[CONTRIBUTION FROM THE BIOCHEMICAL RESEARCH LABORATORY, MASSACHUSETTS GENERAL HOSPITAL, AND THE DEPARTMENT OF BIOLOGICAL CHEMISTRY, HARVARD MEDICAL SCHOOL]

The Nature of the Sulfur in Coenzyme A[1,2]

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The presence of a sulfhydryl group in coenzyme A (CoA) has been established, in the form of β-mercaptoethylamine linked through its amino group to the carboxyl group of pantothenic acid, as in pantetheine. The β-mercaptoethylamine is liberated from CoA by an enzyme in pigeon or chicken liver. This is the only sulfur present in CoA, and by removal of impurities bound in a disulfide linkage, CoA has been obtained containing 25.6% pantothenic acid (about 90% pure).

Among the earliest observations on the chemistry of the pantothenic acid-containing coenzyme A (CoA)[3,4] was the detection of hydrogen sulfide on acidification after treatment with alkali. A nitroprusside test for sulfhydryl groups was obtainable only after reduction, indicating the presence of disulfide linkages. During the later purification of the coenzyme, the sulfur content of the preparations increased parallel to the activity up to a point. This suggestive evidence that sulfur was part of the molecule was supported by enzymatic degradation studies in which CoA was treated with alkaline phosphatase and chromatographed on paper in *n*-butanol/water system.[5,6] All three resulting zones which had growth activity with *Acetobacter suboxydans*[7] showed, when treated with a cyanide–nitroprusside reagent, the presence of disulfide linkages as well.

At this stage in the problem, a growth factor for *Lactobacillus bulgaricus*, discovered by Williams, Hoff-Jørgensen and Snell and named "LBF,"[8] was found to be a derivative of pantothenic acid.[9] It was observed in addition that intestinal phosphatase treatment of CoA liberated LBF.[10] This evidence indicated that LBF constituted a portion of the CoA molecule, and confirmation was obtained in this Laboratory by comparing the positions of the phosphatase degradation products of CoA with that of LBF on paper chromatograms. The factor corresponded to one fragment, which contained pantothenic acid and a sulfur compound, but none of the adenine present in intact CoA.[11] Adenine and its derivatives were located under ultraviolet light (Mineralite).

There was a strong suspicion of a peptidic link between the carboxyl group of pantothenic acid and the sulfur compound, based partly on the hydrolysis of CoA, which gave a marked increase of the ninhydrin reaction in addition to that accounted for by β-alanine. Furthermore, there was the long-standing observation that alkaline phosphatase alone was not enough to liberate free pantothenic acid from its natural bound state,[12] but that treatment with an enzyme found in pigeon or chicken liver was also necessary. It appeared most likely that the bird liver enzyme acted upon a link involving the carboxyl group of pantothenic acid. Meanwhile, Snell, *et al.*,[13] showed that LBF, to which he gave the more specific name of *pantethine* (or *pantetheine*, for the reduced sulfhydryl form), was a derivative of pantothenic acid in which the carboxyl group was linked to the amino group of β-mercaptoethylamine. We have since demonstrated by paper chromatography the appearance of β-mercaptoethylamine disulfide on treatment of CoA with the chicken liver enzyme, whose peptidase type of activity liberates free pantothenic acid. Baddiley[14] also has confirmed the presence of this substance in hydrolysates of CoA. In addition, it has been shown that pantethine and adenosine triphosphate will yield CoA when incubated with pigeon liver acetone powder extract.[15]

The foregoing evidence has drawn attention to the presence of the sulfhydryl group in CoA and has led to a new understanding of the problems involved in purifying the substance, which will be discussed below.

In calculations of purity and composition, the pantothenic acid content of the preparation has so far been used as the reference point, since pantothenate was the first known constituent of CoA, and it is probable that all bound pantothenate in these preparations is present as CoA.[12] The determination of pantothenic acid in CoA samples of various degrees of purity[4] has given average values of 0.7 μg. of pantothenic acid per unit of coenzyme activity. This is equivalent to approximately 310 units per micromole of pantothenate or CoA. Using a molecular weight of 767, based on the constituents of CoA known at present (pantetheine, adenosine, three phosphates), also in good agreement with the value of 800 determined by the

(1) Presented before the Section of Biological Chemistry, XIIth International Congress of Pure and Applied Chemistry, New York, September, 1951.

(2) This investigation was supported in part by a research grant from the National Cancer Institute of the National Institutes of Health, Public Health Service.

(3) F. Lipmann, N. O. Kaplan, G. D. Novelli, L. C. Tuttle and B. M. Guirard, *J. Biol. Chem.*, **167**, 869 (1947).

(4) *Ibid.*, **186**, 235 (1950).

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(7) G. D. Novelli, R. M. Flynn and F. Lipmann, *J. Biol. Chem.*, **177**, 493 (1949).

(8) W. L. Williams, E. Hoff-Jørgensen and E. E. Snell, *ibid.*, **177**, 933 (1949).

(9) R. A. McRorie, P. M. Masley and W. L. Williams, *Arch. Biochem.*, **27**, 471 (1950).

(10) G. M. Brown, J. A. Craig and E. E. Snell, *ibid.*, **27**, 473 (1950).

(11) G. D. Novelli, J. D. Gregory, R. M. Flynn and F. J. Schmetz, Jr., *Federation Proc.*, **10**, 229 (1951).

(12) G. D. Novelli, N. O. Kaplan and F. Lipmann, *J. Biol. Chem.*, **177**, 97 (1949).

(13) E. E. Snell, G. M. Brown, V. J. Peters, J. A. Craig, E. L. Wittle, J. A. Moore, V. M. McGlohon and O. D. Bird, THIS JOURNAL, **72**, 5349 (1950).

(14) J. Baddiley and E. M. Thain, *J. Chem. Soc.*, 2253 (1951).

(15) W. M. Govier and A. J. Gibbons, *Arch. Biochem. Biophys.*, **32**, 347 (1951); T. E. King and F. M. Strong, *J. Biol. Chem.*, **189**, 325 (1951).

Northrup diffusion method,[7] the pure substance should assay about 410 units per mg. and contain 28.60% pantothenic acid.

Experimental

Source of CoA.—The starting material was several batches of CoA of about 40% purity, prepared by Drs. DeVries, Govier and Evans of the research laboratories of the Upjohn Company from cultures of *Streptomyces fradiae*.[6] The purification had been previously carried further in this Laboratory by repeated fractionation on charcoal columns to a level of 60–80%.

Chromatography of Acid Hydrolysate.—The coenzyme was hydrolyzed in 2–3 *N* hydrochloric acid at 100° for three hours. After removal of excess acid, an aliquot equivalent to 0.05 mg. of material was chromatographed on Whatman No. 3 filter paper using the ascending technique. The one-phase solvent was made up of 75 parts *s*-butanol, 15 parts 88% formic acid and 10 parts water by volume.

The papers were dried at room temperature until the odor of formic acid had disappeared, then examined under ultraviolet light to locate the adenine, and sprayed with ninhydrin in the usual manner. The cyanide–nitroprusside test was done on a parallel sample by painting with a solution of 5% sodium cyanide and 5% sodium carbonate in 25% ethanol, then a few minutes later with 2% sodium nitroprusside in 75% ethanol. The zones shown in Fig. 1, sample 1, were obtained, of which only the two with the lowest R_F values (0.04 and 0.08) showed the presence of disulfides. Neither of these corresponded to cystine or any other known amino acid. The β-alanine arises from hydrolysis of pantothenic acid.

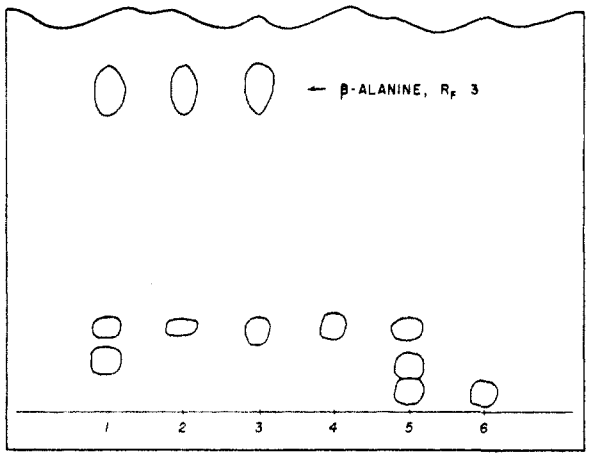

Fig. 1.—Ascending chromatogram of CoA hydrolysates and related compounds on Whatman No. 3 paper; solvent, *s*-butanol, formic acid: samples 1, hydrolysate of 60% pure CoA; 2, hydrolysate of extensively purified CoA; 3, hydrolysate of pantethine; 4, β-mercaptoethylamine disulfide; 5, mixture of disulfides; 6, cystine.

Discussion

The fact that some preparations of CoA which were 60–80% pure still contained, as reported previously,[6] up to two atoms of sulfur per mole of pantothenate, together with the appearance of two sulfur-containing substances on chromatograms, led to some uncertainty concerning the number of sulfur compounds actually associated with CoA. But since a CoA preparation had once been isolated from liver[4] with a ratio of only 1.2 atoms of sulfur per pantothenate, and since the presence of cystine was suggested by microbiological assay and by positive Sullivan tests, a reasonable interpretation now presented itself in terms of the disulfide structure of CoA. This was that CoA might contain a single sulfur component, presumably β-mercaptoethylamine, but that variable amounts of extraneous sulfhydryl compounds such as cysteine might be attached to it through a disulfide linkage by oxidation during isolation. Different proportions of such forms might exist, as no attempts were made to maintain reducing conditions consistently, and any mercaptan present could have been trapped during the isolation procedure. A survey of the sulfur content of various CoA preparations appears in Table I. The atomic ratio of sulfur is based on the figure of 310 units per micromole of CoA.

TABLE I

290-DeV-20: starting material as prepared by the Upjohn Co.; 112d: reduced with zinc and acid, isolated through mercury salt; C123: calcium salt, chromatographed on Duolite CS-100; I-3-A: reduced with hydrogen sulfide, isolated by alcohol precipitation; 133A: reduced with hydrogen sulfide, isolated through lead salt and chromatography; 146: reduced with zinc and acid, isolated through mercury salt.

Preparations	Units/mg.	S, %	Atoms S / Mole CoA
290-DeV-20	150	3.6	2.3
112d	214	4.0	1.8
C123	290	5.2	1.7
I-3-A	187	2.6	1.3
133A	345	4.8	1.3
146	384	4.13	1.04

In all our preparations, the coenzyme is present as disulfide. The determination of coenzyme activity[16] is always done in the presence of cysteine or other reducing agent, the active form being presumably reduced sulfhydryl CoA. Therefore, any foreign material linked through a disulfide bridge would not impair coenzyme activity, but it would be impossible to remove by ordinary fractionation methods. If this were the case, and CoA were partially bound to cysteine, this would imply the presence of two different disulfides, and acid hydrolysis should give rise to two new disulfides, as

$$\text{CoA—S—S—CoA} \xrightarrow{H^+} \text{H}_2\text{NCH}_2\text{CH}_2\text{S—SCH}_2\text{CH}_2\text{NH}_2$$

$$\text{CoA—S—SCH}_2\text{CHCOOH} \xrightarrow{H^+} \text{H}_2\text{NCH}_2\text{CH}_2\text{S—SCH}_2\text{CHCOOH}$$

(with NH_2 on the CHCOOH carbon)

Confirmation of this was obtained by paper chromatography of synthetic β-mercaptoethylamine disulfide (prepared by Dr. M. Soodak) and of the mixed disulfide with cysteine, as shown in Fig. 1. The latter (sample 5) was prepared by oxidation of an alkaline mixture of the two sulfhydryl compounds with air or oxygen; three spots were obtained, one of cystine and the others matching those from hydrolyzed CoA. Hydrolysis of synthetic pantethine[17] (sample 3) gives only β-alanine and the expected single disulfide.

Brown and Snell[5] have demonstrated the possibility of forming a variety of compounds with LBF activity by the disulfide linkage of pantetheine with other mercaptans. In fact, they have shown by paper chromatography[18] that among the major products of alkaline phosphatase action on CoA are pantethine as already mentioned, its mixed disulfide with the contaminating cysteine, and sometimes the disulfide with β-mercaptoethylamine, probably from further degradation of pantethine.

Purification

Purification of CoA.—In efforts to prepare high-activity CoA including removal of disulfide contaminants, we have used fractionation following reduction. Hydrogen sulfide in alkaline solution can be used to reduce the disulfide, but even after several hours the reaction appears not to be complete, since the isolated products still contain a considerable amount of cystine after precipitation of a metal salt and purification with a resin as described below.

The best results were obtained by reduction with zinc and hydrochloric acid as follows: a solution of 10,000 units of CoA (60 units per mg. or better) in 10 ml. of 0.5 *N* hydrochloric acid was stirred with two 50-mg. portions of zinc dust, the second added half-way through the reduction. Certain

(16) N. O. Kaplan and F. Lipmann, *J. Biol. Chem.*, **174**, 37 (1948).
(17) Kindly furnished by Dr. E. E. Snell.
(18) E. E. Snell, private communication.

dark CoA samples gave a heavy, black precipitate on acidification; during reduction, this dissolved and most of the color disappeared. After 30 minutes the suspension of zinc was filtered or centrifuged with a minimum exposure to air, and 2 ml. of 20% mercuric acetate in 2% acetic acid was added with stirring. The resulting tan precipitate was centrifuged and washed with a small volume of water.

Hydrogen sulfide was bubbled for several hours through a suspension of the precipitate in 5 ml. of water, and the mercuric sulfide removed and washed with a little water. The solution and washing (strongly acidic) were combined and aerated briefly to remove most of the hydrogen sulfide. At this stage about 5,000 coenzyme units remained.

The solution was further acidified (if necessary) to below *p*H 1 and applied to a 5 × 185 mm. column of Duolite CS-100 resin (100–200 mesh; reusable repeatedly), which had been washed with 0.2 *N* hydrochloric acid. The sample was followed by acid of this concentration and the optical density of the effluent at 260 mμ examined. A large amount of absorbing impurity appeared quickly, and when the density had fallen to approximately $d = 1$ (about 25 ml.), the eluant was changed to water. As soon as the effluent *p*H rose to 2 or higher, the CoA emerged, and 30 ml. later had virtually ceased. The fractions with coenzyme activity were combined and freeze-dried, leaving a white, cottony product assaying in various runs from 330 to 384 units per mg. The yield was 15–20% of the units in the starting material. The analysis of such a compound, containing 25.6% pantothenic acid, has been reported[19]; this material, on the basis of the figures given above, is about 90% pure. Hydrolysis and paper chromatography gave a pattern as in Fig. 1, sample 2, showing the complete absence of cystine.

(19) J. D. Gregory, G. D. Novelli and F. Lipmann, THIS JOURNAL, **74**, 854 (1952).

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[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

The Chemistry of Tyrocidine. I. Isolation and Characterization of a Single Peptide

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Crystalline tyrocidine hydrochloride has been fractionated by countercurrent distribution and shown to contain three major components. One of these, tyrocidine A, has been purified to the stage where it probably represents a single substance. It has been shown to contain only amino acids and ammonia all of which have been isolated and characterized. An empirical formula $C_{66}H_{87}O_{13}N_{13}\cdot HCl$ has been derived for the molecule. It has been shown to be a cyclic peptide containing one primary amino group, one phenolic hydroxyl group and two primary amide groups. The amino acid residues have been found to be: three of phenylalanine and one each of leucine, valine, tyrosine, proline, ornithine, glutamic acid and aspartic acid.

Introduction

Attempts to fractionate the higher, naturally occurring polypeptides by countercurrent distribution, CCD,[2] have shown that in this class of substances even the first step in structural study, the isolation of a single pure compound to use as a starting point, has seldom been achieved. However, an automatic 220 tube CCD[3] apparatus has now been constructed and it is of considerable interest to determine whether or not a pure polypeptide can be isolated with the aid of this instrument. Tyrocidine has been chosen as a suitable test material.

Tyrocidine was isolated from autolyzed cultures of *B. brevis* by Hotchkiss and Dubos[4] in 1940. It was soon shown to be a basic polypeptide. Since that time it has interested a number of chemists, whose work has been reviewed by Hotchkiss[5] and by Synge.[6] In this early work samples of the peptide were recrystallized until there was no observable change in properties. The criterion of purity then available was thus satisfied. Later these preparations were shown to be mixtures by frontal analysis[7] and by CCD.[2]

The isolation of a pure polypeptide from the family of closely related substances present in tyrocidine has been the first objective in the researches reported here. The second objective has been the characterization of the isolated material, determination of its molecular weight and sufficient structural study to give adequate support to the thesis of purity. The molecular weight studies are reported in an accompanying contribution.[8]

Experimental

We are indebted to the Wallerstein Company for the tyrocidine used in this work. A system made from a mixture of methanol, chloroform and 0.1 *N* hydrochloric acid, volume proportions 2,2,1, appeared to be a satisfactory system for the preliminary fractionation. A 5-g. sample after 673 transfers gave the pattern shown in Fig. 1. Weight determinations were made by the method of Craig, *et al.*[9] Ultraviolet absorption measurements were made directly in the appropriate layer with the Beckman quartz spectrophotometer.

Attention was first focused on the large band, which may be called tyrocidine A, on the left of the pattern of Fig. 1 and an attempt was made to purify it further. For this purpose a second distribution was made with twice the starting charge, 10 g., scattered in 15 tubes. This gave pattern 2a, Fig. 2, at 760 transfers for the part remaining in the train. After removal of the tail material in tubes 0–150 the apparatus was set for recycling.[3] At 1600 transfers pattern 2b was obtained. The experimental band was noticeably too broad but determinations of the partition ratio at various points on the pattern did not show impurity. Six fractions were taken as shown at the top of 2b and separately examined. When solutions of fractions 2–5 in the minimum quantity of methanol were treated with ether until incipiently turbid, beautiful colorless rods separated, total weight 1.9 g. Fractions 1 and 6 yielded no crystalline material.

The recrystallized material from fractions 3 and 4 of

(1) On leave of absence as a Commonwealth Fund Fellow from the University of St. Andrews, Scotland.

(2) L. C. Craig, J. D. Gregory and G. T. Barry, *Cold Spring Harbor Symposia Quantitative Biol.*, **14**, 24 (1949).

(3) L. C. Craig, W. Hausmann, E. H. Ahrens, Jr., and E. J. Harfenist, *Anal. Chem.*, **23**, 1236 (1951).

(4) R. D. Hotchkiss and R. J. Dubos, *J. Biol. Chem.*, **132**, 791 (1940).

(5) R. D. Hotchkiss, *Advances in Enzymology*, **4**, 153 (1944).

(6) R. L. M. Synge, *Quarterly Reviews*, **3**, 245 (1949).

(7) R. L. M. Synge and A. Tiselius, *Acta Chem. Scand.*, **1**, 749 (1947).

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