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Degradation of the Fermentation *L. casei* Factor. II

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In the previous paper[1] data were presented indicating that 2-amino-4-hydroxypteridine-6-carboxylic acid, 2-amino-4-hydroxy-6-methylpteridine and *p*-aminobenzoic acid were degradation products of the fermentation *L. casei* factor. In this paper further degradation products are presented and evidence for the structure of the liver *L. casei* factor is outlined.

When the fermentation *L. casei* factor was dissolved in water at *p*H 4.0 and autoclaved at 120° for six hours, the activity of the compound was destroyed. From this inactive solution a crystalline product was isolated. The analysis, chemical properties, and melting point suggested that the substance was *l*-pyrrolidonecarboxylic acid. However, a cryoscopic determination of the molecular weight offered the possibility of a diketopiperazine of glutamic acid.

Synthetic *l*-pyrrolidonecarboxylic acid caused no depression of the melting point of the unknown compound. Further corroborative evidence of the identity of the degradation product with *l*-pyrrolidonecarboxylic acid was furnished by comparison of their infrared absorption spectra (Fig. 1).

When the fermentation *L. casei* factor was dissolved in water and the resulting solution made 0.5 *N* with sulfur dioxide, the growth-promoting properties of the compound were destroyed. There was a marked increase in the fluorescence of the solution and an aromatic amine was formed that could be detected by the method of Bratton and Marshall.[2]

After removal of the sulfur dioxide, the fluorescent moiety reacted rapidly with such typical aldehyde reagents as hydroxylamine, phenylhydrazine and semicarbazide to form insoluble products. The condensation products exhibited no fluorescence. These derivatives were extremely insoluble and quite unstable but analyses indicated the condensation product with phenylhydrazine to be a hydrazone. If the fluorescent compound arising from sulfurous acid hydrolysis was allowed to stand in dilute alkali anaerobically, approximately equal amounts of 2-amino-4-hydroxypteridine-6-carboxylic acid and a compound which was identified as 2-amino-4-hydroxy-6-methylpteridine were formed. The presence of 2-amino-4-hydroxy-6-methylpteridine was unexpected and its mode of formation is obscure. The ability to form insoluble precipitates with typical aldehyde reagents and the dismutation in dilute alkali indicate the presence of an aldehyde group in the pteridine arising from sulfite cleavage of the fermentation *L. casei* factor.

(1) Stokstad, *et al.*, THIS JOURNAL, **70**, 5 (1948).

(2) Bratton and Marshall, *J. Biol. Chem.*, **128**, 537 (1939).

By appropriate manipulations the aromatic amine fragment could be purified. The aromatic amine nitrogen as determined by the method of Bratton and Marshall was found to be approximately 25% of the total nitrogen. The remaining 75% of the nitrogen could be converted into α-amino acid nitrogen by acid or alkaline hydrolysis. From such hydrolysates the aromatic amine was isolated and identified as *p*-aminobenzoic acid. Microbiological assay of the hydrolysate indicated the presence of 3 moles of glutamic acid. Analytical figures suggested that the amino acids are joined in peptide linkage as *p*-aminobenzoyldiglutamylglutamic acid. The mode of linkage of the glutamic acids will be discussed in a subsequent communication.

After sulfurous acid hydrolysis of the fermentation *L. casei* factor no one or two carbon-containing fragments could be detected. Aerobic ultraviolet inactivation of the fermentation *L. casei* factor which cleaves the compound into a pteridine fraction and an aromatic amine fraction failed to yield any carbon dioxide. The evidence suggests that the carbon content of the fermentation *L. casei* factor is that represented by the degradation products.

The fermentation *L. casei* factor was not measurably inactivated by hydrogenation over regular Raney nickel, Raney nickel containing aluminum, or over palladium on barium sulfate if the hydrogenation was carried out at a *p*H of 7.0 or greater and at atmospheric pressure. If the compound was hydrogenated at *p*H 1.0 or 3.0 and at atmospheric pressure using a palladium–barium sulfate catalyst, there was rapid biological inactivation with the simultaneous formation of an aromatic amine. The ultraviolet absorption at 365 mμ was greatly decreased indicating the reduction of the pteridine to a compound that exhibited no absorption at this wave length. The absorption and fluorescence of the pteridine moiety could be regenerated by oxidation with manganese dioxide in acid solution.

The reductive reaction can be carried out chemically with zinc dust and either dilute hydrochloric or sulfuric acids. From chemically reduced solutions of the fermentation *L. casei* factor, 2-amino-4-hydroxy-6-methylpteridine has been isolated and characterized.

In a summation of the data presented in this and the previous paper of this series the following salient points serve as a guide to the formulation of the structure of the liver *L. casei* factor:

A. Aerobic alkaline hydrolysis, sulfite cleavage and chemical or catalytic reduction each give

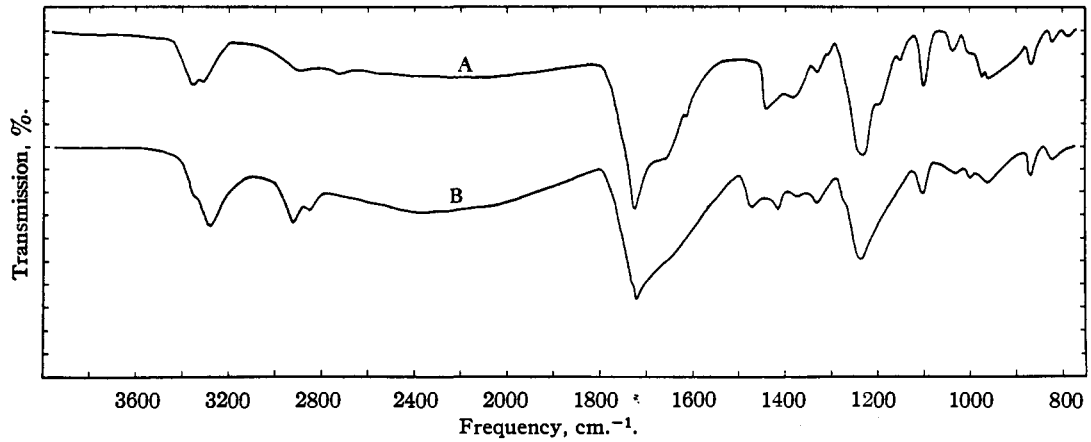

Fig. 1.—Infrared absorption spectra of *l*-pyrrolidonecarboxylic acid: A, synthetic; B, natural.

rise to a pteridine fraction and a primary aromatic amine. This indicates that the point of linkage is probably through the aromatic amine group to the pteridine.

B. A single carbon atom serves as a linkage between the pteridine and aromatic amine. This is indicated by obtaining either 2-amino-4-hydroxypteridine-6-carboxylic acid or 2-amino-4-hydroxy-6-methylpteridine or a compound that could be converted into them as degradation products in several methods of cleavage. Further indicative evidence is the inability to detect any one or two carbon-containing fragments arising from two degradative reactions. The evidence suggests that the one carbon is present as a methylene group. Thus, the necessity of oxygen for the alkaline cleavage is more rational. The reductive formation of 2-amino-4-hydroxy-6-methylpteridine is in accord with this hypothesis.

C. The aromatic amine arising from sulfite-cleavage is a tetrapeptide composed of one mole of *p*-aminobenzoic acid and three moles of glutamic acid. In the previous paper of this series it was observed that in degrading the fermentation *L. casei* factor to the *dl*-liver *L. casei* factor, two moles of α-amino acid nitrogen were liberated. Thus, the liver *L. casei* factor contains one mole of glutamic acid and the fermentation compound three moles of glutamic acid.

Experimental[3,4,5]

Aqueous Hydrolysis of the Fermentation *L. casei* Factor.—Two hundred mg. of the fermentation *L. casei* factor was dissolved in 125 ml. of water and autoclaved at 120° for six hours. The resulting clear solution was allowed to stand at 5° for twenty-four hours. The material that precipitated was centrifuged out and discarded. The water solution was concentrated to dryness and the residue extracted with 4–15 ml. portions of hot absolute ethanol. The ethanol extracts were concentrated to dryness and the residue extracted with 125 ml. of acetone. The acetone was evaporated and the residue sublimed in high vacuum at 148° for six hours. The sublimate was contaminated with a brown pigment that was removed by extracting the sublimate with several 0.1-ml. portions of ice-cold ethanol. The residue was re-sublimed at 138° for six hours. There was obtained a colorless crystalline sublimate (wt. 28 mg.) which melted at 147–148°. A cryoscopic determination of the molecular weight using camphor as the solvent gave a value of 284.

Anal. Found: C, 47.05; H, 5.14; N, 10.93.

Titration data for the compound indicated the presence of a strong carboxylic acid group. The equivalent weight calculated at *p*H 7.0 was 129.2. The compound exhibited no ultraviolet absorption. The substance gave no color with ferric chloride in aqueous or alcoholic solution. The sample adsorbed no hydrogen over Adams platinum oxide catalyst.

The compound contained no α-amino acid nitrogen. However, when 1.794 mg. of the compound was dissolved in 2 ml. of 1 *N* sodium hydroxide and heated at 100° for three hours, the following figures were obtained: total nitrogen, 0.196 mg.; α-amino acid nitrogen before hydrolysis, negative; α-amino acid nitrogen after hydrolysis, 0.174 mg. Because of the small sample used and the accuracy of the Van Slyke determination, these values are indicative of complete conversion of the nitrogen into α-amino acid nitrogen.

A semi-quantitative microbiological determination of the glutamic acid content of a 1 *N* sodium hydroxide hydrolysate of the sublimate was carried out using *Lactobacillus casei* as the test organism. A glutamic acid control was run under identical conditions to determine the extent of racemization. This factor was used in calculating the results. The sublimate analyzed for 81.5% glutamic acid.

l-Pyrrolidonecarboxylic acid was synthesized according to the procedure of Abderhalden and Kautzsch.[6] The *l*-pyrrolidonecarboxylic acid melted not sharply at 144–148°.[7] A mixture of *l*-pyrrolidonecarboxylic acid and the unknown melted at 145–147°.

A cryoscopic determination of the molecular weight of the synthetic *l*-pyrrolidonecarboxylic acid gave a value of 284 indicating polymerization of the compound.

Reaction of the Sulfite-cleaved Pteridine with Phenylhydrazine.—One hundred mg. of the fermentation *L. casei* factor was dissolved in 50 ml. of water with slight warming and a solution of sulfurous acid added. The final volume was 200 ml. and was 0.5 *N* with respect to sulfurous acid. The solution was kept at 32° for sixteen hours. The solu-

(3) All concentrations carried out in vacuum.

(4) All melting points are uncorrected.

(5) The ultraviolet absorption was determined with a Beckman Spectrophotometer. The fluorescence of the pteridines was determined in 0.05 *M* sodium borate buffer with a Pfaltz and Bauer Fluorophotometer.

(6) Abderhalden and Kautzsch, *Z. physiol. Chem.*, **68**, 487 (1910).

(7) On further fractional crystallization the m. p. of the synthetic compound was raised to 160–162°. A lack of material precluded further purification of the isolated *l*-pyrrolidonecarboxylic acid. Partial racemization could account for the low m. p. of the isolated compound (see ref. 3).

tion was concentrated to one-half volume under nitrogen to remove the sulfur dioxide, warmed to 80° and a solution of phenylhydrazine in dilute acetic acid was added. There was an immediate formation of a red precipitate. The solution was warmed for thirty minutes, then cooled and centrifuged. The precipitate was dissolved in a mixture of piperidine and water (0.4:10) and precipitated by the addition of acetic acid. The solution and precipitation were repeated twice.

Anal. Calcd. for $C_{13}H_{11}N_7O$: C, 55.5; H, 3.92. Calcd. for $C_{20}H_{17}N_9O$: C, 48.1; H, 3.41. Found: C, 54.2; H, 3.86.

The analyses indicate the formation of a hydrazone rather than a dihydrazone.

Effect of Alkali on the Sulfite-cleaved Pteridine.—Two hundred and fifty mg. of the fermentation *L. casei* factor was treated with sulfurous acid as described above. After hydrolysis the solution was concentrated to dryness under nitrogen and the residue dissolved in 6.0 ml. of 1 *N* sodium hydroxide. The flask was evacuated and then stored in a vacuum desiccator at 4°. After fourteen days the precipitate which had formed was centrifuged off and washed with 2 *N* sodium hydroxide. The absorption spectra of this precipitate in both 0.1 *N* sodium hydroxide and 0.1 *N* hydrochloric acid were identical with the absorption spectra of the 2-amino-4-hydroxypteridine-6-carboxylic acid. The amount of the 2-amino-4-hydroxypteridine-6-carboxylic acid as determined by ultraviolet absorption was 17 mg.

The solution after removal of the precipitated 2-amino-4-hydroxypteridine-6-carboxylic acid was adjusted to *p*H 7.0 and a volume of 30 ml. and extracted five times with ten volume portions of butanol. The total amount of pteridine extracted by the butanol as measured by fluorescence was 30 mg. The butanol was concentrated and the compound precipitated by the addition of ether. The precipitate was dissolved in 20 ml. of hot water at *p*H 7.0. A small amount of brown insoluble pigment was removed by centrifugation. The solution was cooled and the precipitate collected; yield was 25 mg. The pteridine was crystallized from 2.0 ml. of 5 *N* sodium hydroxide. The crystalline precipitate was collected and recrystallized from 1.3 ml. of 5 *N* sodium hydroxide. The crystals were suspended in water, adjusted to *p*H 7.0 and dissolved by heating. After cooling the pteridine was collected.

Anal. Calcd. for $C_7H_7N_5O_2$: C, 43.5; H, 3.63; N, 36.3. Calcd. for $C_7H_7N_5O$: C, 47.4; H, 3.95; N, 39.5. Found: C, 47.4; H, 4.43; N, 39.6.

A comparison of the ultraviolet absorption spectra of this compound with the absorption spectra of various pteridines indicated its identity with 2-amino-4-hydroxy-6-methylpteridine (Fig. 2). The synthesis of this compound will be described in the following paper of this series. The infrared absorption spectra of the natural and synthetic 2-amino-4-hydroxy-6-methylpteridine are identical (Fig. 3).

The pteridine remaining in the water layer after butanol extraction was precipitated twice by silver at *p*H 5.0 to remove it from the amine in solution. The yield was 10 mg. as determined by ultraviolet absorption. The absorption spectra of this pteridine were similar to those for the 2-amino-4-hydroxypteridine-6-carboxylic acid.

The total amount of pteridines obtained from the fermentation *L. casei* factor that had been cleaved with sulfurous acid and subsequently treated anaerobically with 1 *N* sodium hydroxide is presented in Table I.

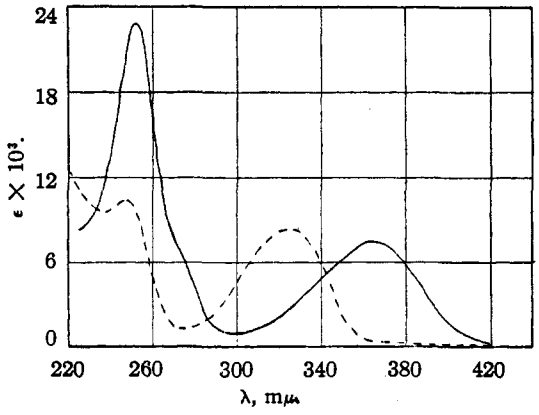

Fig. 2.—Ultraviolet absorption spectra of 2-amino-4-hydroxy-6-methylpteridine: ——, in 0.1 *N* sodium hydroxide; – – – –, in 0.1 *N* hydrochloric acid.

TABLE I

PTERIDINE OBTAINED ON ALKALINE DISMUTATION

	Milligrams	Moles per mole of F. L. C. F.
Fermentation *L. casei* factor used	250	
Amine (calcd. as PABA)	48	0.98
2-Amino-4-hydroxypteridine-6-carboxylic acid		
a. Precipitated by 1.0 *N* sodium hydroxide	17	.23
b. Remaining after extraction with butyl alcohol and precipitated by silver	10	.14
Total	27	.37
2-Amino-4-hydroxy-6-methylpteridine		
a. Total amount extracted by butanol	30	.47

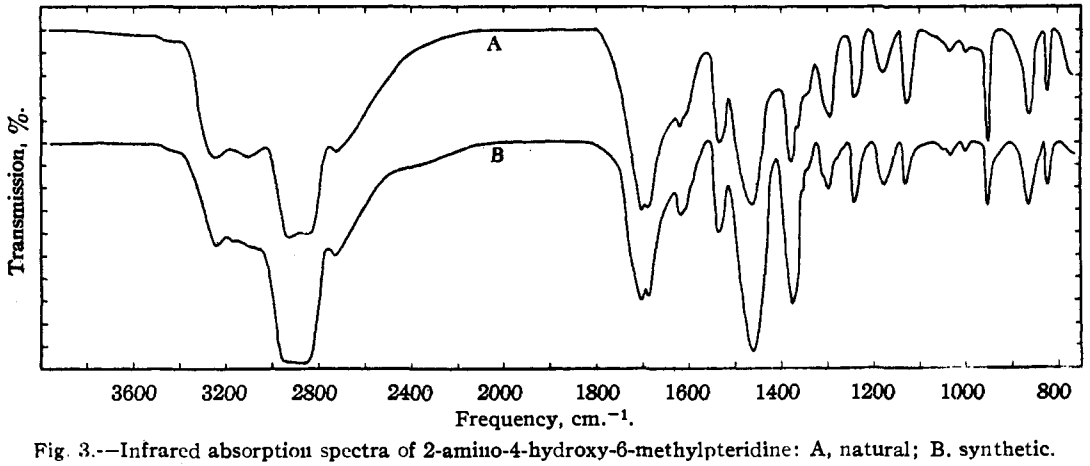

Fig. 3.—Infrared absorption spectra of 2-amino-4-hydroxy-6-methylpteridine: A, natural; B. synthetic.

Purification of the Aromatic Amine.—One gram of the fermentation *L. casei* factor was hydrolyzed with sulfurous acid. After cleavage, the solution was concentrated to one-half volume to remove the sulfur dioxide. The pteridine fraction was removed by precipitation with silver sulfate at *p*H 2.0. Excess silver and sulfate ions were removed from the filtrate; yield was 115 mg. calculated as *p*-aminobenzoic acid. The solution was adjusted to *p*H 2.0 and concentrated to dryness. The residue was dried thoroughly and the amine extracted from the solid with absolute isopropanol. The isopropanol solution was concentrated to dryness and the residue dissolved in 30 ml. of water. Excess lead acetate was added. After standing overnight at 5°, the lead precipitate was collected and washed with 5 ml. of cold water containing a little lead acetate. The precipitate was suspended in water and the lead removed as the sulfide; yield was 96.5 mg. calculated as *p*-aminobenzoic acid. The solution was adjusted to *p*H 2.0 and concentrated to dryness. The amine was extracted into absolute isopropanol. The isopropanol was evaporated and the residue dissolved in 50 ml. of water. Barium hydroxide was added to *p*H 10.0 and the slight precipitate removed. Ethanol was added to 50% concentration and the material kept at 5° overnight. The barium precipitate was collected, dissolved in water, adjusted to *p*H 2.0 and concentrated to dryness. The amine was extracted with isopropanol and then precipitated by the addition of one volume of petroleum ether. The precipitate was centrifuged off, dissolved in 35 ml. of water and concentrated slightly to remove the isopropanol and petroleum ether. Barium hydroxide was added to *p*H 10.0 and ethanol to 50% concentration. After thorough chilling the precipitate was collected, washed thoroughly with 50% ethanol, absolute ethanol and acetone; yield was 71 mg. calculated as *p*-aminobenzoic acid.

Anal. Calcd. for $C_{22}H_{18}N_4O_{11}$: C, 50.5; H, 5.35; N, 10.69. Found: C, 29.42; H, 4.20; N, 6.64; ash, 50.00. Cor. for C in ash: C, 32.46; H, 4.20; N, 6.64; Ba, 34.80. Ba-free: C, 49.8; H, 6.44; N, 10.17.

As the ash is barium carbonate, the carbon values have been corrected for the amount of carbon retained in the ash.

The free acid is extremely soluble in water or alcohol. The compound could not be crystallized from various solvents or combinations thereof.

Aromatic Amine Nitrogen.—Duplicate weighings were dissolved equivalent to 13.62 micrograms of total nitrogen per ml.; 29.1 microgram equivalents of *p*-aminobenzoic acid was found per ml., which is equivalent to 2.97 micrograms of aromatic amine nitrogen. Therefore 22.39% of the total nitrogen was present as aromatic amine nitrogen.

Effect of Acid and Alkaline Hydrolysis on the Amine Fragment.—All samples were hydrolyzed for sixteen hours at 120° in a sealed tube. One normal sodium hydroxide, 5 *N* sodium hydroxide, 1 *N* hydrochloric acid and 2.5 *N* hydrochloric acid liberated 57.6, 73.0, 72.2, and 74%, respectively, of the nitrogen as α-amino acid nitrogen. Glutamic acid analysis of the 2.5 *N* hydrochloric acid hydrolysate was carried out using *Lactobacillus arabinosus*.

Anal. Calcd. for 3 moles of glutamic acid in the amine fragment (as the free acid): 84.25. Found: 81.8, 82.5.

Isolation of *p*-Aminobenzoic Acid from the N_4 Amine.—One hundred and eighty-seven mg. of the amine fragment was dissolved in 6 ml. of 5 *N* sodium hydroxide and hydrolyzed anaerobically for thirteen hours at 120°. The resulting hydrolysate was diluted to 100 ml. and adjusted to *p*H 3.0. The solution was extracted with 100 ml. of ethyl acetate and the ethyl acetate extract washed with 25 ml. of water. The ethyl acetate was removed by distillation and the residue dissolved in 0.5 ml. of hot water. On cooling the compound crystallized as needles. The precipitate was recrystallized from 0.25 ml. of hot water, m. p. 180.5–182.5°. In admixture with an authentic specimen of *p*-aminobenzoic acid (m. p. 183.5–184.5°) it melted at 182.5–183.5°.

Anal. Calcd. for $C_7H_7O_2N$: C, 61.3; H, 5.11. Found: C, 61.5; H, 4.93.

Detection of One or Two Carbon-atom-containing Fragments.—From sulfurous acid hydrolysates of the fermentation *L. casei* factor no carbon dioxide, glyoxal, glycolic aldehyde or ethylene glycol could be detected by appropriately controlled tests. No carbon dioxide was formed on aerobic ultraviolet irradiation of the fermentation *L. casei* factor.

Reductive Cleavage of the Fermentation *L. casei* Factor.—Two hundred and sixteen mg. of the fermentation *L. casei* factor was dissolved in 250 ml. of 0.4 *N* sulfuric acid and 5 g. of zinc dust was added. After twenty-five minutes the excess zinc dust was removed by filtration and 5 g. of manganese dioxide added. After ten minutes of oxidation the solution was filtered and enough dipotassium hydrogen phosphate was added to the filtrate to raise the *p*H to 6.5. The zinc phosphate was centrifuged off, washed with water and discarded. The centrifugate and washings were combined. The distribution of the pteridine between butanol and water at various *p*H values was determined. The results suggested that the compound contained no carboxylic acid group. The solution (*p*H 6.5) was extracted with three ten-volume portions of butanol. The butanol extracts were concentrated and the pteridine precipitated by the addition of ether. The compound was crystallized twice from 4 *N* sodium hydroxide, converted to the free compound and dried.

The ultraviolet absorption spectra of the isolated pteridine were identical with the spectra of an authentic sample of the 2-amino-4-hydroxy-6-methylpteridine.

Acknowledgment.—It is a pleasure to acknowledge the assistance of the Misses E. Boggiano, B. Eames, E. Brogen and A. Buxó for the microbiological assays and the spectrophotometric determinations. The authors extend their appreciation to Dr. R. C. Gore of the Stamford Research Laboratories, American Cyanamid Company, for the infrared determinations, and to Mr. L. Brancone and co-workers for the microanalyses.

The authors are especially indebted to Dr. J. H. Williams for his constant interest and counsel and for his efforts in coördinating the work performed in the various laboratories.

Summary

l-Pyrrolidonecarboxylic acid, 2-amino-4-hydroxypteridine-6-carboxylic acid, 2-amino-4-hydroxy-6-methylpteridine and *p*-aminobenzoic acid have been isolated as degradation products of the fermentation *L. casei* factor. Evidence for the existence of 2-amino-4-hydroxypteridine-6-carboxaldehyde and glutamic acid has been presented.

The significance of these products in formulating the structure of the *L. casei* factors has been discussed.

PEARL RIVER, NEW YORK RECEIVED JANUARY 24, 1947