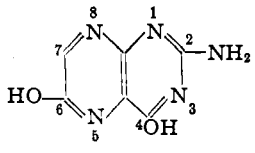
[CONTRIBUTION FROM THE LEDERLE LABORATORIES DIVISION, AMERICAN CYANAMID COMPANY]

The Structure of the Liver *L. casei* Factor

BY J. H. MOWAT, J. H. BOOTHE, B. L. HUTCHINGS, E. L. R. STOKSTAD, C. W. WALLER, R. B. ANGIER, J. SEMB, D. B. COSULICH[1] AND Y. SUBBAROW

Up to the present time the major reported work in the field of pteridine chemistry has been that of Purrmann, Wieland, and their collaborators, who, in their study of the wing pigments of the butterfly, were able to isolate a yellow pigment to which they gave the name xanthopterine and to which they assigned the structure[2] shown in the formula, that is, 2-amino-4,6-dihydroxypteridine.[3]



Earlier reports[4,5,6,7] of the biological relationship between xanthopterine and the *L. casei* factors, as well as a comparison of the ultraviolet absorption spectra of these factors with the spectra of a number of known pteridines, suggested the presence of a pteridine nucleus in the *L. casei* factors. This possibility warranted more serious consideration when we were able to isolate from the fermentation *L. casei* factor a number of degradation products having pteridine-like absorption curves and having chemical properties consistent with such a hypothesis.

The first of these pteridine-like compounds (V) whose isolation and properties have been described in Paper III[8] of this series, after cleavage with chlorine water and hydrochloric acid gave a positive test for guanidine,[8] thus indicating the presence of a 2-aminopyrimidine. Furthermore, a titration curve indicated the presence of two acidic groups of *pKa* 3.9 and 7.7, probably a carboxyl group and another less acidic group of the enolic type.

Elementary analyses[9] indicated a compound having an empirical formula $C_7H_5O_3N_5$. Neither methoxyl groups nor N-methyl groups could be detected.

Decarboxylation of a few milligrams of the material gave a product which appeared to be identical with 2-amino-4-hydroxypteridine (II), prepared from 2,4,5-triamino-6-hydroxypyrimidine[10] (I) and glyoxal.

The total synthesis of (V) was then accomplished by chlorination and reduction of the previously known iso-xanthopterinecarboxylic acid (III)[11] and also by the condensation of 2,4,5-triamino-6-hydroxypyrimidine (I) and ethyl α-bromo-β,β-diethoxypropionate.[12] These syntheses established the nature of the ring skeleton and the positions of the 2-amino group and the 4-hydroxyl group, and by directly relating the position of the carboxyl group to that of iso-xanthopterinecarboxylic acid (III), showed that the carboxyl group was attached to the pyrazine ring, very probably in the 6-position. The position of the carboxyl group required further proof, however, since the structures postulated by Purrmann[2,11] for xanthopterine and iso-xanthopterinecarboxylic acid were still open to question.

A method of degrading substituted lumazines to pyrazines has been described by Weijlard, Tishler and Erickson[13] and by following this procedure, using 2-amino-4-hydroxy-7-methylpteridine (XIII) we obtained 2-amino-6-methylpyrazine. Subsequently this work was repeated, using the corresponding 2-amino-4-hydroxy-6-methylpteridine (VII), and the product was found to be identical with an authentic sample of 2-amino-5-methylpyrazine (IX) prepared from the known 2,5-dimethylpyrazine (X).

The structure of the so-called "fluorescent dibasic acid[8] obtained from the fermentation *L. casei* factor after aerobic alkaline hydrolysis was therefore conclusively proven to be 2-amino-4-hydroxypteridine-6-carboxylic acid (V).

Another pteridine-like compound[8,14] isolated from hydrolysates of the fermentation *L. casei* factor gave an analysis leading to the empirical formula $C_7H_7ON_5$, a formula which corresponded to that of 2-amino-4-hydroxy-6-methylpteridine. This assumption was proved to be correct by the synthesis of an identical compound through the following series of reactions.

(1) Calco Chemical Division, American Cyanamid Company, Bound Brook, New Jersey.

(2) Purrmann, *Ann.*, **546**, 98 (1940).

(3) The system of numbering recommended by "Chemical Abstracts" and the "Ring Index" will be used instead of the older system of numbering which has been used by Purrmann and his collaborators.

(4) Mitchell, THIS JOURNAL, **66**, 274 (1944).

(5) Wright and Welch, *Am. J. Med. Sci.*, **206**, 128 (1943).

(6) Totter, *et al.*, *Federation Proc.*, **2**, 72 (1943).

(7) Wright, Skeggs and Welch, *ibid.*, **3**, 88 (1944).

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

(9) For some time we experienced considerable difficulty in obtaining correct and reproducible analysis of the pteridines, particularly in the case of nitrogen determinations. Wieland and Purrmann, *Ann.*, **545**, 163 (1940), have reported similar difficulties. An investigation of this problem by our microanalytical department appears to have resulted in a satisfactory procedure which may be the subject of a later communication.

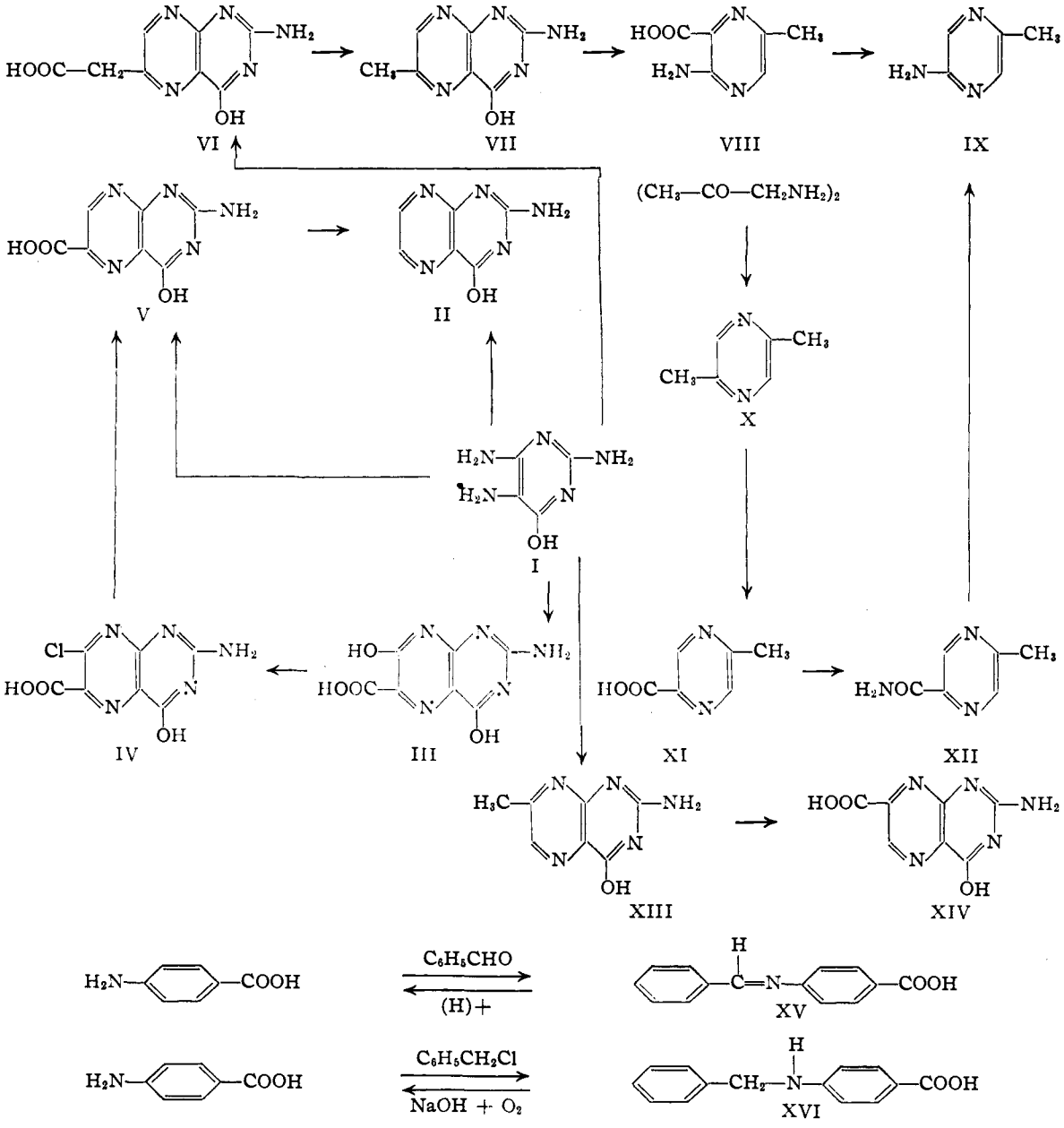
(10) Traube, *Ber.*, **33**, 1371 (1900).

(11) Purrmann, *Ann.*, **548**, 284 (1941).

(12) Direct halogenation of ethyl-β,β-diethoxypropionate appeared to give a mixture of the mono- and di-halogen compounds which could not be readily separated, but which was satisfactory for the synthesis described. The pure mono-halogenated compound has been prepared recently by Oroshnik and Spoerri, THIS JOURNAL, **67**, 721 (1945), using another method.

(13) Weijlard, Tishler and Erickson, THIS JOURNAL, **67**, 802 (1945).

(14) Hutchings, *et al.*, *ibid.*, **70**, 1 (1948).



Methyl γ,γ-dimethoxyacetoacetate, prepared from methyl dimethoxyacetate and methyl acetate by the procedure of Johnson and Mikeska,[15] was condensed with 2,4,5-triamino-6-hydroxypyrimidine (I), to give 2-amino-4-hydroxypteridine-6-acetic acid (VI), (Fig. 5) which could be easily oxidized with alkaline potassium permanganate to 2-amino-4-hydroxypteridine-6-carboxylic acid (V). Decarboxylation of (VI) gave the desired 2-amino-4-hydroxy-6-methylpteridine (VII).

The isomeric 2-amino-4-hydroxy-7-methylpteridine (XIII) was prepared by the condensation of 2,4,5-triamino-6-hydroxypyrimidine (I) with the acetal of methyl glyoxal.[16] This 7-methylpteridine was then converted to the corresponding 2-amino-4-hydroxypteridine-7-carboxylic acid (XIV) by oxidation with alkaline potassium permanganate.

The ultraviolet absorption curves of the 6- and 7-methylpteridines are shown in Figs. 1 and 2.

Since the pteridine nucleus appears to be stable in the presence of alkaline potassium permanganate, various side chains in the 6 or 7 position may usually be oxidized to the corresponding pteridinecarboxylic acid which may then be identified by comparing its ultraviolet absorption spectrum

(15) Johnson and Mikeska, THIS JOURNAL, **41**, 812 (1919).

(16) Madelung and Oberwegner, *Ber.*, **65**, 940 (1932).

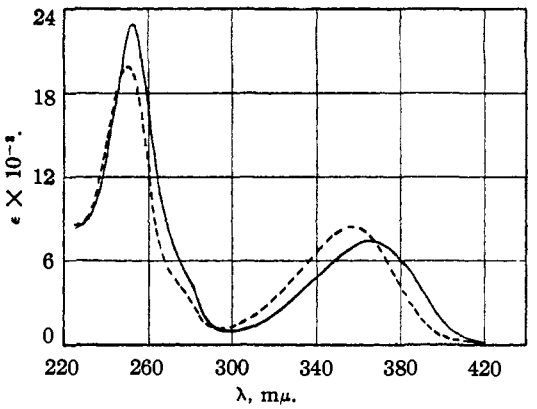


Fig. 1.—Ultraviolet absorption spectra in 0.1 *N* sodium hydroxide: ——, 2-amino-4-hydroxy-6-methylpteridine; - - - -, 2-amino-4-hydroxy-7-methylpteridine.

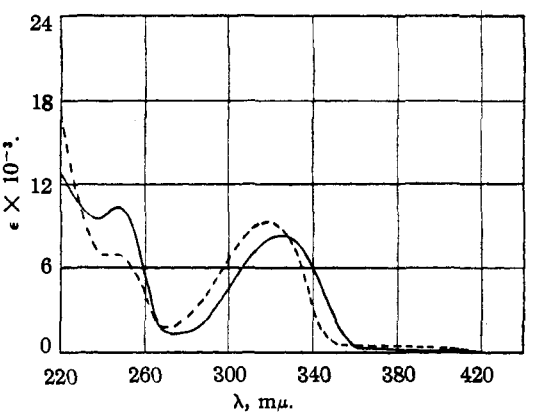


Fig. 2.—Ultraviolet absorption spectra in 0.1 *N* hydrochloric acid: ——, 2-amino-4-hydroxy-6-methylpteridine; - - - - -, 2-amino-4-hydroxy-7-methylpteridine.

with that of a known sample. The ultraviolet absorption curves of these pteridinecarboxylic acids are shown in Figs. 3 and 4.

The structure of the cleavage products of the *L. casei* factors having been determined, it then became necessary to consider the linkages by which these fragments were joined. Evidence has been presented in a previous paper[14] of this series which indicates that the pteridine ring system must be joined at the 6-position to the amino group of *p*-aminobenzoylglutamic acid (or its homologs) through a one-carbon atom linkage. Two simple model compounds were prepared, namely, N-(benzylidene)-*p*-aminobenzoic acid[17] (XV), and N-(benzyl)-*p*-aminobenzoic acid (XVI). The anil linkage of (XV) was found to cleave with extreme rapidity in the presence of even traces of weak acids, whereas the methylene linkage of (XVI) was stable when subjected to acids or to anaerobic alkaline hydrolysis but was rapidly cleaved by aerobic alkaline hydrolysis, just as in the case of the *L. casei* factors. Such a linkage also appears to explain the role of oxygen in the aerobic alkaline hydrolysis, since it has been reported[18] that compounds of this type are cleaved by hydrolysis in the presence of hydrogen acceptors.

(17) Reddelien and Danilof, *Ber.*, **54**, 3132 (1921).

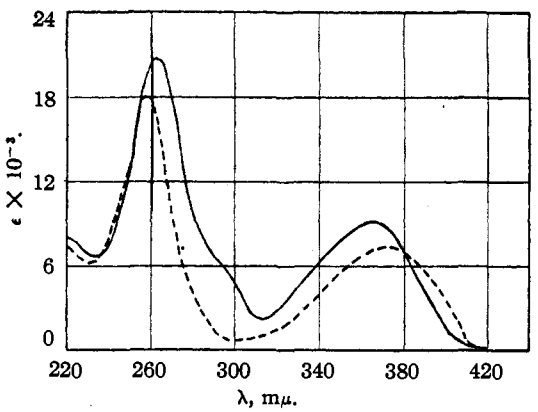


Fig. 3.—Ultraviolet absorption spectra in 0.1 *N* sodium hydroxide: ——, 2-amino-4-hydroxypteridine-6-carboxylic acid; - - - -, 2-amino-4-hydroxypteridine-7-carboxylic acid.

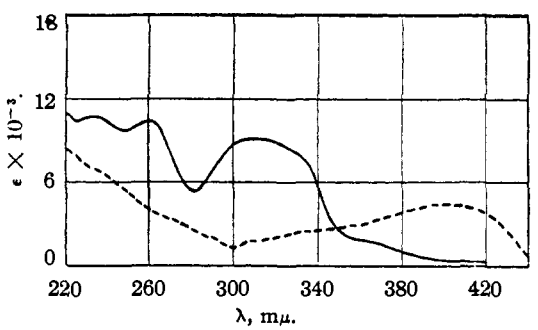


Fig. 4.—Ultraviolet absorption spectra in 0.1 *N* hydrochloric acid: ——, 2-amino-4-hydroxypteridine-6-carboxylic acid; - - - - - -, 2-amino-4-hydroxypteridine-7-carboxylic acid.

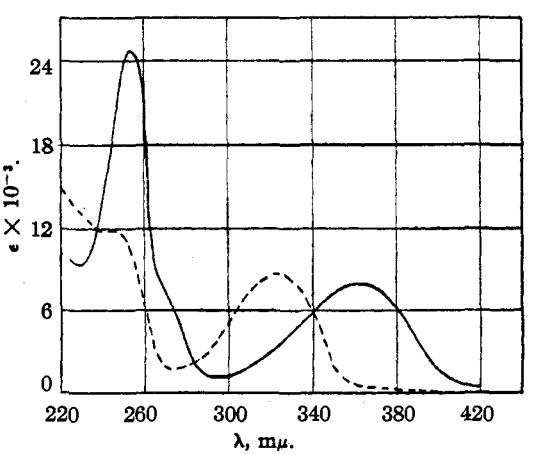
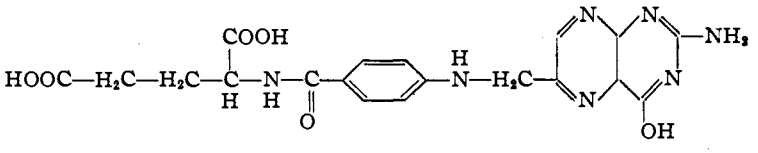


Fig. 5.—Ultraviolet absorption spectra of 2-amino-4-hydroxypteridine-6-acetic acid: ——, in 0.1 *N* sodium hydroxide; - - - - -, in 0.1 *N* hydrochloric acid

(18) Gilman, "Organic Chemistry, An Advanced Treatise," John Wiley and Sons, Inc., New York, N. Y., 1938, p. 881.

In view of these considerations the structure of the liver *L. casei* factor was therefore postulated to be N-[4-{[(2-amino-4-hydroxy-6-pteridyl)-methyl]-amino}-benzoyl]-*l*(+)-glutamic acid, that is



The validity of this formula has been proved by the synthesis of this compound by a number of methods, several of which are described in the following communications.

Experimental

2-Amino-4-hydroxypteridine (II).—A solution of 14.1 g. of 2,4,5-triamino-6-hydroxypyrimidine[10] in 200 cc. of 1 *N* hydrochloric acid was heated to 70° and treated with 22 cc. of a 26.5% solution of glyoxal. After twenty-four hours the precipitate was collected, washed with water, alcohol and ether and dried; weight was 13.4 g. This material was dissolved in a mixture of 50 cc. of water and 10 cc. of 10 *N* sodium hydroxide solution, filtered and then diluted with an equal volume of 10 *N* sodium hydroxide. The sodium salt crystallized rapidly. The sodium salt was collected, washed with alkali and again dissolved in water. Acidification to *p*H 3 precipitated the crude 2-amino-4-hydroxypteridine; weight was 13 g. A portion of this material was recrystallized several times from alkali and then precipitated at *p*H 3, thoroughly washed with hot water, alcohol and ether, and dried *in vacuo*.

Anal. Calcd. for $C_6H_5ON_5$: C, 44.10; H, 3.07; N, 42.94. Found: C, 43.65, 44.04; H, 2.82, 3.50; N, 42.88, 42.75.

2-Amino-4-hydroxy-7-chloropteridine-6-carboxylic Acid (IV).—A mixture of 0.59 g. of 2-amino-4,7-dihydroxypteridine-6-carboxylic acid (iso-xanthopterinecarboxylic acid),[11] (III) 3.0 g. of phosphorus pentachloride and 30 cc. of phosphorus oxychloride was heated on the steam-bath under a reflux condenser for twenty minutes. The mixture was then refluxed over a gas burner for about thirty minutes. In order to effect complete solution of the starting material, an additional 15 cc. of phosphorus oxychloride was then added and refluxing was continued for fifteen minutes. The hot solution was filtered and evaporated to dryness *in vacuo*. The residue was then triturated with 5 cc. of cold water for about forty-five minutes. The solid product was collected on a filter and washed with a little water and finally with a little ether; weight was 0.56 g.

Anal. Calcd. for $C_7H_4O_3N_5Cl$: Cl, 14.7. Found: Cl, 13.9. Attempts to further purify a portion of this material resulted in the loss of halogen.

2-Amino-4-hydroxypteridine-6-carboxylic Acid (V): **A.**—A mixture of 1.5 g. of 2-amino-4-hydroxy-7-chloropteridine-6-carboxylic acid, 21 cc. of glacial acetic acid and 21 cc. of hydriodic acid (sp. gr. 1.7) was heated on the steam-bath with stirring for five minutes. The cooled solution was diluted with 150 cc. of cold water. After a short time, the precipitate was collected in the centrifuge, washed with water and then with very dilute sulfurous acid. The product was then washed with water, alcohol and ether and dried; wt. 0.400 g. This material was dissolved in about 30 cc. of very dilute sodium hydroxide solution, and decolorized with charcoal. The clarified solution was acidified to *p*H 2 and the precipitate was collected and recrystallized several times from 2 *N* sodium hydroxide solution. The pure, crystalline sodium salt was then dissolved in warm water and converted to the free acid by acidification to *p*H 2. After thorough washing with water, alcohol and ether, the product was dried *in vacuo*. Weight was 0.060 g. *Anal.* Calcd. for $C_7H_5O_3N_5$: C, 40.58; H, 2.42; N, 33.82. Found: C, 40.24; H, 2.80; N, 33.37.

B.—A mixture of 100 g. of 2,4,5-triamino-6-hydroxypyrimidine, 192 g. of ethyl α-bromo-β,β-diethoxypropionate,[12] 112 g. of silver carbonate and 2500 cc. of absolute alcohol was refluxed under an atmosphere of nitrogen for five hours. After distilling off most of the alcohol the residue was suspended in a mixture of 2500 cc. of water and 250 cc. of concentrated hydrochloric acid, refluxed for one hour and then filtered while hot. To the filtrate was added a solution of 125 g. of iodine in 800 cc. of alcohol and the mixture was then adjusted to *p*H 2 by the addition of ammonium hydroxide. After thorough cooling, the precipitate was collected in the centrifuge and washed once with dilute sulfurous acid and then with water. This crude product was dissolved in 1 liter of hot water containing 16 g. of sodium hydroxide, decolorized with charcoal and then precipitated by acidification of the solution to *p*H 2. The precipitate was collected, washed with water, alcohol, and ether and dried; weight was 15.5 g. Further purification was effected by crystallization from 1 *N* sodium hydroxide. The pure, crystalline sodium salt was then converted to the free acid. *Anal.* Calcd. for $C_7H_5O_3N_5$: C, 40.58; H, 2.42; N, 33.82. Found: C, 40.54; H, 2.75; N, 33.80.

2-Amino-4-hydroxypteridine-6-acetic Acid (VI).—A stirred mixture of 7.5 g. of 2,4,5-triamino-6-hydroxypyrimidine, 50 cc. of glacial acetic acid and 9.0 g. of methyl γ,γ-dimethoxyacetoacetate[13] (b. p. 92° (5 mm.)) was heated on the steam-bath for twenty minutes; then 50 cc. of water was added and the mixture was heated for an additional thirty minutes. After cooling thoroughly, the precipitate was collected, washed with a little water and then dissolved in 250 cc. of very dilute sodium hydroxide solution. The solution was clarified with charcoal and the crude product was precipitated by the addition of acetic acid; weight was 6.1 g. A portion of this crude material (1.0 g.) was dissolved in 10 cc. of dilute sodium hydroxide solution, filtered, and then treated with an equal volume of 10 *N* sodium hydroxide solution. After cooling, the crystalline sodium salt was collected, washed with 5 *N* sodium hydroxide solution and then recrystallized twice more by the same procedure. The pure, crystalline sodium salt was then dissolved in water at *p*H 10–12, decolorized with charcoal and the free acid was precipitated by the addition of hydrochloric acid to *p*H 2. The washed and dried product weighed 0.355 g. *Anal.* Calcd. for $C_8H_7O_3N_5$: C, 43.44; H, 3.17; N, 31.67. Found: C, 43.75; H, 3.82; N, 32.05.

Oxidation of 2-Amino-4-hydroxypteridine-6-acetic Acid.—A stirred solution of 0.11 g. of 2-amino-4-hydroxypteridine-6-acetic acid, 0.25 cc. of 10 *N* sodium hydroxide solution and 10 cc. of water was heated in a water-bath to a temperature of about 75°. To this hot solution was then added 5 cc. of a 0.2 *M* solution of potassium permanganate during ninety minutes. After stirring at 75° for an additional thirty minutes, the mixture was cooled and treated with a few drops of sulfurous acid to destroy any excess permanganate. The manganese dioxide was removed by filtration and the oxidation product was precipitated by acidification to *p*H 2. The precipitate was collected, washed with water, and recrystallized from about 5 cc. of 2 *N* sodium hydroxide solution. The crystalline sodium salt was then dissolved in water and acidified. The product was collected, washed with water, alcohol and ether and dried; weight was 0.060 g. This material was identical with the previously described 2-amino-4-hydroxypteridine-6-carboxylic acid.

2-Amino-4-hydroxy-6-methylpteridine (VII).—A dry, finely powdered sample of 2-amino-4-hydroxypteridine-6-acetic acid (0.200 g.) was heated for about seven hours at 280° in an atmosphere of nitrogen. The residue was dissolved in 150 cc. of dilute sodium hydroxide solution, decolorized with charcoal and then adjusted to *p*H 7.0. The mixture was then boiled for a few minutes and filtered

through a heated funnel. The filtrate was thoroughly cooled and the precipitate was collected; weight was 0.105 g. This material was again dissolved in very dilute alkali, adjusted to *p*H 7.0, heated to boiling, filtered and allowed to cool. The precipitate was collected, washed with cold water and finally recrystallized from 90 cc. of hot water; weight was 0.068 g. *Anal.* Calcd. for $C_7H_7ON_5$: C, 47.46; H, 3.96; N, 39.55. Found: C, 47.82; H, 4.75; N, 39.56.

2-Amino-4-hydroxy-7-methylpteridine (XIII).—A mixture of 0.5 g. of 2,4,5-triamino-6-hydroxypyrimidine and 0.6 g. of methylglyoxal diethyl acetal[16] was dissolved in 25 cc. of warm 4 *N* hydrochloric acid. After heating on the steam-bath for ninety minutes the solution was cooled and neutralized to *p*H 3.0. The precipitate was collected in the centrifuge, washed with water, alcohol and ether and dried; weight was 0.6 g. This crude product was dissolved in 30 cc. of dilute sodium hydroxide solution and then treated with an equal volume of 10 *N* sodium hydroxide solution. The crystalline precipitate was collected and recrystallized twice more by the same procedure. The crystalline sodium salt was then dissolved in water, decolorized with charcoal and precipitated at *p*H 3.0. *Anal.* Calcd. for $C_7H_7ON_5$: C, 47.46; H, 3.96; N, 39.55. Found: C, 47.18; H, 4.45; N, 39.42.

2-Amino-4-hydroxypteridine-7-carboxylic Acid (XIV).—A stirred solution of 0.200 g. of 2-amino-4-hydroxy-7-methylpteridine in 20 cc. of water containing 0.3 cc. of 10 *N* sodium hydroxide solution was heated in a water-bath to 90° and treated with small portions of a 0.2 molar solution of potassium permanganate until no further reaction occurred. The excess potassium permanganate was destroyed with a little sodium sulfite and the manganese dioxide was removed by filtration. The filtrate was adjusted to *p*H 2.0 and the precipitate was collected, washed with water, methanol and ether; weight was 0.195 g. This material was purified by crystallization from alkali as previously described and finally converted to the free acid. *Anal.* Calcd. for $C_7H_5O_3N_5$: C, 40.58; H, 2.42; N, 33.82. Found: C, 40.50; H, 3.11; N, 33.30, 33.49. This compound appears to be very hygroscopic and rapidly takes up water to form a very stable monohydrate.

2-Amino-5-methylpyrazine-3-carboxylic Acid (VIII).—A mixture of 7.0 g. of 2-amino-4-hydroxy-6-methylpteridine (VII), 22 cc. of water and 16 cc. of 10 *N* sodium hydroxide solution was heated in a sealed bomb at 170° for twenty hours. After being removed from the bomb, the reaction mixture was heated to effect solution and filtered while still hot. The hot filtrate was then acidified to *p*H 2.5 with hydrochloric acid, and on cooling the crude product crystallized; weight was 4.1 g. This material was recrystallized from 45 cc. of hot water after clarification with charcoal; weight was 3.2 g. An analytical sample was recrystallized twice more from hot water, m. p. 171.5–172.0°. *Anal.* Calcd. for $C_6H_7O_2N_3$: C, 47.05; H, 4.60; N, 27.45. Found: C, 46.75; H, 4.49; N, 27.40.

2-Amino-5-methylpyrazine (IX).—A solution of 1.0 g. of 2-amino-5-methylpyrazine-3-carboxylic acid (VIII) in 5 cc. of butyl cellosolve was refluxed for forty-five minutes. The solution was then clarified with charcoal, diluted with about 40 cc. of heptane and cooled. The precipitate was filtered off and discarded and the filtrate was then treated with gaseous hydrogen chloride. The precipitated hydrochloride was collected, dissolved in water and made alkaline by the addition of sodium hydroxide solution. This solution was extracted with ether several times. The ether solution was evaporated to dryness and the yellow crystalline residue (0.3 g.) was then recrystallized from a mixture of ether and petroleum ether and then sublimed twice *in vacuo* at 70° and 1–2 mm. pressure. The white sublimate melted at 116–117°. This melting point was not depressed by mixture with a sample of 2-amino-5-methylpyrazine prepared from 2-methylpyrazine-5-carboxylic acid by the method of Weijlard, Tishler and Erickson.[13] *Anal.* Calcd. for $C_5H_7N_3$: C, 55.00; H, 6.47; N, 38.53. Found: C, 55.58; H, 6.68; N, 38.40.

N-(Benzyl)-*p*-aminobenzoic Acid (XVI).—A mixture of 1.4 g. of *p*-aminobenzoic acid, 0.7 g. of potassium carbonate, 20 cc. of absolute ethanol and 1.3 cc. of benzyl chloride was refluxed on the steam-bath for several hours and then allowed to stand overnight at room temperature. The reaction mixture was diluted with water and the precipitate was collected and washed with water, then with a little cold alcohol and then again with water. The material may be recrystallized from hot, aqueous ethanol. *Anal.* Calcd. for $C_{14}H_{13}O_2N$: C, 74.00; H, 5.77; N, 6.16. Found: C, 74.10; H, 5.40; N, 6.13.

Acknowledgment.—We wish to acknowledge the technical assistance of Miss Barbara Eames and Miss Edith Brogan. The microanalyses were carried out by Mr. Louis Brancone and coworkers. 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

The structure of the pteridine nucleus of the *L. casei* factors has been established and a structure for the liver *L. casei* factor consistent with all of the experimental evidence has been postulated.

Experimental methods and reference compounds of known structure have been described which allow rapid and certain distinction between pteridines which are isomeric in the 6- and the 7-positions.

PEARL RIVER, NEW YORK RECEIVED JANUARY 24, 1947