[CONTRIBUTION FROM THE CHEMICAL LABORATORIES OF OREGON STATE COLLEGE]

Pantothenic Acid. III. Analysis and Determination of Constituent Groups[1]

BY ROGER J. WILLIAMS, HARRY H. WEINSTOCK, JR., EWALD ROHRMANN, JOHN H. TRUESDAIL, HERSCHEL K. MITCHELL AND CURTIS E. MEYER

Due to the peculiar difficulties involved in the purification of pantothenic acid[2] we have been led to use certain unconventional methods of attack in connection with the study of the chemistry of this substance.

We will consider data obtained not only by examination and analysis of our most potent concentrates, but also by studying the behavior of the physiologically active principle itself under various treatments. It can be safely assumed that an alteration in the chemical structure of a physiologically potent substance will destroy or at least materially modify its physiological effects. Especially safe does this assumption seem in the case of a substance of low molecular weight. On the above assumption it is possible to judge the presence of specific active groups in pantothenic acid by treating it even in relatively crude form with various reagents which will bring about modification in structure and in physiological activity, in case the suspected groups are present.

Oxidation Equivalent Analysis.—This mode of study was originated in this Laboratory,[3-5] in connection with the investigation of pantothenic acid and has been of value as indicated below.

Numerous determinations on pantothenic acid preparations of various potencies have shown that all of the material with which we have dealt during the later stages of fractionation is highly oxygenated, similar in this respect to pantothenic acid. Expressed in terms of milligrams of oxygen required by one milligram of the calcium salts, the oxidation equivalent values of materials of potencies[2] 5000–11,000 have lain between 1.05 and 1.18. This is indicated in Table I. The latter value, or within 1% of this value, was consistently obtained on our best preparations. Analysis was made by the iodate method.[4] For complete oxidation of this material and to avoid side reactions it was found desirable to introduce two modifications of the procedure as previously described, first the use of 80% sulfuric acid instead of more concentrated acid, and second to increase the heating up to ninety minutes at 190°

TABLE I

"Potency" of prepn.	Weight, mg.	Oxidation equiv. (mg. O/mg. material)
6,900	0.888	1.048
8,050	1.524	1.122
8,950	1.861	1.17
10,050	1.125	1.176
11,100	1.230	1.185

Because of the slow drift in the oxidation equivalent values with purification, it seems safe to conclude that the value 1.18 cannot be far from the true value even if it is admitted that the material analyzed was not highly pure.

On the basis of three analyses, two for nitrogen and one for calcium, the molecular weight of free pantothenic acid is calculated to be 195, 201 and 209, respectively. Using any one of these figures and the method of calculation previously outlined,[3] we find that no formula agrees even approximately with the data except those in which five atoms of oxygen are present. With this as a basis the only formulas deducible for the free pantothenic acid are $C_8H_{15}O_5N$ and $C_8H_{13}O_5N$. The former agrees better with the oxidation equivalent data.

Elementary Analysis.—The most potent preparation so far obtained was subjected to elementary analysis[6] with the results indicated below. Previous tests on less pure samples had shown the absence of sulfur, phosphorus and halogen.

Anal. Calcd. for $(C_8H_{14}O_5N)_2Ca$: C, 42.8; H, 6.25; N, 6.25; Ca, 8.92. Calcd. for $(C_8H_{12}O_5N)_2Ca$: C, 43.2; H, 5.4; N, 6.3; Ca, 9.0. Found: C, 42.06, 42.77; H, 6.30, 6.32; N, 6.54, 6.35; Ca, 8.77.

The data correspond well to the composition $(C_8H_{14}O_5N)_2Ca$. This requires an oxidation equivalent of 1.21, and agrees with the first formula deduced from oxidation equivalent data. The molecular weight 205 for the free acid corresponds substantially with that of the physiologically active principle as determined by its rate of diffusion.

(1) Original manuscript received June 8, 1938.

(2) Williams, Truesdail, Weinstock, Rohrmann, Lyman and McBurney, THIS JOURNAL, **60**, 2719 (1938).

(3) R. J. Williams, *ibid.*, **59**, 288 (1937).

(4) Williams, Rohrmann and Christensen, *ibid.*, **59**, 291 (1937).

(5) Christensen, Williams and King, *ibid.*, **59**, 293 (1937).

(6) We are indebted to Dr. Randolph T. Major and Mr. D. F. Hayman of Merck & Co., for the carbon and hydrogen analyses.

Since the foregoing analyses seem to establish the molecular formula for pantothenic acid as $C_8H_{15}O_5N$ we shall present briefly the evidence as to the presence or absence of various characteristic groups, and the number of such groups in the molecule.

Carboxyl Group.—The behavior of pantothenic acid in an electrical field[7] has indicated the ionization constant to be approximately 3.9×10^{-5}. This corresponds in strength approximately to that of a β- or γ-hydroxycarboxylic acid. Since no other elements than carbon, hydrogen, oxygen and nitrogen are present in pantothenic acid, the presence of the carboxyl group is strongly indicated.

The esterification of pantothenic acid and subsequent hydrolysis can now be accomplished without appreciable loss. The concentrate is allowed to stand in a large excess of methanol 0.17 N in sulfuric acid for one hour at 30°, whereupon the physiological activity disappears practically completely. On allowing the resulting (neutralized) ester solution to stand for one or two hours in 0.05 N sodium carbonate solution at 30°, the active principle is nearly quantitatively recovered (95–99%). Pantothenic acid likewise can be esterified with diazomethane, and the original activity almost wholly recovered on hydrolysis.

On electrometric titration with calcium hydroxide by means of a glass electrode, pantothenic acid was found to contain only one acidic group per equivalent weight of 205, in agreement with the analytical figures for the calcium salt.

Amino and Imino Groups.—Van Slyke analysis of samples of pantothenic acid preparations showed the absence of significant amounts of amino nitrogen (Table II).

TABLE II

"Potency" of prepn.	Weight analyzed, mg.	Ml. N at S. C. (above blank)	% amino N[a]
1000	10	0.14	0.85
1000	10	.04	.25
1600	10	.04	.25
1600	10	.035	.2
7760	2.5	.03	.75

[a] These values are subject to a considerable variation since the measurement of very small amounts of gas was involved.

Moreover, the active principle was found not to be destroyed when treated with nitrous acid under Van Slyke conditions, and allowing the action to proceed longer as for the ϵ-amino group in lysine gave the same results.

The absence of both amino and imino groups was likewise indicated by the fact that the active principle was not destroyed (78% recovered) by treatment with p-bromobenzenesulfonyl chloride, and by the lack of marked basic properties on the part of pantothenic acid. When a solution of methyl pantothenate was treated with an excess of methyl iodide at 30° for forty hours and subsequently hydrolyzed, the physiologically active principle was recovered practically completely (96–98%). This was interpreted to mean the absence of a tertiary nitrogen.

Amide Group.—That pantothenic acid possesses very feeble basic properties was shown in electrolytic experiments in which it, along with glucose and aspartic acid as reference compounds, was placed at the outset in the anode compartment of a five-cell fractional electrical transport apparatus.[8] Approximately a 11,000 volt potential was applied for twenty-four hours. It was found that the weakly basic aspartic acid had moved into the next cell (toward the cathode) to the extent of 10.8% (as determined by micro kjeldahl). A physiological test indicated a transfer of 11.8% of the pantothenic acid. That this was actually an electrical transport and not a diffusion was shown by the fact that less than 2% of the glucose had moved from the anode compartment.

Electrolysis of methyl pantothenate indicated an even more pronounced migration toward the cathode, considerable of the ester having been found to have moved from the anode to the cathode cell (pH 8.0). This was determined by the physiological activity after hydrolysis.

When heated in alkaline solution pantothenic acid preparations fail to yield ammonia, indicating the absence of a simple amide group. A substituted amide group remains a possibility, and in fact a probability since such a group would exhibit weak basic properties. It is difficult to postulate another type of group which would meet this and other requirements. Its destruction with acid and alkali[9] is in accord with the supposed existence of an amide linkage.

Methoxy and Methyl Imino Groups.—Micro Zeisel determinations on pantothenic acid preparations of potency 10,000 indicated no distilled

(7) Williams and Moser, THIS JOURNAL, **56**, 169 (1934).

(8) R. J. Williams, *J. Biol. Chem.*, **110**, 589 (1935).

(9) Williams, Lyman, Goodyear, Truesdail and Holaday, THIS JOURNAL, **55**, 2912 (1933).

iodide, even when the temperature was raised up to 350–400°. The absence of methoxyl, methylimino and homologous groups was therefore shown.

Olefinic Unsaturation.—Catalytic hydrogenation experiments[9] have been repeated several times on highly concentrated preparations without substantial loss of activity both at room temperature and at 80°. When a pantothenic acid solution was treated with a large excess of bromine in carbon tetrachloride and allowed to stand for one hour at room temperature, practically all of the physiological activity was recovered. The absence of olefinic unsaturation was therefore indicated.

Aldehyde and Ketone Groups.—Hydrogenation experiments with Adams catalyst using ferric chloride as an activator[10] failed to cause destruction of the active principle. Treatment with sodium and sodium amalgam likewise failed to destroy the physiological activity. Oxidation experiments using ammoniacal silver solution, hydrogen peroxide, permanganate, etc., indicated that pantothenic acid is relatively difficult to oxidize. Treatment of pantothenic acid with phenylhydrazine in the presence of sodium acetate at 85° for two hours, resulted in only a slight loss of physiological activity. These experiments all point to the absence of aldehyde and ketone groups.

Aromatic Nucleus.—Through the kindness of Professor F. C. Koch, of the University of Chicago, a pantothenic acid preparation, potency 8500, was studied from the standpoint of its ultraviolet absorption spectrum. No bands appeared such as would have been present had the material been aromatic in character. The analytical data likewise do not correspond to those of an aromatic compound.

Hydroxyl Groups.—A considerable body of evidence has been accumulated on the question of the presence and number of hydroxyl groups.

The non-volatility of both the acid and the methyl ester indicated groups of this nature. While the ester of the active principle has been distilled in a molecular still in high vacuum, such distillation was very slow.

The hydrophilic nature of the acid and ester suggests the presence of hydroxyl groups. Even the methyl ester is distributed between water and ether in favor of the water.

(10) Adams, *et al.*, THIS JOURNAL, **45**, 1071, 3029 (1923).

More definite evidence has been obtained by the use of various reagents, which (excluding the presence of an amino group) could be considered to be more or less specific for hydroxyl groups. In every case destruction of the activity was obtained. The results are summarized in Table III.

TABLE III

Reagent	Time of reaction, hours	%. destruction
Acetic anhydride	24	95
Acetyl chloride	24	90
Thionyl chloride	0.4	85
Phosphorus pentachloride	.6	100
α-Naphthyl isocyanate	24	90
Phenyl isocyanate	24	95
Chloroacetyl chloride	24	90
Acetic acid[a]	10	<2

[a] In all these reactions 1–2 mg. of calcium salt was dissolved in 0.01 cc. of glacial acetic acid, and treated with a large excess of the reagent at room temperature.

In the case of the chloroacetyl chloride, 30–60% of the original activity could be recovered by allowing the product to stand five hours with 0.15 *N* sodium carbonate solution at 30°. This indicated that an actual esterification was taking place, rather than some deep-seated reaction.

The solution of dry calcium pantothenate (potency 10,000) in deuterium oxide and subsequent drying resulted in a substantial increase in weight, corresponding to the presence of from two to four active hydrogen atoms. Subsequent solution in ordinary water and drying brought the weight back to approximately the original value. Due to the non-crystalline character of the product, this could not be made the basis for a satisfactory quantitative determination of active hydrogen, but it served as a positive qualitative test.

Using a method recently developed in this Laboratory[11] 3.350 mg. of calcium pantothenate, potency 10,500 (determined by later methods to be about 85% pure), yielded, after nineteen hours of heating with hydriodic acid at 141°, 0.0133 milliequivalent of substituted hydroxyl and 0.0204 milliequivalent of reduced hydroxyl. The total hydroxyl was, therefore, 0.0337 milliequivalent. The theoretical value for pure calcium pantothenate with the formula given above and two hydroxyl groups is 0.0299 milliequivalent. Considering the impurity of the sample analyzed, this was regarded as a reasonably satisfactory

(11) Mitchell and Williams, *ibid.*, **60**, 2723 (1938).

check with the supposed existence of two hydroxyl groups.

Further evidence for the existence of at least two hydroxyl groups is based upon the observation that the physiological activity of pantothenic acid was 98% destroyed by allowing it to stand in the presence of acetaldehyde–hydrochloric acid (1%) solution for sixteen hours at room temperature. This activity was in part recovered (50%) by acidic hydrolysis. Similar destruction and recovery was effected by using acetone or benzaldehyde in place of acetaldehyde. This strongly suggests that condensation products similar to the well-known acetone glucose were formed. This type of condensation can take place with α, β-, α, γ- or α, δ-glycols.[12]

The basic property of pantothenic acid is so low that the argument previously presented[13] still holds. Pantothenic acid is not strong enough as an acid to be an alpha hydroxy acid. Furthermore, the ferric chloride test for α-hydroxy acids[14,15] was applied to purified calcium pantothenate with negative results.

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Summary

Oxidation equivalent analysis data and combustion analysis data agree with the formula $(C_8H_{14}O_5N)_2Ca$ for the calcium salt of pantothenic acid. Studies directed toward the determination of constituent groups indicate the presence in the molecule of one carboxyl group, two hydroxyl groups and probably a substituted amide group. The absence of amino, imino, tertiary amine, simple amide, methoxyl, methyl imino, olefinic, aldehydic, ketonic and aromatic groups is likewise indicated.

(12) Hibbert, *et al.*, THIS JOURNAL, **46**, 1286, (1924).
(13) Williams and Moser, *ibid.*, **56**, 169 (1934).
(14) Mulliken, "Identification of Pure Organic Compounds," John Wiley and Sons, Inc., New York, N. Y., 1910, Vol. I, p. 78.
(15) Berg, *Bull. soc. chim.*, [3] 11, 883 (1894).

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The Reaction of Carbon Suboxide with Methylmagnesium Iodide

BY JOHN H. BILLMAN AND CARL M. SMITH

A survey of the reactions of carbon suboxide[1] shows that on treatment with water, alcohols, thiols, ammonia, amines, hydroxylamine, hydrazines, and halogens, the expected derivatives of malonic acid are formed in each case. However, its reaction with organomagnesium halides, where its dual ketene nature should lead to interesting products, has not been investigated previously.

It might be expected that carbon suboxide would react with two moles of Grignard reagent to give an intermediate addition product which on hydrolysis would produce a 1,3-diketone.

$$O{=}C{=}C{=}C{=}O + 2RMgX \longrightarrow$$

$$R{-}C(OMgX){=}C{=}C(OMgX){-}R \xrightarrow[\text{ketonization}]{\text{dil. acid and}}$$

$$R{-}\overset{O}{\overset{\|}{C}}{-}CH_2{-}\overset{O}{\overset{\|}{C}}{-}R$$

However, actually on treating an ether solution of methylmagnesium iodide with carbon suboxide in dry ether, no acetylacetone could be obtained from the addition product even though the Grignard reagent was present in excess throughout the major portion of the reaction. The only crystalline solid that was isolated melted at 155°. Analysis and molecular weight gave an empirical formula of $C_{12}H_{12}O_6$. The chemical properties of this compound suggested that it might be triacetophloroglucinol. Accordingly a sample of the compound described by Heller[2] and by Göschke and Tambor[3] as triacetophloroglucinol was synthesized. A comparison of the synthetic and the isolated compound was made and they were found to be identical.

It was noted that during the reaction a precipitate formed. This addition compound upon hydrolysis was converted to triacetophloroglucinol. Because of the formation of this latter compound it is obvious that only one mole of methylmagnesium iodide reacted with each mole

(1) Reyerson and Kobe, *Chem. Rev.*, **7**, 479 (1930).
(2) Heller, *Ber.*, **45**, 418 (1912).
(3) Göschke and Tambor, *ibid.*, **45**, 1237 (1912).