[CONTRIBUTION FROM THE CHEMISTRY LABORATORIES OF OREGON STATE COLLEGE]

Pantothenic Acid. V. Evidence for Structure of Non-β-Alanine Portion

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The determination of the structure of pantothenic acid has proved an unusually difficult task due principally to the extraordinary difficulties concerned in its isolation in pure form.[1] These difficulties are responsible for the fact that the substance itself had not at any time prior to its synthesis[2] been obtained in a form pure enough to yield the correct analysis; and this has made it necessary to attack the problem in unconventional ways and to use very small samples for study. During the course of this investigation a number of new methods have been devised to help solve the problem. In addition to the two new micro methods for α- and β-hydroxy acids alluded to in this article, other methods have been devised: (1) a method for accurately determining microorganisms in suspension,[3] (2) the micro determination of active hydrogen with deuterium oxide,[4] (3) the micro determination of hydroxyl groups with hydriodic acid,[5] (4) a micro method for carbonyl groups (unpublished), (5) selective oxidation with a new reagent, iodic acid,[6] (6) oxidation equivalent analysis whereby an empirical formula can be determined using a fraction of a milligram of pure material,[7,8,9] (7) fractional electrical transport in high potential field without the use of diaphragms[10] which is applicable both to colloids and non-colloids and which has been developed to a high degree by Tiselius in connection with protein separations.

Studies involving the use of several of these methods and others have shown pantothenic acid to be a "peptide" of β-alanine and a hydroxy acid. Analysis of calcium pantothenate of about 90% purity[11] indicated that the non-β-alanine portion was a five carbon acid with two hydroxyl groups. Since a partial synthesis of pantothenic acid using β-alanine had been accomplished,[12] it became evident that the crux of the problem remaining lay in the structure of the non-β-alanine fragment of the pantothenic acid molecule. Evidence regarding this structure follows.

α-Hydroxy Acid.—The ferric chloride test[13] for α-hydroxy acids was positive when applied to high potency pantothenic acid *after hydrolysis* with dilute sodium hydroxide.

In order to confirm this indication a semi-quantitative micro method for determination of α-hydroxy acids was developed based on the reactions:

$$\mathrm{RCHOHCOOH} \xrightarrow[\Delta]{\mathrm{H_2SO_4}} \mathrm{RCHO} + \mathrm{HCOOH}$$

$$\mathrm{HCOOH} \xrightarrow[\Delta]{\mathrm{H_2SO_4}} \mathrm{CO} + \mathrm{H_2O}$$

During a one-hour period of heating to 140° in an atmosphere of carbon dioxide, the carbon monoxide was swept out of the reaction vessel with carbon dioxide and measured in a micro nitrometer over concentrated potassium hydroxide. In order to make sure the gas evolved was actually carbon monoxide, it was in all crucial cases absorbed in cuprous chloride in hydrochloric acid. Table I sums up results. The data indicated strongly the formation of an α-hydroxy acid.

TABLE I

CARBON MONOXIDE YIELDS FROM HYDROXY ACIDS

Compound	Sample, mg.	Millimoles: Sample	Millimoles: CO obtained
α-Hydroxy-γ-butyrolactone	2.0	0.0196	0.0104
β-Hydroxybutyric acid	1.5	.0150	.0002
Lactic acid	1.5	.0167	.0152
Calcium pantothenate (potency 7760)	2.08	.0060[a]	.0056
(potency 700)	11.0	.006[b]	.0140

[a] Assuming 65% pure, mol. wt. 225. [b] Assuming 6% pure.

Lactonization.—The ferric chloride test discussed above was negative when the pantothenic acid was hydrolyzed with acid. This was interpreted to indicate the presence of an α-hydroxy lactone, since such lactones (unhydrolyzed) do not give the ferric chloride test. Acid hydrolyzed pantothenic acid gave the test after alkaline treatment.

Further evidence for the presence of a potential lactone was simultaneously obtained by quantitative acid and basic hydrolyses of approximately 90% pure calcium salt of pantothenic acid. The first sample was treated in a

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(1) Williams, *et al.*, THIS JOURNAL, **60**, 2719 (1938).
(2) Williams and Major, *Science*, **91**, 2358 (1940).
(3) Williams, McAlister and Roehm, *J. Biol. Chem.*, **83**, 315 (1929).
(4) Williams, THIS JOURNAL, **58**, 1819 (1936).
(5) Mitchell and Williams, *ibid.*, **60**, 2723 (1938).
(6) Woods and Williams, *ibid.*, **59**, 1408 (1937).
(7) Williams, *ibid.*, **59**, 288 (1937).
(8) Williams, Rohrman and Christensen, *ibid.*, **59**, 291 (1937).
(9) Christensen, Williams and King, *ibid.*, **59**, 293 (1937).
(10) Williams, *J. Biol. Chem.*, **110**, 589 (1935).
(11) Williams, *et al.*, THIS JOURNAL, **61**, 454 (1939).
(12) Williams, *Science*, **89**, 486 (1939).
(13) Berg, *Bull. soc. chim.*, [3] **11**, 883 (1894).

sealed tube with 0.042 mol of 0.5 *N* sulfuric acid for three hours at 100°. The tube was then broken and the acid titrated with standard alkali. The second sample was treated in a sealed tube at 80° for fifteen minutes with 0.5 *N* sodium hydroxide. The tube was broken under acid and back titrated. Results are indicated in Table II.

TABLE II

TITRATION OF HYDROLYZED PANTOTHENIC ACID SAMPLES

Method of hydrolysis	Sample, mg.	Milliequiv. sample	Milliequiv. NaOH used by sample minus blank
(1) acid	1.54	0.0061	−0.00541
(2) base	1.55	.0062	.0

The fact that less sodium hydroxide was used than in the blank (acid treatment) means that approximately one equivalent of calcium ion was freed during the hydrolysis. Thus the cleavage products must have been essentially neutral compounds, *viz.*, β-alanine and a lactone. In the basic hydrolysis more alkali is required because the lactone ring breaks. The conversion of the hydroxy lactone into the salt of the hydroxy acid and the reverse was followed quantitatively on several samples. Partial synthesis experiments, described in a separate publication,[14] also confirmed the lactone character of this cleavage product.

Physiological Test—Streptococcus lactis-125.—In connection with the tests on suspected synthetic lactones it was desirable to have a test organism that was unaffected by the presence of β-alanine, since it was advantageous to use an excess of this reagent in the condensation. For this purpose an assay method was developed using the organism *Streptococcus lactis*. The medium and technique were similar to those of Snell, Strong and Peterson.[15]

β-Hydroxyl Group.—This determination is qualitative and is based on the following reaction

$$\mathrm{R{-}CHOHCH_2COOH} \xrightarrow[\Delta]{\mathrm{H_2SO_4}} \mathrm{R{-}CH{=}CHCOOH}$$

The dehydration (requiring an α-hydrogen) was followed by oxidation with potassium permanganate in acetone solution. Samples were heated in sealed tubes for one and one-half hours with 0.05 ml. of 25% sulfuric acid. The permanganate titration was carried out at room temperature in 5 ml. of 50% acetone solution. Table III sums up results obtained.

TABLE III

PERMANGANATE TITRATION OF HYDROXY ACIDS AFTER DEHYDRATION

	Sample, mg.	Moles $KMnO_4$/ mole compound
β-Hydroxy-γ-butyrolactone	3.0	2.0
α-Hydroxy-γ-butyrolactone	1.9	0
Hydracrylic acid	0.5	4.1
β-Hydroxybutyric acid	.73	5.3
Lactic acid	1.6	0
Erythronic lactone	2.0	1.1
α,β-Dihydroxybutyric acid	2.4	0.3
α,β-Dihydroxyisobutyric acid	1.5	0
α,β,β′-Trihydroxyisobutyric acid	1.5	0
α-Hydroxy-β-methyl-γ-butyrolactone	1.5	0.02
Pantothenic acid (potency 7700)	0.81	0
Pantothenic acid (potency 8900)	2.1	0.03

The data point to the absence of a β-hydroxyl group (accompanied by an α-hydrogen atom) in hydrolyzed pantothenic acid.

Selective Oxidations.—The well-known reaction of Criegee, the oxidation of 1,2-glycols with lead tetraacetate, was applied to pantothenic acid. In a typical experiment, 1.7 mg. of calcium pantothenate, potency 850 (1440 mg. units) was dissolved in 0.05 ml. of glacial acetic acid and heated for one hour with 10 mg. of lead tetraacetate. Water was added and the precipitate removed by centrifugation. A physiological test showed the recovery of 960 to 1100 mg. (70–80%) of the original activity. Higher potency samples gave similar results.

The reagent studied by Malaprade[16] for the oxidation of 1,2-glycols, periodic acid, on application to pantothenic acid concentrates gave negative results as determined by back titration of the periodate. Known 1,2-glycols were oxidized quantitatively under the conditions used. The selective oxidation method using iodic acid[6] gave negative results with pantothenic acid concentrates.

Qualitative experiments indicated the standard iodoform reaction to be negative on high potency calcium pantothenate concentrates. A semi-quantitative test was carried out as follows. Two 0.4-mg. samples of calcium pantothenate (potency 8900) were dissolved in 0.015 ml. of normal sodium hydroxide solution. Iodine solution (0.005 ml. of 0.35 *M*) was added to one of the samples. After standing for thirty minutes at 30° both samples were neutralized and tested physiologically. The recovery of activity was 86 and 89% for the control and the hypoiodite treated sample, respectively.

Synthetic Compounds.—Four hydroxy lactone structures with five carbon atoms were considered as possibilities for the non-nitrogenous portion of pantothenic acid. Accordingly these were synthesized,[17] condensed with β-alanine, and tested physiologically on *S. lactis*. Table IV summarizes the results. Erythronic lactone and α-hydroxy-γ-butyrolactone are included for comparison. The condensation procedure involved simply allowing an excess of β-alanine ethyl ester to react with the lactone under the conditions specified. Subsequently the reaction product was hydrolyzed for ninety minutes at room temperature with 0.3 *N* sodium carbonate solution and tested for physiological activity.

For a 100% yield of activity from one mg. of the correct lactone about 20,000 mg. units would be obtained. The yields were therefore significant but less than 0.5%. It was concluded that these lactones were similar to the natural one obtainable from pantothenic acid.

Purification.—For further work it became desirable to prepare additional amounts of concentrates with high potency. A repetition of the procedure using solvent fractionation of the calcium salts[1] was attemtped on further batches with potency 5000–6000. The results were un-

(14) Williams, Mitchell, Weinstock and Snell, THIS JOURNAL, **62**, 1784 (1940).

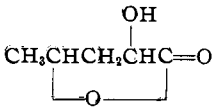
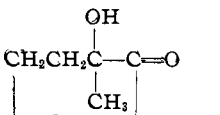
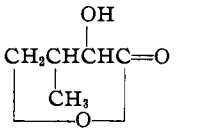
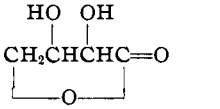
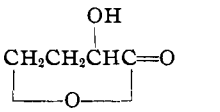
(15) Snell, *et al.*, *J. Bact.*, **38**, 293 (1939).

(16) M. L. Malaprade, *Bull. soc. chim.*, [4] **43**, 686 (1928); [5] **1**, 833 (1934).

(17) The synthetic studies here concerned will be described in a separate publication by one of us (H. H. W.).

TABLE IV

ACTIVITY OF SYNTHETIC PRODUCTS

Substance	Mg. of lactone	Time of reaction	Temp. of reaction	Activity in mgu.
	0.3	18	5	2–8
	.8	24	30	40–80
	.4	18	5	10
	1.7	24	30	0
	0.6	18	5	8.5
	.4	18	5	12–24
	1.0	1.5	30	5–25
	0.5	18	5	0
	1.5	18	5	0

satisfactory and we were not able to make any very substantial progress. Our total supply of material of approximately 90% purity was included in the fractions previously described and amounted to less than 100 milligrams. Apparently this material contained impurities which made the analyses correspond to a compound with one less carbon atom than is actually present.

While we have presented previously[1] a brief outline of an extended method which yielded nearly pure pantothenic acid, it is expensive and cumbersome. We are giving below a convenient method for preparing concentrates containing 10–25% barium pantothenate.[18]

All operations are described for a 1 kilo batch of liver extract. The raw material was the fraction of aqueous liver extract which is soluble in stronger than 90% alcohol.[19] Aqueous liver extract is not recommended for this purpose because of the large amounts of colloidal matter which it contains.

One kilo of liver extract was dissolved in 12 liters of water and 200 ml. of concentrated ammonium hydroxide (enough to bring the *p*H above 9.5) was added. Four hundred grams of decolorizing Norit was added, and the mixture was stirred for thirty minutes and filtered. The filtrate and washings were acidified to approximately *p*H 3 with sulfuric acid. The solution was diluted to 25 liters and 650 g. of Norit A (Pfanstiehl, or a comparable quality) was added. The mixture was stirred for an hour, filtered and washed with water. The Norit was re-suspended in 4 liters of water, 40 ml. of concentrated ammonium hydroxide was added, the mixture was stirred for thirty minutes, and filtered. This elution was repeated twice. The combined eluates were adjusted with sulfuric acid to approximately *p*H 3, and 200 g. of Norit A (Pfanstiehl, or a comparable quality) was added. The mixture was stirred for an hour, filtered, and the Norit was washed thoroughly with water, then eluted four times by stirring for thirty minutes each time with 1 liter of alcohol plus 5 ml. of pyridine.

The combined eluates were concentrated under reduced pressure to approximately 50 ml. Twenty-five grams of barium carbonate was added, and after a few minutes powdered barium hydroxide was cautiously added until the thick, sirupy material was approximately at *p*H 8. The mixture was then poured into 600 ml. of alcohol. After some time, the precipitate was filtered out and washed with alcohol. In order to remove more completely the activity from the precipitate, it was again suspended in 50 ml. of water and poured into 600 ml. of alcohol. After the precipitate had settled it was filtered off and washed as before. The two combined filtrates were concentrated under reduced pressure to a thick sirup and thoroughly mixed with 50 ml. of absolute alcohol. The precipitate at this stage was filtered off, dissolved in water, concentrated under reduced pressure, and worked up with absolute alcohol as before. The combined filtrates were concentrated under reduced pressure to a sirup.[20]

Further purification may be effected by adding acetone to the above sirup, stirring well, and filtering off the white granular solid which contains most of the active barium salt. The final acetone-insoluble barium salts are approximately 10–15% pure. Barium is best removed by adding sodium sulfate or sulfuric acid to the solution until no more precipitate forms, and filtering. Where sulfuric acid is used, a strongly acid solution is obtained even before all the barium is removed, and such solutions cannot be subjected to high temperature without loss of activity.

The solution of free acids thus obtained (10–15% solids) is adjusted to *p*H 1.5–2.5 with sulfuric acid and extracted continuously with ether for forty-eight hours. The ether extract contains most of the physiological activity and the concentrate obtained is approximately 20 to 25% pure pantothenic acid.

Methyl Acetyl Pantothenate.—This derivative was prepared as follows. Twenty-six mg. of calcium pantothenate (potency 6500) was treated with a mixture of 0.5 ml. of pyridine and 5 ml. of acetic anhydride in a sealed tube for one hour and fifteen minutes at 100°. The product was evaporated in vacuum, taken up in 0.5 ml. of methanol and treated in an ice-bath with an ether solution of diazomethane (prepared from 1 g. of methyl nitroso urea). After standing for two hours the solution was evaporated, taken up in 5 ml. of water and extracted five times with an equal volume of ether. The extract was subjected to distillation in a molecular still at 10^{-4} to 10^{-5} mm. pressure, giving 8 mg. of slightly yellow, viscous liquid. Further purification was carried out by high vacuum distillation in a straight 6-mm. Pyrex tube with a temperature gradient applied by means of a close fitting copper tube jacket heated at one end.

By this means several samples were obtained that were believed to be nearly pure, having potencies (after hydroly-

(18) This method was developed by one of us (E. E. S.) at the University of Wisconsin in collaboration with Drs. D. W. Woolley and F. M. Strong.

(19) Obtainable from Wilson and Company, Inc., Chicago.

(20) The alcohol-insoluble barium salts contain from 1 to 4% of pantothenic acid.

sis) from 10,500 to 11,500. Calculated as calcium salt this corresponds to a potency of about 12,500.

For the physiological tests the derivative was hydrolyzed to pantothenic acid by allowing to stand for one hour with 1 *N* alcoholic potassium hydroxide at room temperature.

Analyses of this material seemed to show definitely the presence of *one* rather than *two* acetyl groups. Acetic acid found: 23, 26, 26%. This was interpreted to mean that if two hydroxyl groups were present as indicated above, one of them must be unreactive under the acetylation conditions used.

Low yields, limited supplies of suitable material and unexpected decomposition during distillation as well as lack of time prevented extending this study to a satisfactory conclusion.

Discussion

Evidence has been presented for the formation of an α-hydroxylactone as a cleavage product of pantothenic acid. It was presumed to be a γ- or δ-lactone, and the former was indicated by the previously reported[11] condensation with acetone, acetaldehyde and benzaldehyde which would necessitate the formation of seven-membered rings in case the second hydroxyl was in the δ-position.

By the time the work reported in this paper had been completed the vitamin properties of pantothenic acid had been recognized,[21,22] and an increased interest in the compound was manifest. A co-operative arrangement was entered into whereby all of the material in this paper and other unpublished results were turned over to the Merck Research Laboratories, where the work was continued and the exact structure of the lactone determined.

We wish to express our keen appreciation to Professor J. W. E. Glattfeld of the University of Chicago for supplying us with generous samples of a number of hydroxy acids and lactones, to the Rockefeller Foundation for support of the research and to our collaborators in the Merck Research Laboratories, who have carried the work ahead to a successful identification of the lactone.

Summary

1. The presence of an α-hydroxyl group in the non-β-alanine portion of pantothenic acid has been shown.

2. This cleavage product is capable of spontaneous lactonization under acid conditions and is indicated to be an α-hydroxy-γ-lactone.

3. There are no adjacent hydroxyl groups in the pantothenic acid molecule and the absence of a β-hydroxy group in the lactone portion is indicated.

4. The groups CH_3CO— and CH_3CHOH— are not present in pantothenic acid.

5 The synthetic β-alanine derivatives of several α-hydroxy-γ-lactones: *i.e.*, α-hydroxy-γ-*n*-valero-lactone, α-hydroxy-β-methyl-γ-butyrolactone, and α-hydroxy-α-methyl-γ-butyrolactone, show definite but slight physiological activity.

6. A rapid method for the preparation of a concentrate containing about 20% pantothenic acid is described.

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(21) Jukes, THIS JOURNAL, **61**, 975 (1939).

(22) Woolley, Waisman and Elvehjem, *ibid.*, **61**, 977 (1939).

[CONTRIBUTION FROM THE RESEARCH LABORATORY OF MERCK & CO., INC.]

Pantothenic Acid. VI. The Isolation and Structure of the Lactone Moiety

BY ERIC T. STILLER, JOHN C. KERESZTESY AND JACOB FINKELSTEIN

It has been reported by Williams and his coworkers[1,2,3] that β-alanine is an essential constituent of the molecule of pantothenic acid. They further showed that this amino acid was combined by means of an amide linkage with a dihydroxy acid which was capable of ready lactonization.

These findings were confirmed for the chick anti-dermatitis factor by Woolley, Waisman and Elvehjem,[4] thus identifying pantothenic acid with a member of the vitamin B complex. This latter fact was also established by Jukes.[5]

Pantothenic acid from natural sources has been found to be extremely difficult to purify and so far the natural vitamin has not been isolated in a state of purity. This lack of success is probably due to its hydrophilic nature and also to the lack of suitable precipitating reagents.

(1) Williams, Weinstock, Jr., and Mitchell, Abstracts, Division of Organic Chemistry, Amer. Chem. Soc., Milwaukee, Wis., 1938.

(2) Weinstock, Jr., Mitchell, Pratt and Williams, THIS JOURNAL, **61**, 1421 (1939).

(3) Mitchell, Weinstock, Jr., Snell, Stanbery and Williams, *ibid.*, **62**, 1776 (1940).

(4) Woolley, Waisman and Elvehjem, *J. Biol. Chem.*, **129**, 673 (1939); THIS JOURNAL, **61**, 977 (1939).

(5) Jukes, *ibid.*, **61**, 975 (1939).