was phosphorylated with dibenzylphosphorochloridate at −20° and, after reduction of the initially obtained neutral product with sodium in liquid ammonia, the analytically pure barium salt of DL-pantetheine-4′ phosphate was obtained in 78% yield by direct precipitation with ether from methyl alcohol. This yield is to be compared with that (23%) obtained by Baddiley and Thain[14] after several precipitations of different metallic salts.[19,20]

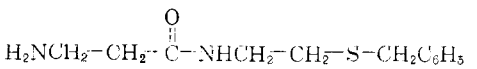
(17) D. M. Brown, L. J. Haynes and A. R. Todd, *J. Chem. Soc.*, 3299 (1950).

(18) An alternative preparation of D-pantetheine-4′ phosphate involving phosphorylation of S-benzyl pantetheine with dibenzylphosphorochloridate has been described by J. M. Osbond [British Pateut 749,715; *Chem. Abs.*, **51**, 2853 (1957)]. We are grateful to Dr. Osbond for sending us a sample of his product.

As mentioned above, much effort was expended on alternative approaches to the synthesis of optically pure D-pantetheine-4′ phosphate. While this phase of the work did not meet with success, the chemistry encountered in the field may be of interest and is placed on record. In the first step of the synthesis in Chart II, completely racemized 2-*O*-benzylpantoyl lactone (XI) was obtained from the optically pure starting material, pantoyl lactone (X).[21] This result was not unexpected under the basic conditions used. The aim of alternative approaches was to avoid racemization[21] at this center by using suitably protected intermediates during assembly of the pantetheine skeleton. Thus, in one approach 2-O-tetrahydropyranylpantoyl lactone (XIV) was prepared in good yield by the acid-catalyzed addition of dihydropyran to pantoyl lactone. Attempted condensation of this derivative with N-(β-alanyl)-S-benzyl-2-mercaptoethylamine (XV), however,



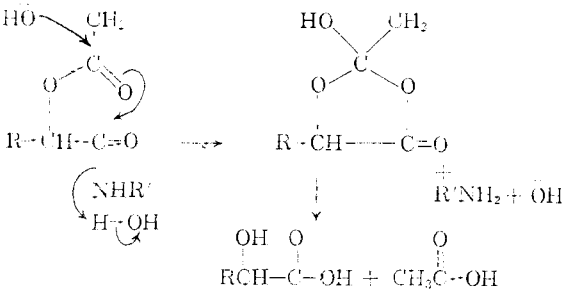
XIV



XV

failed and the starting materials were recovered almost quantitatively. Similarly, in our hands, the reaction of crystalline 2-O-*p*-nitrobenzoyl-(−)-pantoyl lactone[22] and the sodium salt of β-alanine did not proceed satisfactorily either under the conditions of Harris, *et al.*,[23] or on prolonged refluxing of a homogeneous methyl alcoholic solution.[24]

In another approach, methyl pantothenate was converted to crystalline 4′-O-tritylpantothenic acid (XVI) in 77% yield by reaction with triphenylmethyl (trityl) chloride in pyridine followed by alkaline hydrolysis of the methyl ester. Acetylation gave the 2′-*O*-acetyl derivative (XVII) as an amorphous solid after chromatography on silicic acid. Condensation of the latter with S-benzyl-2-mercaptoethylamine gave S-benzyl-4′-*O*-trityl-2′-*O*-acetyl pantetheine (XVIII) as a microcrystalline powder in 78% yield following chromatography on silicic acid, and the next step would have been removal of the trityl group followed by phosphorylation of the 4′-hydroxyl group. However, the entire approach was abandoned when it was discovered that the 2′-acetyloxyl group had a great labilizing effect on the adjacent amide bond. Thus, under the very mildly alkaline conditions tried to remove the acetyl group in 4′-*O*-trityl-2′-*O*-acetyl pantothenic acid (XVII), cleavage of the amide bond occurred with consequent release of β-alanine.[25]



The Synthesis of Adenosine-2′,3′-cyclic Phosphate 5′-Phosphoromorpholidate (III).—Chart III shows the reactions used to prepare this key intermediate. The preparation of the mixed adenosine-2′,5′- and -3′,5′-diphosphates (XXI and XXII) by the direct phosphorylation of adenosine with an excess of dibenzylphosphorochloridate has been described by Cramer, *et al.*,[26] and by Baddiley, *et al.*[27] Both groups of workers used treatment with lithium chloride in hot 2-ethoxyethanol to effect monodebenzylations and the process caused complications due to the partial formation of 2-ethoxyethyl phosphate esters. In our experiments adenosine was phosphorylated with an excess of dibenzylphosphorochloridate and the total products treated with 33% acetic acid to effect partial debenzylation. Subsequent hydrogenolysis and purification gave the diphosphates in 70% yield.[28]

(19) These were evidently aimed at removal of the pantothenic acid phosphates which contaminated the products obtained by Baddiley and Thain.[14] In our experiments, the directly obtained pantetheine-4′ phosphate was free from these contaminants. Only the presence of a trace of the disulfide form could be detected on chromatograms.

(20) Furthermore, our experience with the paper chromatography of pantetheine derivatives is at variance with the observations recorded by the English workers. They routinely used ammoniacal solvent systems for chromatography of the sulfhydryl compounds and obtained nitroprusside (sulfhydryl) positive spots. In this Laboratory the use of ammoniacal solvents invariably led to extensive oxidation of the sulfhydryl compounds to the disulfide form. Acidic solvent systems (see Experimental) have proved satisfactory in the present work.

(21) Pantoyl lactone itself is remarkably resistant to racemization under alkaline conditions [J. Weijland and J. P. Messerly, U. S. Patent 2,377,390, *Chem. Abs.*, **40**, 89 (1946)], probably due to the ionization of the 2-hydroxyl group. The racemization observed, thus, occurs subsequent to the substitution of the hydroxyl group.

(22) E. T. Stiller, S. A. Harris, J. Finkelstein, J. C. Keresztesy and K. Folkers, THIS JOURNAL, **62**, 1785 (1940).

(23) S. A. Harris, G. A. Boyack and K. Folkers, *ibid.*, **63**, 2662 (1941).

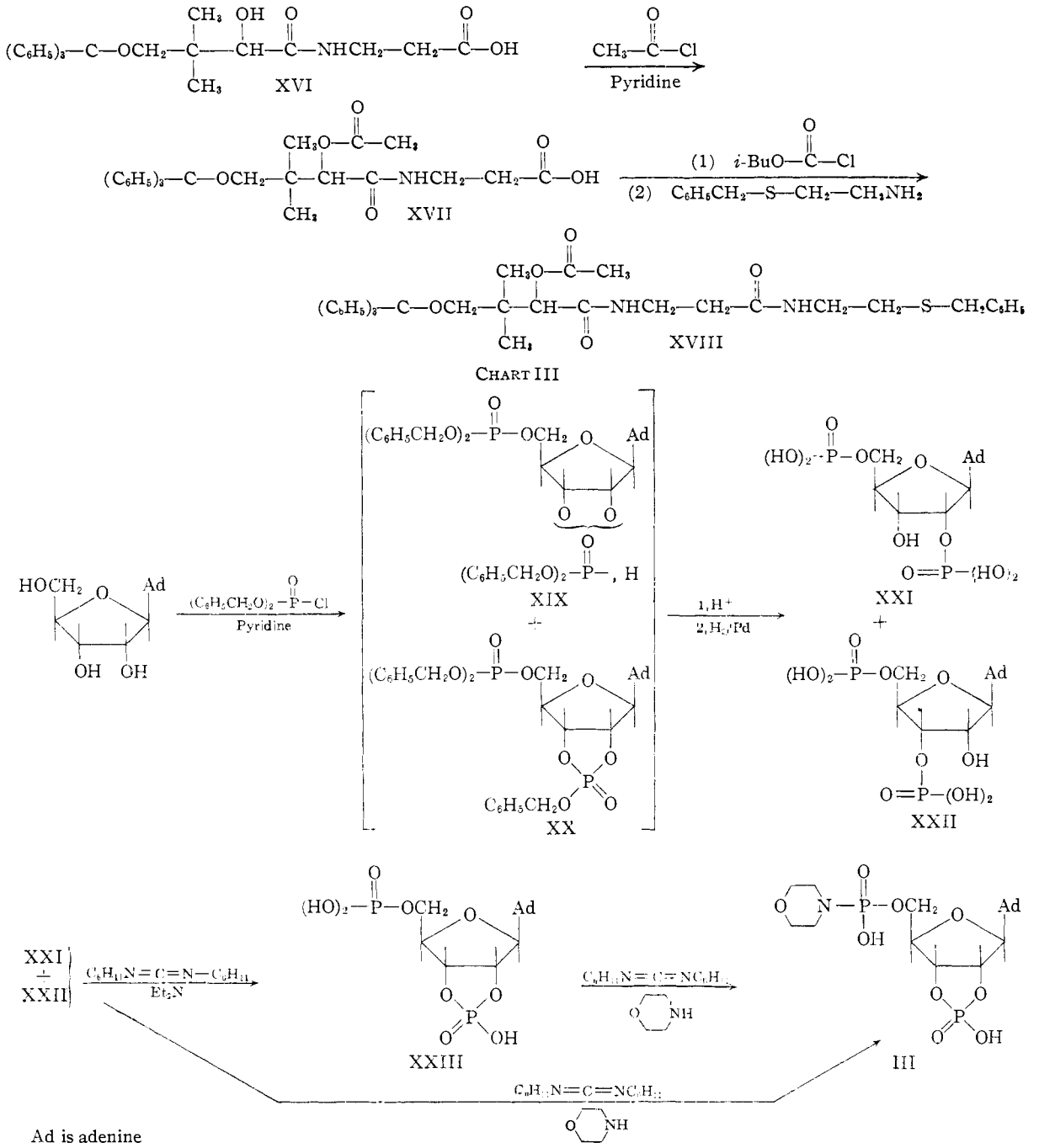
(24) Even if further effort had given the desired 2-O-*p*-nitrobenzoylpantothenic acid, it is doubtful, in view of the subsequent work reported below on 2′-*O*-acetyl derivatives, if it could have led to a successful synthesis of pantetheine derivatives.

(25) Some known aspects of the hydrolytic properties of pantothenic acid derivatives may be noted. Pantothenic acid itself is markedly labile to alkali. On the other hand, 4′-*O*-trityl pantothenic acid is stable to alkali, as was demonstrated during its preparation from methyl 4′-*O*-trityl pantothenate by alcoholic potassium hydroxide treatment. Similarly, pantothenic acid -4′ phosphate is also alkali-stable [J. Baddiley and E. M. Thain, *J. Chem. Soc.*, 246 (1951)]. The labilization of the amide bond in pantothenic acid thus appears to be due to the intramolecular attack by the anion of the 4′-hydroxyl group resulting in the formation of the stable five-membered pantoyl lactone. It is reasonable to suggest that the acetylation of 2′-hydroxyl group in the 4′-*O*-trityl pantothenic acid exerts a similar intramolecular effect *via* a five-membered ring as shown below for alkaline conditions.

(26) F. Cramer, G. W. Kenner, N. A. Hughes and A. R. Todd, *ibid.*, 3297 (1957).

(27) J. Baddiley, J. G. Buchanan and R. Letters, *ibid.*, 1000 (1958).

(28) The phosphorylation of the unprotected nucleoside using an excess of the phosphorylating agent would be expected to give the 2′(or 3′),5′ diphosphates as the major products. The first point of attack on the nucleoside would be the primary 5′-hydroxyl function. Thus, the monophosphate fraction (14%) isolated consisted entirely



(The yields reported by the earlier workers were 32 and 26%, respectively.) The diphosphates, as was expected, contained the 2′,5′- and 3′,5′-isomers (XXI and XXII) in equal amounts. A small yield (6%) of a triphosphate, presumably adenosine-2′,3′,5′-triphosphate, was also obtained.[28]

The mixed diphosphates (XXI and XXII) on treatment with dicyclohexylcarbodiimide in the presence of triethylamine gave adenosine-2′,3′-cyclic phosphate 5′-phosphate (XXIII) in high yield. This result was to be expected on the basis of the previous studies of the reactions of carbodiimides.[29] The 5′-phosphate group would enter into bimolecular reactions more slowly and this would explain the minor side products which were also formed. The pure XXIII was isolated by ion exchange chromatography on a DEAE-cellulose column in 84% yield.[30] Subsequent treatment of XXIII with dicyclohexylcarbodiimide and morpholine[1] rapidly gave the 5′-phosphoromorpholidate

of the 5′-isomer. The second point of attack would be the 2′ or 3′-hydroxyl group. Attack on either of these positions may cause steric hindrance to the subsequent attack on the adjacent hydroxyl group. Furthermore, it is very probable that the resulting acyclic neutral esters (XIX) undergo a rapid transesterification reaction to form the cyclic ester (XX) [ref. 10 and D. M. Brown, D. I. Magrath and A. R. Todd, *J. Chem. Soc.*, 4396 (1955)].

(29) M. Smith, J. G. Moffatt and H. G. Khorana, THIS JOURNAL, **80**, 6204 (1958).

(30) The same compound has been prepared by a rather more complex route by A. M. Michelson [*J. Chem. Soc.*, 2055 (1958)].

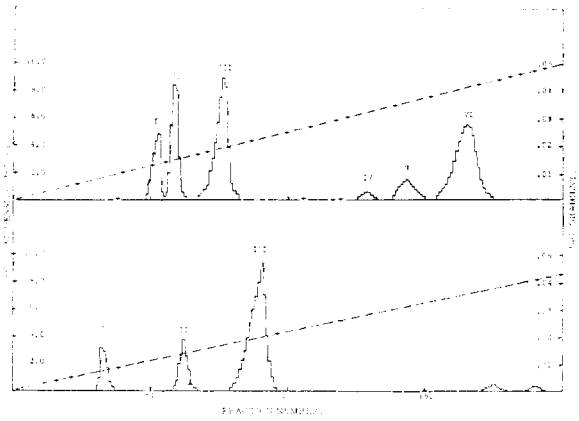


Fig. 1.—Products of the reaction of adenosine-5′ phosphoromorpholidate with pantetheine-4′ phosphate. Chromatography on a DEAE-cellulose column using a linear salt gradient as shown. Top, direct chromatography of the reaction mixture: peak I, unidentified; peak II, adenosine-5′ phosphate; peak III, 3′-dephospho-Coenzyme A; peak IV, unidentified; peak V, mixed disulfide of 3′-dephospho-Coenzyme A and pantetheine-4′ phosphate; peak VI, oxidized (disulfide)-3′-dephospho-Co nzyme A. Bottom, chromatography after reduction with 2-mercaptoethanol: peaks I, II and III, as in (a) above.

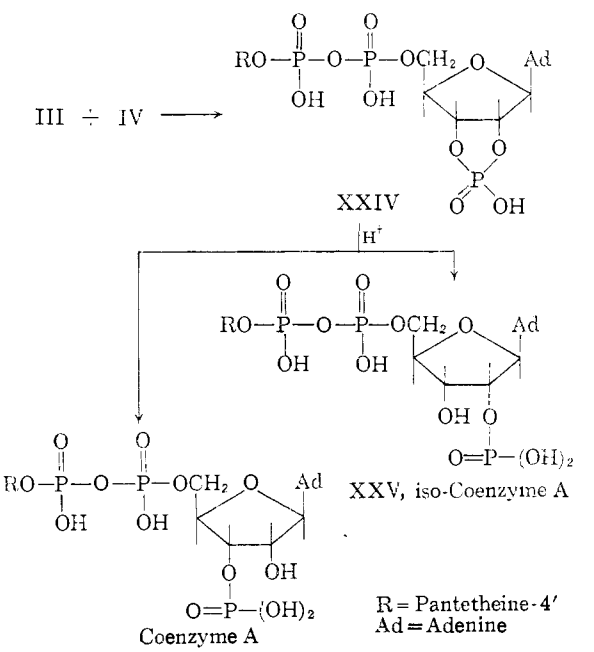
(III). More conveniently, the desired III was obtained chromatographically and analytically pure in 91–98% yield by the direct treatment of the mixed diphosphates with dicyclohexylcarbodiimide and morpholine. As in the previous work,[1] III was isolated as its *bis*-(4-morpholine N,N′-dicyclohexylcarboxamidinium) salt. In one out of the many runs performed, the product was not formed quantitatively and ion exchange chromatography was used to separate the contaminating adenosine-2′,3′-cyclic phosphate 5′-phosphate (XXIII) (see Experimental).

The structure of III was confirmed by hydrolysis of the cyclic phosphate with 0.5 *N* sodium hydroxide at room temperature, followed by treatment of the product[31] with prostatic phosphomonoesterase. The resulting adenosine-5′-phosphoromorpholidate (V) was identical with the preparation synthesized and characterized previously.[1] The synthesis of the key intermediate (III) starting with adenosine is, thus, relatively simple and efficient, the over-all yield being around 65%.

The Synthesis of 3′-Dephospho-Coenzyme A and Coenzyme A.—DL-Pantetheine-4′ phosphate reacted rapidly with adenosine-5′-phosphoromorpholidate in anhydrous pyridine and anion exchange chromatography of the reaction mixture gave the elution diagram shown in Fig. 1a. Peak I was not identified; peak II was adenosine-5′ phosphate. Peak III gave a positive sulfhydryl group reaction and was identified as the desired 3′-dephospho-Coenzyme A. Thus the ratio of its phosphorus to adenosine content was found to be 1.98, and it was readily degraded by crude rattle snake venom to adenosine, inorganic phosphate and pantetheine-4′-phosphate. Furthermore, in its chromatographic and electrophoretic behavior it was identical with a sample of the same substance prepared by treatment of the commercially available Coenzyme A with prostatic phosphomonoesterase. Peaks V and VI both contained disulfide groups and by application of the techniques used for the identification of peak III as well as by reduction of the disulfide groups were shown to be, respectively, the mixed disulfide between pantetheine-4′-phosphate and 3′-dephospho-Coenzyme A and the disulfide (oxidized) form of the latter itself.

In all the subsequent work the complications arising from the oxidation of sulfhydryl groups during pyrophosphate synthesis were mostly overcome by a reductive step given prior to ion exchange chromatography.[32] Of the various reducing agents (sodium borohydride, sodium amalgam, cysteine, glutathione) examined, 2-mercaptoethanol was the most convenient and efficient. The elution pattern thus obtained on repeating a preparation of 3′-dephospho-Coenzyme A is shown in Fig. 1b. The desired product was isolated as the pure lithium salt in 63% yield.[33]

Attention was then turned to the synthesis of Coenzyme A itself. The reaction of the bis-(4-morpholine N,N′-dicyclohexylcarboxamidinium) salt of III with three equivalents of D-pantetheine-4′ phosphate in anhydrous pyridine gave XXIV (anhydro-Coenzyme A) which was



(31) Treatment of this ring-opened product with dicyclohexylcarbodiimide in the presence of triethylamine[29] reformed III quantitatively.

(32) Under the acidic conditions used for chromatography, reoxidation of the sulfhydryl compounds is negligible.

(33) While the chemical characterization of this preparation, in which the pantetheine moiety was racemic, left little doubt as to its identity or homogeneity, a quantitative enzymic assay has not been possible because of the lack of availability of sufficiently pure 3′-dephospho-Coenzyme A kinase. Nevertheless, incubation of the synthetic sample with a partially purified preparation from hog liver [*cf.* T. P. Wang and N. O. Kaplan, *J. Biol. Chem.*, **206**, 311 (1954)] and adenosine-5′ triphosphate led to the formation of Coenzyme A, as shown by the phosphotransacetylase assay.[34] We are grateful to Dr. W. E. Razzell for these experiments.

(34) E. R. Stadtman in "Methods in Enzymology," Vol. I, Academic Press, Inc., New York, N. Y., 1955, p. 596

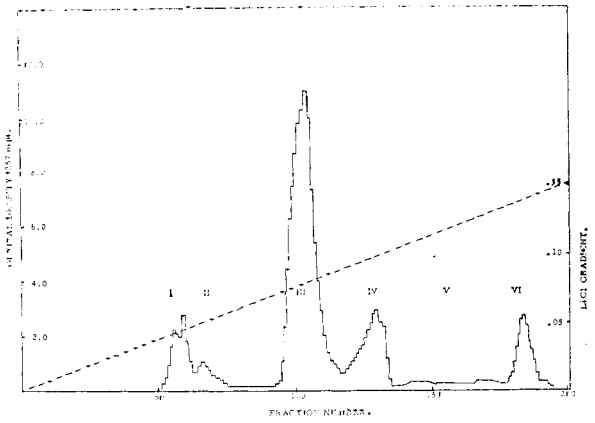
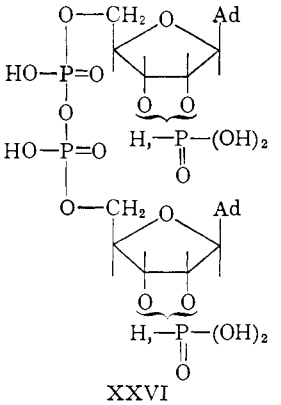


Fig. 2.—Products of the reaction of adenosine-2′,3′-cyclic phosphate 5′-phosphoromorpholidate with pantetheine-4′ phosphate, after acidic hydrolysis and reduction with 2-mercaptoethanol. Chromatography on a DEAE-cellulose column as in text. Peak I, adenosine-2′ (or 3′), 5′ diphosphates; peak II, unidentified; peak III, Coenzyme A + *iso*-Coenzyme A; peak IV, P^1,P^2-*bis*-(2′(3′)-phosphoryladenosine-5′) pyrophosphate (XXVI); peak VI, oxidized Coenzyme A.

not isolated.[35] Instead, the total reaction mixture was treated first with acid to open the cyclic phosphate ring and then with 2-mercaptoethanol. Ion exchange chromatography gave the elution pattern shown in Fig. 2. Peak I (9%) consisted of a mixture of adenosine-2′(3′),5′-diphosphates, while peak II (3.5%) has not been identified. Peak IV (17%) was a chromatographically homogeneous, sulfur-free compound which has been identified (Experimental section) as the pyrophosphate represented by the structure XXVI. Peak V (6.6%) contained three components, one of which evidently was the mixed disulfide between Coenzyme A (or *iso*-Coenzyme A, XXV) and D-pantetheine-4′ phosphate.



XXVI

Peak III (55.4%) contained a mixture of Coenzyme A and *iso*-Coenzyme A, and peak VI (9.7%) contained these compounds in the oxidized (disulfide) forms. The total yield was thus 65%, based on III. Since the degradation by crude snake venom of peak III and separation of the

(35) In one experiment XXIV was purified by anion exchange chromatography at *p*H 4. The substance was inactive in the phosphotransacetylase assay,[34] but the product formed after acidic treatment to open the cyclic phosphate ring was active.

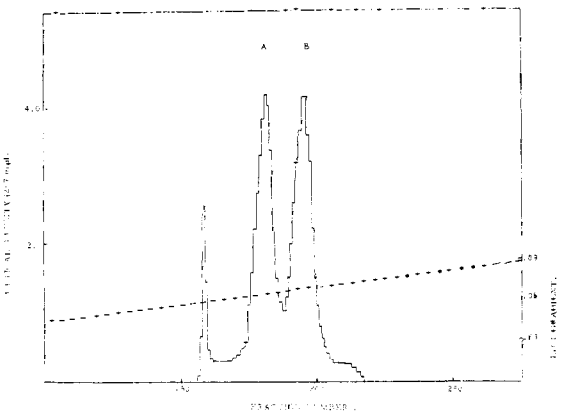


Fig. 3.—Rechromatography of peak III of Fig. 2 on an ECTEOLA-cellulose column using a linear gradient as shown. Peak A, *iso*-Coenzyme A; peak B, Coenzyme A.

resulting adenosine diphosphates showed the latter to be an equal mixture of the 2′,5′ and 3′,5′ isomers, the ring opening of XXIV had, as expected, given Coenzyme A and *iso*-Coenzyme A in equal amounts. In further work, peaks III and VI were combined, and after repeating the reduction step were rechromatographed on an ECTEOLA cellulose column using a shallow linear gradient of salt for elution. Two major peaks, largely resolved from one another, were obtained (Fig. 3). A comparable separation could not be achieved when a DEAE-cellulose column was used for rechromatography. Peaks A and B, as isolated after careful pooling of the fractions to avoid mutual contamination, were chromatographically and electrophoretically identical with Coenzyme A. Each contained a small amount of the disulfide form but single spots were obtained on chromatography after reduction. Peak A was attacked by crude rattle snake venom to give pantetheine-4′ phosphate and adenosine-2′,5′ diphosphate (XXI), only a trace of the isomeric 3′,5′-diphosphate (XXII) being present in the latter. Peak B, on similar degradation, gave pantetheine-4′ phosphate and the ultraviolet absorbing product was entirely adenosine-3′,5′ diphosphate (XXII). The two peaks are thus respectively *iso*-Coenzyme A and Coenzyme A and the order of their elution is consistent with previous results on the ion exchange chromatography of nucleoside-2′ and -3′ phosphates, in particular, adenosine-2′ and -3′ phosphates[36] as well as the 2′,5′ and 3′,5′-diphosphates.[27]

The alkaline hydrolysis of adenosine-2′,3′-cyclic phosphate has previously been found[37,38] to give 65–70% of the 3′-phosphate, and it was, therefore, of interest to attempt alkaline ring-opening of XXIV so as to increase the proportion of the desired Coenzyme A. However, the alkaline conditions[39] that were found to be necessary for the

(36) W. E. Cohn in "The Nucleic Acids," Vol. I, E. Chargaff and J. N. Davidson, Editors, Academic Press, Inc., New York, N. Y., 1955, p. 211.

(37) C. A. Dekker, unpublished work; *cf.*, C. A. Dekker and H. G. Khorana, THIS JOURNAL, **76**, 3522 (1954).

(38) L. A. Heppel, P. R. Whitfeld and R. Markham, *Biochem. J.*, **60**, 20 (1955).

(39) The cyclic phosphate ring in both XXIV and adenosine-2′,3′-cyclic phosphate 5′-phosphoromorpholidate (III) was more stable to alkali than that in adenosine-2′3′-cyclic phosphate. Thus, while the

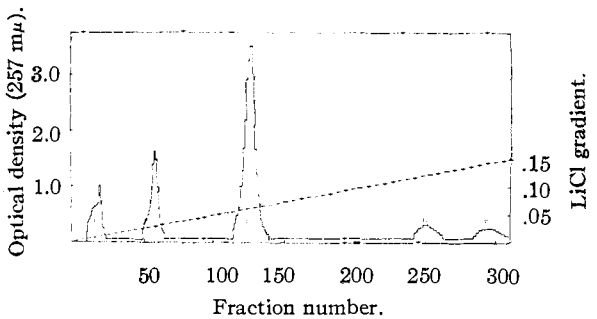


Fig. 4.—Chromatography of commercial Coenzyme A on a DEAE cellulose column. Peak III, Coenzyme A; for comments on other peaks see Experimental text.

completion of the ring opening in XXIV caused considerable breakdown of the pyrophosphate linkage.

The enzymic assay of Coenzyme A is usually done by the use of the enzyme phosphotransacetylase[34] and requires prior calibration against a standard sample of Coenzyme A. The best preparations which are commercially available are claimed to be 75% pure on a weight basis and accepting this value, the synthetic sample (peak B, above) showed, on the basis of its ultraviolet absorption, an activity of 139%.[40] It was therefore clear that the commercial preparations, several of which were tested, were in fact less than 75% pure and one 25 mg. lot was therefore subjected to chromatography on a DEAE cellulose column (Fig. 4). While detailed observations on the eluted fractions are recorded in the Experimental section, Coenzyme A was eluted in peak III of Fig. 4 and was evidently pure. This preparation was then used as the standard in another assay of the synthetic product, the concentrations of both compounds being determined by ultraviolet absorption. Peak B (Fig. 3) was now found to have 96% activity. Peak A of Fig. 3 (*iso*-Coenzyme A) showed 1.8% activity, a result which is in agreement with the slight contamination by Coenzyme A revealed by the snake venom degradation.

The work reported above provides synthetic confirmation of the structure of Coenzyme A and the total synthetic steps used are relatively simple and efficient, except for the lack of specificity in the cyclic phosphate ring opening of XXIV. The latter compound and *iso*-Coenzyme A may themselves prove of interest in the study of the specificities and properties of the various enzymic systems normally requiring Coenzyme A.

The synthesis of Coenzyme A and the work reported in the accompanying papers bring to a close the program of research initiated in this Laboratory some seven years ago[41] on the development of methods for the synthesis of unsymmetrical pyrophosphates of biological interest. The methods now available are believed to be satisfactory and completely general so that the synthesis of any nucleotide coenzyme or related pyrophosphate may be undertaken in the laboratory with confidence.

latter was completely hydrolyzed by 0.1 *N* sodium hydroxide at room temperature in 1 hr., III required 2.5 hr. under the same conditions. The increased resistance to alkali is probably due to the electrostatic effect of the negative charge on the 5′-position.

(40) The activity response with the synthetic sample was linear with concentration up to 0.015 μmole in the standard assay. With higher concentrations, however, some inhibition was noted (about 10% decrease with 0.03 μmole). The cause of this is unknown.

(41) H. G. Khorana, THIS JOURNAL, **76**, 3517 (1954).

Experimental[42]

Chromatography.—Paper chromatography by the descending technique was done on Whatman No. 1 paper using these systems: Solvent I, ethyl alcohol: 0.5 *N* ammonium acetate buffer *p*H 3.8 (5:2); Solvent II, ethyl alcohol: 1 *N* ammonium acetate (*p*H 7.5) (5:2); Solvent III, *n*-butyl alcohol: acetic acid: water (5:2:3); Solvent IV, isopropyl alcohol: concd. ammonia: water (7:1:2); Solvent V, saturated ammonium sulfate:isopropyl alcohol: 1 *N* sodium acetate (80:2:18); Solvent VI, isobutyric acid: 1 *N* ammonium hydroxide: 0.1 *M* tetrasodium ethylenediamine tetraacetic acid (100:60:1.6). Paper electrophoresis was done using Whatman No. 3 MM paper impregnated with either 0.05 *M* triethylammonium bicarbonate (*p*H 7.5) or 0.05 *M* ammonium acetate buffer (*p*H 3.5) in an apparatus similar to that described by Markham and Smith.[43]

Phosphorus-containing compounds were located on chromatograms with the Hanes and Isherwood spray[44] followed by ultraviolet irradiation.[45] Sulfhydryl compounds were located with the nitroprusside spray of Toennies and Kolb[46] or with 0.025% aqueous 2,6-dichlorophenolindophenol.[47] Compounds containing amino or amide functions were located using the chlorine–starch–iodide method of Ryden and Smith.[48]

Enzyme Experiments.—Crude *Crotalus adamanteus* venom was obtained commercially[49] and used directly in 0.5 *M* tris-hydroxymethyl aminomethane buffer at *p*H 8.0. Venom phosphodiesterase (free of monoesterase) was purified as described previously[50] and used in the same buffer as the crude venom. Prostatic phosphomonoesterase was purified by the method of Boman[51] and was completely free of diesterase activity. It was used in 0.5 *M* acetate buffer at *p*H 5.5. Phosphotransacetylase was a commercial product.[52]

D-Pantetheine-4′ Phosphate (IV).—D-Pantethine[53] (831 mg., 1.5 mmole) was dried by three evaporations of its solutions in anhydrous pyridine (10 ml. each), dissolved in pyridine (30 ml.) and frozen in a Dry-Ice bath. A solution of dibenzyl phosphorochloridate[54] (from 1.33 ml., 6 mmole of dibenzyl phosphite and 810 mg. of N-chlorosuccinimide) in benzene (10 ml.) was then added and the solution was thawed and rapidly refrozen. It was then allowed to stand overnight in the deep freeze (−18°). Water (5 ml.) was added and after 15 minutes the pale yellow solution was carefully evaporated[55] *in vacuo* with a bath temperature less than 35°. The residue was dissolved in ethyl acetate[56] and extracted three times each with 2 *N* sulfuric acid, 10% sodium bicarbonate and saturated sodium sulfate.[57] The organic phase was then dried over sodium sulfate and the solvent evaporated *in vacuo* giving a thick syrup which was dissolved in glacial acetic acid (10 ml.) and heated on a

(42) Melting points are uncorrected and elemental analyses were performed by W. Manser, Herrliberg, Switzerland.

(43) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552 (1952).

(44) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).

(45) R. S. Bandurski and B. Axelrod, *J. Biol. Chem.*, **193**, 405 (1951).

(46) G. Toennies and J. J. Kolb, *Anal. Chem.*, **23**, 823 (1951).

(47) We are indebted to Dr. J. M. Siegel and Pabst Laboratories, Milwaukee, for pointing out the use of this spray.

(48) H. N. Ryden and P. W. G. Smith, *Nature*, **169**, 922 (1952).

(49) Ross Allen's Reptile Farm, Silver Springs, Florida.

(50) W. E. Razzell and H. G. Khorana, *J. Biol. Chem.*, **234**, 2105 (1959).

(51) H. G. Boman, *Arkiv Kemi*, **12**, 453 (1958).

(52) Sigma Chemical Co., St. Louis, Mo.

(53) We are grateful to Dr. O. D. Bird of Parke, Davis and Co., Detroit, for a sample of this compound.

(54) G. W. Kenner, A. R. Todd and F. J. Weymouth, *J. Chem. Soc.*, 3675 (1952). In the preparation of dibenzyl phosphite, pyridine was used in place of dimethylaniline. [O. M. Friedman, D. L. Klass and A. M. Seligman, THIS JOURNAL, **76**, 916 (1954)].

(55) Excessive heating must be avoided in order to minimize the formation of pantetheine-2′,4′ cyclic phosphate.

(56) Part of the product, mostly pyridine hydrochloride, did not dissolve in ethyl acetate alone.

(57) Emulsions result if water alone is used.

TABLE I

R_f VALUES OF COMPOUNDS[58]

Compound	R_f Solvent III
Pantothenic acid	0.72
4′-*O*-Tritylpantothenic acid	.92
4′-*O*-Trityl-2′-*O*-acetylpantothenic acid	.94
S-Benzyl-4′-*O*-trityl-2′-*O*-acetylpantetheine	.98
Pantetheine	.78
Pantethine	.76
β-Alanine	.42
S-Benzyl-2-mercaptoethylamine	.73
β-Alanyl-S-benzyl-2-mercaptoethylamine	.74
Pantetheine-4′ phosphate (SH)	.47
Pantetheine-4′ phosphate (SS)	.33
Pantetheine-2′,4′ cyclic phosphate (SH)	.50
Pantetheine-2′,4′ cyclic phosphate (SS)	.33

	R_f Solvent: I	II	VI
Adenosine-5′ phosphate	0.43	0.19	0.54
Adenosine-2′(3′),5′ diphosphate	.34	.04	.37
Adenosine-2′,3′,5′ triphosphate	.25	.02	.24
Adenosine-2′,3′ cyclic phosphate 5′-phosphate	.43	.14	.43
Adenosine-2′,3′ cyclic phosphate 5′-phosphoromorpholidate	.54	.49	.65
Adenosine-2′(3′) phosphate 5′-phosphoromorpholidate	.44	.24	.50
Dephospho CoA (SH)	.57	.52	.68
Dephospho CoA (SS)	.32	.20	.56
Dephospho CoA-S-S-pantetheine-4′ phosphate	.51	.28	.58
Coenzyme A (SH)	.50	.21	.55
Coenzyme A (SS)	.21	.02	.31
P^1,P^2-Bis-(2′(3′)phosphoryladenosine-5′) pyrophosphate	.40	.01	.38
P^1,P^2-Di-adenosine-5′ pyrophosphate	.22	.18	.54

steam-bath for 1 hr. after the gradual addition of water (10 ml.) over the first five minutes. The acetic acid was then evaporated under reduced pressure and the residue repeatedly evaporated with methyl alcohol until the odour of acetic acid was no longer noticeable. The residue was dissolved in liquid ammonia (25 ml.) and a solution of sodium in ammonia was added until a blue colour persisted. Dry methyl alcohol was then added to destroy the blue colour after which the ammonia was evaporated by gentle warming leaving a dry white powder. An aqueous slurry of Amberlite-IR 120 (H^+) resin was quickly added to this residue until the solution was acidic and the entire mixture was then applied to the top of a 2 × 10 cm. column of fresh IR 120 (H^+) resin. After all acidic material had been washed from the resin with water, the eluate was brought to *p*H 7.5 with barium hydroxide and the precipitate removed by centrifugation. The aqueous solution was evaporated *in vacuo* to a volume of roughly 10 ml., the *p*H was readjusted to 7.5 with barium hydroxide and a small precipitate was removed. The remaining water was evaporated *in vacuo* and the residue dried on an oil pump. The resulting clear glass was dissolved in methyl alcohol, separated from a trace of insoluble material and concentrated to a volume of roughly 3 ml. The addition of dry, peroxide-free ether[59] (30 ml.) gave a white precipitate which was washed with ether and dried *in vacuo* at room temperature giving 880 mg. (44%) of the barium salt of D-pantetheine-4′ phosphate as the heptahydrate. Various preparations had $[\alpha]^{24}D = +11.7°$ to 12.8°[60] and all contained traces of pantetheine-2′,4′ cyclic phosphate and the disulfide form of pantetheine-4′ phosphate. After drying *in vacuo* at 100° the anhydrous compound was obtained.

Anal. Calcd. for $C_{11}H_{21}O_7N_2PS\cdot Ba$: C, 26.77; H, 4.29; N, 5.68; P, 6.27. Found: C, 26.34; H, 4.49; N, 5.38; P, 6.21.

D-Pantetheine-2′,4′ Cyclic Phosphate (VIII).—A preparation of D-pantetheine-4′ phosphate in which the acetic acid hydrolysis prior to treatment with sodium in ammonia was omitted was shown by chromatography in Solvent III to be heavily contaminated with the 2′,4′ cyclic phosphate. The mixture of barium salts[61] (475 mg.) was passed through a column of Amberlite IR-120 resin (H^+) and then adjusted to *p*H 7.5 with triethylamine. The solution was directly applied to a 2 × 30 cm. column of DEAE cellulose[62] in the carbonate form and after washing with water the column was eluted using a linear gradient technique. The reservoir contained 1 liter of 0.5 *M* triethylammonium bicarbonate (*p*H 7.5) and the mixing vessel 1 liter of a 0.005 *M* solution of the same buffer. Fractions of 10 ml. each were collected at a flow rate of 1 ml. per minute. The various phosphorus and sulfhydryl containing compounds were located by spotting the fractions on filter paper and spraying with the Hanes and Isherwood reagent[44] or with aqueous 0.025% 2,6-dichlorophenolindophenol.[47] Phosphorus and SH containing compounds were found in tubes 25–31 and in 33–39. The first peak was pooled and evaporated to dryness.[63] The residue was dissolved in methyl alcohol (2 ml.) and 2-mercaptoethanol (0.5 ml.) was added. After 4 hr. at room temperature the solvent was evaporated and the residual liquid evacuated on an oil pump. It was then dissolved in water, passed through a 1 × 5 cm. column of Amberlite IR-120 (H^+) resin, and the effluent was brought to *p*H 4.5 with barium hydroxide. The solution was extracted four times with ether and evaporated to dryness *in vacuo*. The residue was then dissolved in methyl alcohol (2 ml.) and precipitation caused by the addition of peroxide-free ether (25 ml.). The resulting barium salt of pantetheine-2′,4′-cyclic phosphate (180 mg.) was completely free of the acyclic-4′ phosphate as shown by chromatography in Solvent III, by electrophoresis at *p*H 7.5 and by its complete resistance to prostatic phosphomonoesterase.

Anal. Calcd. for $C_{11}H_{20}N_2O_8PS\cdot Ba_{1/2}\cdot 0.5\ H_2O$: C, 31.69; H, 5.32; N, 6.72. Found: C, 31.59; H, 5.37; N, 6.29.

DL-2-*O*-Benzyl-pantoyl Lactone (XI).—D(−)-Pantoyl lactone was benzylated with sodium ethoxide and benzyl chloride in dry xylene by the method of Baddiley and Thain.[21] After short path distillation (b.p. 87° at 10^{-4} mm.) from a modified Hickman flask[64] the compound was obtained as somewhat oily crystals. Crystallization from ether at −78° gave dry needles melting sharply at 49° (Baddiley and Thain[21] reported m.p. 46–47°). The product was completely racemic.

S-Benzyl-2-mercaptoethylamine Hydrochloride.—Ethyleneimine (4.2 g., 0.1 mole) was added dropwise to a chilled (ice–salt bath) solution of α-toluene-thiol (12.4 g., 0.1 mole) in methyl alcohol. After the vigorous reaction had subsided, the solution was kept overnight at room temperature. After removing the solvent *in vacuo* the mobile residue (16.2 g.) was dissolved in ether (200 ml.) and saturated with dry hydrogen chloride. The fine white crystals which separated were removed, washed with ether and dried *in vacuo* giving 17.5 g. (87%) of S-benzyl-2-mercaptoethylamine hydrochloride. The compound could be recrystallized as chunky needles from chloroform–methyl alcohol but the melting point (110–121°) remained unchanged. Baddiley and Thain[65] report m.p. 119–121° and Walton, *et al.*,[66] report 120–136° for the same compound prepared by different routes.

(58) The relative mobilities in this table should be reproducible since all compounds were run simultaneously.

(59) In the presence of peroxides serious oxidation of the sulfhydryl compound to the disulfide results.

(60) Calculated for the anhydrous barium salt. Baddiley and Thain (ref. 14) report +10.8°; Osbond (ref. 18) reports +14.6°.

(61) Small amounts of the corresponding disulfides were also present.

(62) Brown Co., Berlin, New Hampshire. Type 20, Reagent grade.

(63) Quite a lot of the cyclic phosphate was chromatographically (Solvent III) shown to be present in the disulfide form.

(64) K. C. D. Hickman, *Chem. Revs.*, **34**, 51 (1944).

(65) J. Baddiley and E. M. Thain, *J. Chem. Soc.*, 800 (1952).

(66) E. Walton, A. N. Wilson, F. W. Holley and K. Folkers, THIS JOURNAL, **76**, 1146 (1954).

DL-2′-*O*,*S*-**Dibenzyl Pantetheine.**—The compound was prepared essentially according to Baddiley and Thain[14] except that the final product was purified by chromatography on an alumina column (60 g. of alumina for 4.03 g. of the reaction product). An unidentified forepeak (850 mg.) was eluted with benzene–ether (1–1) and the desired product (3.1 g. 60%) was subsequently eluted with ether containing 10% methyl alcohol.

DL-**Pantetheine-4′ Phosphate (IV).**—DL-2′-*O*,*S*-Dibenzylpantetheine (1.15 g., 2.5 mmole) was rendered anhydrous by three evaporations of its solutions in dry pyridine (10 ml. each) and finally taken up in fresh pyridine (10 ml.). This solution was frozen in a Dry Ice–acetone bath and a freshly prepared solution of dibenzyl phosphorochloridate[55] (5 mmole) in benzene (10 ml.) was added. The mixture was thawed until homogeneous, refrozen and stored overnight at −20°. Water (1 ml.) was then added to the very pale yellow solution and after fifteen minutes at room temperature the solvent was evaporated *in vacuo*. After removal of residual pyridine by several evaporations with methyl alcohol, the final residue was dissolved in ethyl acetate (25 ml.) and extracted three times each with 2*N* sulfuric acid, saturated sodium bicarbonate and saturated sodium sulfate. The ethyl acetate solution was then dried over sodium sulfate and evaporated to dryness giving 1.68 g. of a viscous syrup which was dissolved in liquid ammonia (roughly 25 ml.) containing dry methyl alcohol (0.1 ml.). A solution of sodium in liquid ammonia was added portionwise until a stable blue colour resulted. The mixture was then worked up in the same way as was described above for D-pantetheine-4′ phosphate and finally gave 1.08 g. (78%) of the barium salt of DL-pantetheine-4′ phosphate as the trihydrate.

Anal. Calcd. for $C_{11}H_{21}N_2O_7PSBa \cdot 3H_2O$: P, 5.66; SH, 6.05. Found: P, 5.61; SH, 6.08[67]

After drying *in vacuo* at 100° the anhydrous material was obtained.

Calcd. for $C_{11}H_{21}N_2O_7PSBa$: C, 26.77; H, 4.29; N, 5.68. Found: C, 26.78; H, 4.50; N, 5.44.

Paper chromatography in Solvent III followed by phosphorus spray showed a very intense spot[68] (*Rf* 0.52) and a trace of the disulfide form (*Rf* 0.31).

4′-*O*-**Tritylpantothenic Acid (XVI).**—Calcium D-pantothenate (2.38 g., 10 mmole) was converted to the free acid by passage through a column of Amberlite IR-120 (H^+) resin and evaporated to dryness. It was then dissolved in methyl alcohol and an ice cold ethereal solution of diazomethane was added until a yellow color persisted. After evaporation of the solvent *in vacuo* the clear residual gum of methyl D-pantothenate was dried by three evaporations with pyridine and dissolved in anhydrous pyridine (50 ml.). Trityl chloride (4.2 g., 15 mmole) was added and the resulting solution was heated on a boiling water bath for 4 hr. After removal of the solvent *in vacuo* the residue was dissolved in benzene (25 ml.) and filtered from insoluble pyridine hydrochloride. The benzene was then evaporated and the syrup dissolved in methanolic potassium hydroxide (100 ml. of 0.3 *N*). After 1 hr. at room temperature with occasional warming the solvent was evaporated and on addition of ether (50 ml.) and water (50 ml.) to the residue a stable, milky emulsion formed which separated to give two clear layers on shaking with Dowex 50 (H^+) resin (20 ml.). The aqueous layer was extracted once more with ether and the combined ether extracts were dried over sodium sulfate and evaporated to give a sticky froth. This was dissolved in benzene (25 ml.) and on addition of petroleum ether 4′-*O*-tritylpantothenic acid (3.7 g., 77%) separated as clusters of fine needles melting at 137.5–138°.

Anal. Calcd. for $C_{28}H_{31}NO_5$: C, 72.90; H, 6.78; N, 3.04. Found: C, 72.83; H, 6.96; N, 3.01.

The direct tritylation of unesterified pantothenic acid gave the same product but in only 31–40% yields.

2′-*O*-**Acetyl-4′-*O*-tritylpantothenic Acid (XVII).**—A solution of 4-*O*-tritylpantothenic acid (1.38 g., 3 mmole) in anhydrous pyridine (10 ml.) was chilled to 0° and shaken well with acetyl chloride (1.08 ml., 15 mmole) for 1 hr. After addition of water (2 ml.) the solvent was evaporated, the residue dissolved in benzene and extracted five times with 0.1 *N* hydrochloric acid. After one extraction with water the benzene solution was dried and evaporated giving 1.7 g. of a dry froth which was redissolved in benzene and chromatographed on a column of silicic acid (50 g.). A small amount of triphenylcarbinol was eluted with benzene, and 2′-*O*-acetyl-4′-*O*-trityl pantothenic acid (1.3 g., 86%) was eluted with benzene containing 20% ether. The compound could not be obtained crystalline and the elemental analysis was not completely satisfactory, but the compound served well for the next step.

Anal. Calcd. for $C_{30}H_{33}NO_6$: C, 71.60; H, 6.60; N, 2.78. Found: C, 73.06; H, 6.84; N, 3.22.

Hydrolysis with 80% acetic acid at 100° for twenty minutes gave presumably 2′-*O*-acetylpantothenic acid as the sole product. The latter moved on paper chromatograms in Solvent III just ahead of pantothenic acid.

S-**Benzyl-4′-*O*-trityl-2′-*O*-acetylpantetheine (XVIII).**—2′-*O*-Acetyl-4′-*O*-trityl pantothenic acid (1.096 g., 2.18 mmole) was dissolved in freshly purified N,N-dimethylformamide[69] containing N-methylmorpholine (0.24 ml., 2.18 mmole) and chilled to −15°. Isobutyl chloroformate (0.29 ml., 2.2 mmole) was added dropwise and the solution maintained at −15° for ten minutes prior to the addition of a dried solution of S-benzyl-2-mercaptoethylamine (from 600 mg., 2.9 mmole, of the hydrochloride) in dichloromethane (5 ml.). The mixture was kept at −5° for twenty minutes and then at room temperature overnight. After evaporation of the solvent *in vacuo* the residue was dissolved in ether (25 ml.) and extracted three times each with 0.1 *M* acetate buffer (*p*H 4.0), 0.1 *M* phosphate buffer (*p*H 7.5) and water. The ether solution was dried over sodium sulfate and evaporated to give 1.41 g. of a froth which was chromatographed on a column of silicic acid (40 g.). A partially crystalline impurity was first eluted with benzene–ether (4:1) and the product (1.1 g., 78%) subsequently with benzene–ether (1:1) as a microcrystalline powder that could not be recrystallized. (Another unidentified compound (250 mg.) was obtained with ether–methyl alcohol.)

Anal. Calcd. for $C_{39}H_{44}N_2O_6S$: C, 71.75; H, 6.79; N, 4.29. Found: C, 72.03; H, 6.86; N, 3.93.

N-(N-**Phthaloyl-β-alanyl)-S-benzyl-2-mercaptoethylamine.**—N-Phthaloyl-β-alanine[70] (4.38 g., 20 mmole) was dissolved in purified N,N-dimethylformamide[69] (10 ml.) containing N-methylmorpholine (2.2 ml., 20 mmole) and the solution cooled to −15°. Isobutyl chloroformate (2.65 ml., 20 mmole) was added dropwise and was followed, after ten minutes at −15°, by a dried solution of S-benzyl-2-mercaptoethylamine (from 5.1 g., 25 mmole, of the hydrochloride) in dichloromethane (10 ml.). A vigorous evolution of carbon dioxide resulted, and the mixture was held at −5° for twenty minutes and then at room temperature overnight. After evaporation of the solvent *in vacuo* the residue was dissolved in dichloromethane and extracted three times each with 2*N* sulfuric acid, 10% sodium bicarbonate and water. Evaporation of the dried solution gave a crystalline mass that was recrystallized from ethyl acetate giving fine needles of m.p. 124–131°. One further crystallization from aqueous ethyl alcohol gave N-(N-phthaloyl-β-alanyl)-S-benzyl-2-mercaptoethylamine (4.1 g., 56%) as fine needles melting at 133.5–134.0°.[71]

Anal. Calcd. for $C_{20}H_{20}N_2O_3S$: C, 65.22; H, 5.47; N, 7.61. Found: C, 65.22; H, 5.63; N, 7.68.

2-*O*-**Tetrahydropyranyl-pantoyl Lactone (XIV).**—(−) Pantoyl lactone (2.60 g., 20 mmole) was dissolved in dichloromethane (15 ml.) containing 2,3-dihydropyran[72] (5.0 ml., 60 mmole) and anhydrous hydrogen chloride in dioxane (0.1 ml. of 5 *N*). After 2 hr. at room temperature the solvent was removed *in vacuo* and the oily residue evacuated to constant weight (4.5 g.) on an oil pump. Most of this residue was chromatographed on 90 g. of silicic acid. The compound

(67) Sulfhydryl analyses were done essentially by the method of J. X. Khym, R. Shapira and D. G. Doherty, [THIS JOURNAL, **79**, 5663 (1957)] using cysteine as a standard and following the colour change at 600 mμ with a Cary recording spectrophotometer.

(68) It is characteristic of compounds containing both sulfhydryl and phosphate functions that they became visible as blue spots during drying of the chromatogram at 90° after spraying with the Hanes and Isherwood reagent (ref. 44)

(69) Freshly distilled from phthalic acid after shaking with solid sodium bicarbonate [D. R. Lundberg and P. Doty, THIS JOURNAL, **79**, 3962 (1957)].

(70) R. A. Turner, *ibid.*, **75**, 2388 (1953).

(71) Walton, *et al.* (ref. 66), report m.p. 113–123° for this compound as prepared by a different route.

(72) Freshly distilled from sodium hydroxide.

(3.4 g., 81%) appeared as a broad peak using petroleum ether containing 35–70% benzene, the infrared spectrum remaining unchanged throughout the peak. A portion was distilled in a short path apparatus as a single fraction of b.p. 60° at 10^{-3} mm.

Anal. Calcd. for $C_{11}H_{13}O_4$: C, 61.65; H, 8.46. Found: C, 61.69; H, 8.62.

Attempted Preparation of S-Benzyl-2′-*O*-tetrahydropyranylpantetheine.—Treatment of N-(N-phthaloyl-β-alanyl)-S-benzyl-2-mercaptoethylamine with hydrazine in ethyl alcohol according to Walton, *et al.*,[66] gave N-(β-alanyl)-S-benzyl-2-mercaptoethylamine hydrochloride in 70% yield as needles (m.p. 162–164°) from ether–petroleum ether. Treatment of the hydrochloride (476 mg., 2 mmole) in water with 3.5 *N* sodium hydroxide (1 ml.) followed by extraction with dichloromethane gave 410 mg. of the crystalline free base after washing with water and evaporation of the solvent. This was heated under reflux for 2 hr. in methyl alcohol (15 ml.) containing 2-*O*-tetrahydropyranylpantoyl lactone (402 mg., 2 mmoles). After evaporation of the solvent the residue was dissolved in dichloromethane and extracted three times with *p*H 4 acetate buffer and once with water. The dried organic phase gave on evaporation 376 mg., (94%) of unreacted 2-*O*-tetrahydropyranylpantoyl lactone.

DL-3′-Dephospho Coenzyme A[73] (I; R = H).—Barium DL-pantetheine-4′ phosphate (460 mg., 0.83 mmole) was passed through a 1 × 4 cm. column of Amberlite IR-120 (H^+) resin into an excess of pyridine. The solution was then taken to dryness *in vacuo* and dried by three evaporations with anhydrous pyridine. Separately 4-morpholine N,N′-dicyclohexylcarboxamidinium adenosine-5′ phosphoromorpholidate[1] (252 mg., 0.33 mmole) was dissolved in pyridine and dried by two evaporations with pyridine. The pantetheine phosphate solution was then added to that of the morpholidate and the mixture was evaporated to dryness twice with anhydrous pyridine *in vacuo* and with readmission of dry air. The final residue was dissolved in anhydrous pyridine (7 ml.) and left overnight at room temperature.[74,75] The solvent was then removed *in vacuo* and residual pyridine removed by several evaporations with methyl alcohol. The residue was dissolved in water (5 ml.), the *p*H adjusted to 4.5 and 2-mercaptoethanol (5 ml.) added. After 3 hr. at room temperature the solution was diluted with water (50 ml.) and applied to a 30 × 3.5 cm. column of ECTEOLA cellulose[76] in the chloride form. The column was washed with water until no further ultraviolet absorbing material appeared and then eluted using a linear salt gradient. The mixing vessel contained 4 liters of 0.003 *N* hydrochloric acid and the reservoir 4 liters of 0.07 *N* lithium chloride in 0.003 *N* hydrochloric acid. A flow rate of 2 ml. per minute was maintained and fractions of 20 ml. were collected. The elution curve is shown in Fig. 1b. Each peak was adjusted to *p*H 4.5 with lithium hydroxide and evaporated to dryness *in vacuo* at 40°. The resulting syrups were evacuated on an oil pump until they became dry white solids which were then dissolved in methyl alcohol (3 ml.). The addition of acetone (30 ml.) and peroxide-free ether (3 ml.) gave white precipitates which were retreated with methyl alcohol and acetone until the supernatant gave a negative chloride test. The products were then dried *in vacuo* at room temperature. Peak III (3100 optical density units, 62% yield, 148 mg.) was chromatographically pure 3′-dephospho Coenzyme A. Peaks V and VI were combined, precipitated reduced for 3 hr. with 50% aqueous 2-mercaptoethanol and chromatographed on a small ECTEOLA (Cl^-) column as above giving a further 14 mg. of the desired product. The combined isolated yield was 162 mg. (63%) of the pentahydrate of dilithium 3′-dephospho Coenzyme A.

Anal. Calcd. for $C_{21}H_{33}N_7O_{13}P_2SLi_2 \cdot 5H_2O$: P, 7.84; SH, 4.19; Adenosine:phosphorus:sulfhydryl = 1:2:1. Found: P, 7.94; sulfhydryl, 4.22; Adenosine:phosphorus:sulfhydryl = 1.00:2.01:0.97.

On drying *in vacuo* at 100° the dihydrate was obtained. Calcd. for $C_{21}H_{33}N_7O_{13}P_2SLi_2 \cdot 2H_2O$: C, 34.31; H, 5.07; N, 13.35. Found: C, 34.51; H, 5.41; N, 12.74.

Adenosine-2′(3′),5′ Diphosphate (XXI and XXII).—Adenosine (2.67 g., 10 mmole, dried overnight *in vacuo* at 100°) was dissolved in boiling anhydrous pyridine (150 ml.) and the solution quickly frozen in a Dry Ice-bath. A solution of dibenzyl phosphorochloridate (from 45 mmole, 10 ml., of dibenzyl phosphite and 6.0 g. of N-chlorosuccinimide) in 100 ml. of dry benzene was added, and the mixture was quickly thawed, refrozen and stored for 18 hr. at −20°. Water (10 ml.) was added and after 20 minutes the solvent was removed *in vacuo* at 40°. After removal of residual pyridine by several evaporations with methyl alcohol the residue was dissolved in glacial acetic acid (25 ml.) and water (50 ml.) was added. The mixture was heated for 1 hr. in a boiling water-bath. After evaporation *in vacuo* residual acetic acid was removed by two evaporations with water. The oily residue was dissolved in water and passed through a 2.2 × 20 cm. column of Amberlite IR-120 (H^+) resin. The column was alternately washed with water and methyl alcohol[77] (total of 2 l.) until the optical density of the effluent was negligible. Lithium hydroxide was added until a homogeneous solution (*p*H 5) was obtained and the volume was then reduced to 100 ml. Palladium oxide on barium sulfate catalyst[78] (2 g.) was added and the mixture hydrogenated at room temperature until hydrogen uptake ceased (3 hr.). The solution was adjusted to *p*H 11 with lithium hydroxide, and after 1 hr. at 4° the insoluble precipitate and catalyst were removed by centrifugation and washed several times with 0.001 *N* lithium hydroxide. The combined aqueous solution was directly applied to a 3.3 × 30 cm. column of Dowex 2 (Cl^-) resin which was then washed well with water. Adenosine-5′ phosphate[28] (21800 optical density units at 257 mμ, 14.4%) was eluted with 0.03 *N* lithium chloride in 0.003 *N* hydrochloric acid. A mixture of adenosine-2′(or 3′), 5′ diphosphates (105,000 optical density units at 257 mμ, 70%) was eluted with 0.075 *N* lithium chloride in 0.003 *N* hydrochloric acid, and adenosine-2′,3′,5′ triphosphate (9,200 optical density units at 257 mμ, 6.1%) was eluted with 0.2 *N* lithium chloride in 0.003 *N* hydrochloric acid.

The pooled diphosphate peak was adjusted to *p*H 4.5 with lithium hydroxide, evaporated to dryness and dried to a white solid on an oil pump. The solid was thoroughly stirred with methyl alcohol (50 ml.) and acetone (250 ml.) was added. The nucleotides were collected by centrifugation and repeatedly treated with methyl alcohol and acetone until the supernatant was free of chloride ions. The lithium salt of adenosine-2′(3′),5′ diphosphate (3.83 g., 70% as the pentahydrate) was obtained as a fine white powder that was chromatographically and electrophoretically pure.

Anal. Calcd. for $C_{10}H_{13}N_5O_{10}P_2Li_2 \cdot 5H_2O$: P, 11.45; Adenosine: P = 1:2. Found: P, 11.27; Adenosine: P = 1:1.97. On drying *in vacuo* at 100° the dihydrate was obtained. Calcd. for $C_{10}H_{13}N_5O_{10}P_2Li_2 \cdot 2H_2O$: C, 25.03; H, 3.61; N, 14.74. Found: C, 25.02; H, 3.92; N, 15.02.

Adenosine-2′,3′,5′ Triphosphate.—The triphosphate peak from the preceding experiment was worked up in the same way as described for the diphosphate and gave the chromatographically and electrophoretically pure lithium salt (400 mg., 5.7% as the decahydrate).

Anal. Calcd. for $C_{10}H_{13}N_5O_{13}P_3Li_3 \cdot 10H_2O$: P, 13.18; Adenosine: P = 1:3. Found: P, 13.3; Adenosine: P = 1:3.00. On drying *in vacuo* at 100° the dihydrate was obtained. Calcd. for $C_{10}H_{13}N_5O_{13}P_3Li_3 \cdot 2H_2O$: C, 21.32; H, 3.05; N, 12.49. Found: C, 21.63; H, 3.44; N, 12.19.

Adenosine-2′,3′-cyclic Phosphate 5′-Phosphate (XXIII).—An aqueous solution of dilithium adenosine-2′(3′),5′ diphosphate (269 mg., 0.5 mmole) was passed through a 1 × 5 cm. column of Dowex 50 resin (triethylammonium form), and after evaporation of the total effluent the residue was dissolved in a mixture of water (5 ml.) and *t*-butyl alcohol (10 ml.). Dicyclohexylcarbodiimide (515 mg., 2.5 mmole) and purified triethylamine (0.21 ml., 1.5 mmole) were added and the mixture refluxed for 3 hr. After evaporation of the solvent the residue was dissolved in water, extracted

(73) In this compound DL refers only to the asymmetric carbon in the pantothenic acid fragment.

(74) Chromatographically it was found that the reaction was complete in less than 6 hr.

(75) A small precipitate separated after fifteen minutes and was shown in a separate experiment to contain considerable 3′-dephospho Coenzyme A.

(76) E. A. Peterson and H. A. Sober, THIS JOURNAL, **78**, 751 (1956).

(77) Washing with methyl alcohol during the early stages is necessary since dibenzyl phosphoric acid oils out and is only slowly removed with water alone.

(78) R. Kuhn and H. J. Haas, *Angew. Chem.*, **67**, 785 (1955).

three times with ether and applied to a 2 × 12 cm. column of DEAE cellulose in the carbonate form. After washing with water the column was eluted using a linear gradient technique. The reservoir contained 1 liter of 0.1 *M* triethylammonium bicarbonate (*p*H 7.5), and the mixing vessel contained 1 liter of 0.005 *M* triethylammonium bicarbonate. A large peak was eluted at roughly 0.06 *M* salt and contained 6,000 optical density units at 257 mμ (84%). The pooled peak was evaporated to dryness, freed from traces of triethylammonium bicarbonate by a further evaporation with methyl alcohol and dissolved in ethyl alcohol (5 ml.). The addition of 1 *M* calcium chloride in ethyl alcohol (1.2 ml.) and acetone (15 ml.) gave a white precipitate. It was collected by centrifugation and washed several times with ethyl alcohol and acetone until the supernatant was free of chloride ions. After drying *in vacuo* at room temperature 211 mg. (84%) of the calcium salt of XXIII was obtained as the dihydrate.

Anal. Calcd. for $C_{10}H_{10}N_5O_9P_2Ca_{1.5}\cdot 2H_2O$: C, 24.40; H, 2.87; N, 14.23. Found: C, 24.69; H, 2.90; N, 13.25.

Adenosine-2′,3′-cyclic Phosphate 5′-Phosphoromorpholidate (III).—(a) The lithium salt of adenosine-2′(3′),5′ diphosphate (538 mg., 1 mmole) was dissolved in water and passed through a 1 × 10 cm. column of Dowex-50 resin (morpholinium form). After washing the column free of the nucleotide with water the total effluent was taken to dryness and the residue dissolved in a mixture of water (10 ml.) and *t*-butyl alcohol (25 ml.) containing morpholine (0.7 ml., 8 mmole) and dicyclohexylcarbodiimide (2.06 g., 10 mmole). After refluxing the mixture[79] for 3 hr., paper electrophoresis at *p*H 7.5 showed only a single product with a mobility of 0.78 relative to the diphosphates (XXI + XXII).[80] The *t*-butyl alcohol was largely evaporated *in vacuo* and the remaining aqueous mixture diluted to 25 ml. with water. After one extraction with ether, some insoluble material was removed by filtration and the filtrate was extracted twice more with ether. The solvent was then removed *in vacuo* and the residual white foam dried on an oil pump for 30 minutes before being dissolved in the minimum volume of methyl alcohol. The volume was then carefully reduced *in vacuo* to roughly 3 ml. in a 40 ml. centrifuge tube and dry ether was added. On stirring the resulting gum with fresh, dry ether it changed to a white powder which was washed twice with ether and dried *in vacuo* at room temperature to give bis-(4-morpholine N,N′-dicyclohexylcarboxamidinium)-adenosine-2′,3′-cyclic phosphate 5′-phosphoromorpholidate (1.06 g., 91%) as the dihydrate. In several preparations the yields varied between 90 and 98%.

Anal. Calcd. for $C_{48}H_{82}N_{12}O_{11}P_2\cdot 2H_2O$: C, 52.40; H, 7.88; N, 15.27; P, 5.62. Found: C, 52.27; H, 7.87; N, 14.96; P, 5.46.

(b) In one preparation on a 1 mmole scale the reaction did not go to completion and the product was purified by ion exchange chromatography. The reaction was worked up as above, dissolved in water and applied to a 2.2 × 15 cm. column of Dowex 2 ($CO_3^=$) resin. After washing with water elution was begun using a linear salt gradient technique. The mixing vessel contained 3.5 liters of 0.005 *M* triethylammonium bicarbonate and the reservoir 3.5 liters of 0.4 *M* triethylammonium bicarbonate. A large peak was eluted with roughly 0.25 *M* salt and was evaporated to dryness. After two further evaporations with methyl alcohol the residue was dissolved in methyl alcohol (10 ml.) containing 4-morpholine N,N′-dicyclohexylcarboxamidine (586 mg., 2 mmole). The solvent was evaporated and the resulting froth was thoroughly evacuated and then dissolved in methyl alcohol (3 ml.). The addition of ether gave a gum that was triturated several times with fresh ether giving a product identical to that described above.

Coenzyme A.—Bis-(4-morpholine N,N′-dicyclohexylcarboxamidinium)-adenosine-2′,3′-cyclic phosphate 5′-phosphoromorpholidate (221 mg., 0.2 mmole) was dried by three evaporations with anhydrous pyridine (5 ml.). Separately the barium salt of (+)-pantetheine-4′ phosphate (368 mg. of hexahydrate, 0.6 mmole) was converted to the pyridine salt with Dowex-50 (pyridinium) resin and evaporated to dryness. The residue was dried by three evaporations with pyridine (10 ml.) and added to the morpholidate. The mixture was evaporated *in vacuo* twice more with anhydrous pyridine and with readmission of dry air and finally dissolved in fresh pyridine (10 ml.). After 15 hr. at room temperature the solvent was evaporated *in vacuo* and residual pyridine removed by three evaporations with water (5 ml. each time). The residue was dissolved in 0.1 *N* hydrochloric acid (10 ml.) and after 1 hr. at room temperature the solvent was evaporated *in vacuo*. After two further evaporations with methyl alcohol (5 ml.), water (3 ml.) was added and the *p*H was adjusted to 6.0 with ammonium hydroxide. 2-Mercaptoethanol (4 ml.) was then added and the clear solution was left overnight.[81] It was then diluted with water (50 ml.) and applied to a column (2 × 30 cm.) of DEAE cellulose in the chloride form. The column was washed with water until the optical density of the effluent was negligible. Elution was then commenced using a linear gradient technique. The mixing vessel contained 1.5 liters of 0.003 *N* hydrochloric acid and the reservoir contained 1.5 liters of 0.15 *N* lithium chloride in 0.003 *N* hydrochloric acid.[82] Fifteen ml. fractions were collected at a flow rate of 1.5 ml. per minute. The elution curve is shown in Fig. 2. Peak I (280 optical density units at 257 mμ, 9.3%) was adenosine-2′(3′),5′-diphosphate; Peak II (3.5%) has not been identified; Peak III (1660 optical density units, 55.4%) was a mixture of Coenzyme A and iso-Coenzyme A; Peak IV (17%) was P¹,P′-bis-(2′(3′)-*O*-phosphoryladenosine-5′) pyrophosphate (XXVI), (see below); Peak V (6.6%) contained the mixed disulfide between Coenzyme A and pantetheine-4′ phosphate and two other compounds; Peak VI (9.7%) was oxidized Coenzyme A. Each pooled peak was adjusted to *p*H 4.5 with lithium hydroxide and evaporated to dryness *in vacuo*. After drying the residue from each peak on the oil pump, it was well stirred with methyl alcohol (5 ml.) and acetone (50 ml.). The resulting precipitates were repeatedly treated with methyl alcohol and acetone until the supernatants were free of chloride ions and then dried *in vacuo* at room temperature. Peaks III–VI were obtained in yields of 84, 18, 7 and 14 mg., respectively. The total yield of pure Coenzyme A and *iso*-Coenzyme A (Peak III and VI together) was 1,950 optical density units (65%) which together gave 98 mg. of isolated lithium salt.

A portion of the mixture of III and VI (1,500 optical density units, 0.1 mmole) was dissolved in 50% aqueous 2-mercaptoethanol, and the solution after being kept overnight at room temperature was applied to a 3.5 × 50 cm. column of ECTEOLA cellulose in the chloride form. After washing with water the nucleotides were eluted with a linear salt gradient. The mixing vessel contained 2.5 liters of 0.03 *N* lithium chloride in 0.003 *N* hydrochloric acid and the reservoir contained 2.5 liters of 0.10 *N* lithium chloride in 0.003 *N* hydrochloric acid. The elution curve is shown in Fig. 3. Fractions 185–189 in the overlapping region were discarded and the remainder of peaks A and B were separately pooled and worked up to give the lithium salts (23 and 25 mg., respectively) as described above. Further elution of the column with 0.15 *N* lithium chloride in 0.003 *N* hydrochloric acid gave an additional 120 optical density units of the oxidized forms of Coenzyme A and *iso*-Coenzyme A as a single peak.

Characterization of Synthetic Coenzyme A.—(a) *iso*-Coenzyme A (Peak A, Fig. 3) (XXV).—The ratio of phosphorus to adenosine was found to be 2.97 (theory 3.00) and the compound was chromatographically and electrophoretically identical with natural reduced Coenzyme A. Incubation of a sample with crude *Crotalus adamanteus* venom followed by chromatography in Solvent V showed adenosine-2′,5′ diphosphate and only a trace of the 3′, 5′-diphosphate to be the only nucleotides present. Assayed by the phosphotransacetylase method[34] using purified commercial Coenzyme A (see below) as the standard *iso*-Coenzyme A had an activity of 1.8%.

Anal. Calcd. for $C_{21}H_{33}N_7O_{16}P_3SLi_3\cdot 6H_2O$: C, 28.84; H, 5.08; N, 10.98. Found: C, 28.52; H, 5.08; N, 10.39.

(79) A small lower phase initially separated but the mixture soon became homogeneous.

(80) In shorter times the intermediate product (adenosine-2′,3′-cyclic phosphate 5′-phosphate, relative mobility, 0.92) was present.

(81) Paper chromatography in Solvent I showed the reduction to be complete in 3 hr.

(82) The low salt concentrations necessary for elution of Coenzyme A and Dephospho Coenzyme A from cellulose anion exchangers are to be contrasted with those necessary when using conventional resins. See, *e.g.*, E. R. Stadtman and A. Kornberg, *J. Biol. Chem.*, **203**, 47 (1953); O. Brenner-Holzach, R. Adler and P. Leuthardt, *Helv. Chim. Acta*, **39**, 1770 (1956).

(b) Coenzyme A (Peak B, Fig. 3).—The ratio of phosphorus to adenosine was found to be 2.96 and the compound was chromatographically and electrophoretically identical with natural Coenzyme A. Degradation with crude rattlesnake venom gave adenosine-3′,5′-diphosphate as the only detectable nucleotide. Enzymatically assayed as above the compound gave a linear response of activity with concentration for 0.0041 and 0.0082 μmole (optical density) and gave an activity of 139% in each case assuming a purity of 75% (by weight) for commercial Coenzyme A. Using the purified commercial product (see below) as the standard and assuming 100% activity on the basis of its adenosine content, the synthetic sample had 96% activity.

Anal. Calcd. for $C_{21}H_{33}N_7O_{16}P_3SLi_3 \cdot 6H_2O$: C, 28.41; H, 5.08; N, 10.98. Found (after drying at 100°): C, 28.52; H, 4.98; N, 9.87.

Purification of Commercial Coenzyme A.—The contents of a freshly opened 25 mg. bottle of "75%" Coenzyme A[83] were dissolved in water (3 ml.) and adjusted to *p*H 6.0 with ammonium hydroxide. 2-Mercaptoethanol (3 ml.) was added and the mixture stored at room temperature for 4 hr. after which time it was diluted with water (15 ml.) and applied directly to a 2 × 22 cm. column of DEAE cellulose in the chloride form. After washing the column with water until no further ultraviolet absorbing material was present in the washing, elution was commenced using a linear salt gradient. The mixing vessel contained 1.5 liters of 0.003 *N* hydrochloric acid and the reservoir contained 1.5 liters of 0.15 *N* lithium chloride in 0.003 *N* hydrochloric acid. Ten ml. fractions were collected at the rate of 1 ml. per minute. Three distinct peaks and two small ones were detected by ultraviolet absorption at 257 mμ (Fig. 4). Peak I had λ_{max} 239 mμ and a second small maximum at 283 mμ (ϵ 239/ϵ 283 = 5.5 at *p*H 2.7) and was obviously not a nucleotide. Peak II contained at least two superimposed compounds one having λ_{max} 243 mμ, and the other λ_{max} 255 mμ. Peak III (273 optical density units at 257 mμ, 18 μmole) was reduced Coenzyme A. Peaks IV and V were too small for identification but, from its position, IV is probably oxidized Coenzyme A.

Peak III was adjusted to *p*H 4.0 with lithium hydroxide and worked up as described for the synthetic material to give 16 mg. of lithium salt which was chromatographically shown (Solvent I) to contain only reduced Coenzyme A and a little of the disulfide form. The material was somewhat hydrated, two preparations having equivalent weights of 960 and 1050 by ultraviolet absorption.

Assayed by the phosphotransacetylase method against the same "75%" standard as used for the synthetic material, it now showed 143% activity on the basis of its adenosine content.

Characterization of P¹,P′Bis-(−2′(or 3′)-phosphoryladenosine-5′) Pyrophosphate (XXVI).—Incubation of the sulfur-free product from peak IV (Fig. 3) with crude rattle snake venom rapidly gave adenosine-2′(3′),5′-diphosphate as the only phosphorus containing product. On incubation with purified prostatic phosphomonoesterase it was slowly (~ 75% in 24 hr.) dephosphorylated to give initially P¹-2′(3′)-phosphoryladenosine-5′ P²-adenosine-5′-pyrophosphate (XXVI, with loss of one phosphomonoester group) and subsequently di-adenosine-5′-pyrophosphate which were isolated in Solvents VI and I, respectively. The chromatographically isolated initial dephosphorylation product was rapidly degraded by crude venom, giving equal amounts of adenosine-2′(3′),5′-diphosphate, adenosine and inorganic phosphate. These results are all consistent with the structure assigned (XXVI) to this product.

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[CONTRIBUTION FROM THE CHEMISTRY DIVISION OF THE BRITISH COLUMBIA RESEARCH COUNCIL, VANCOUVER, B. C.]

Studies on Polynucleotides. VIII.[1] Experiments on the Polymerization of Mononucleotides. Improved Preparation and Separation of Linear Thymidine Polynucleotides. Synthesis of Corresponding Members Terminated in Deoxycytidine Residues[2]

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Treatment of a molar anhydrous pyridine solution of a mixture of 3′-*O*-acetylthymidine-5′ phosphate (25%) and thymidine-5′ phosphate (75%) with dicyclohexylcarbodiimide at room temperature for six days gives linear thymidine polynucleotides as the major products. Members containing up to eleven units in a chain have been purified and characterized, smaller amounts of somewhat higher polynucleotides also being present in the polymerization mixtures. Procedures developed for the purification include chromatography of the total mixture on a DEAE-cellulose (carbonate) column using the volatile triethylammonium bicarbonate as the eluent and rechromatography of the major peaks under similar conditions. Polymerization of a mixture of N,3′-*O*-diacetyldeoxycytidine-5′ phosphate (25%) and thymidine-5′ phosphate (75%) gives products from which thymidine polynucleotides bearing deoxycytidine residues at one end were isolated pure and characterized. The procedures developed for their purification involved, first, chromatography on DEAE-cellulose (carbonate) columns followed by rechromatography of the major peaks at acidic *p*H using the anion exchanger in the chloride form.

Introduction

The development of methods for the polymerization of mononucleotides and the separation and characterization of the resulting polymers forms a part of the program of synthetic work in the polynucleotide field which is in progress in this Laboratory.[4-6] The range of simple polymers thus obtained offers obvious advantages for a variety of chemical, physico-chemical and enzymic studies in the nucleic acids field. The polymerizations of thymidine-5′ phosphate and the isomeric 3′-phosphate by reaction with dicyclohexylcarbodiimide in anhydrous pyridine have previously been reported.[7,8] While the extension of these initial studies to other mononucleotides and, indeed, in a number of directions is clearly desirable,[9] many

(1) Paper VII, H. G. Khorana, THIS JOURNAL, **81**, 4657 (1959).

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