from hot methanol yielded a preparation whose activity could not be increased by further purification. The solubility of the methyl ester in methanol is approximately 60 micrograms per ml. at 2°. The results obtained by methanol fractionation are shown in Table I. The analysis of two preparations of the methyl ester as obtained by this method are as follows

	C	H	N (Dumas)
Sample 1	53.0	4.6	21.2
Sample 2	53.1	5.1	20.5

The free acid was prepared by saponifying 2.0 mg. of a nearly pure preparation of the ester with 5.0 ml. of 0.01 *N* sodium hydroxide. The solution was treated with 4 mg. of Darco. On acidifying the filtrate to *p*H 3.0, the free acid precipitated out, which was then redissolved by heating to 90°. On cooling slowly the free acid of the *L. casei* factor crystallized out. The solubility of the free acid at *p*H 3.0 at 2° is approximately 10 micrograms per ml. and more than 0.5 mg. per ml. at 100°. These were recrystallized once from 0.1 *n* sodium chloride and two times from water.

The crystal form of the free acid is shown in Fig. 1.

The extinction coefficients in 0.1 *N* sodium hydroxide were determined with a Beckman spectrophotometer and found to be as follows: $E^{1\%}_{1\ cm.}$ 255 mμ, 565; 282 mμ, 350; 365 mμ, 195.

The amount required per ml. of medium for half maximum growth was 0.00007 microgram for *L. casei* and 0.0003 for *S. faecalis* R. This amount required for half maximum growth is not an accurately reproducible value and varies from one experiment to another.

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Summary

The isolation of the *Lactobacillus casei* factor from liver has been described. This was accomplished by the use of adsorption and elution of the free acid on Norit, esterification and extraction of the methyl ester with *n*-butanol. The methyl ester was chromatographed on Superfiltrol using aqueous acetone as the developer. Final purification of the ester was accomplished by fractional precipitation from water and methanol. The free acid was crystallized from hot water.

PEARL RIVER, NEW YORK RECEIVED JANUARY 24, 1947

[CONTRIBUTION FROM LEDERLE LABORATORIES DIVISION, AMERICAN CYANAMID COMPANY]

The Degradation of the Fermentation *Lactobacillus casei* Factor. I

BY E. L. R. STOKSTAD, BRIAN L. HUTCHINGS, JOHN H. MOWAT, JAMES H. BOOTHE, COY W. WALLER, ROBERT B. ANGIER, JOSEPH SEMB AND Y. SUBBAROW

The isolation of two *Lactobacillus casei* factors, one from liver[1,2] and one from a fermentation product[3,4] has been described.

These two *L. casei* factors differ in biological activities and physical properties. Extinction coefficients indicated that the fermentation *L. casei* factor has the larger molecular weight. The structure and synthesis of the liver *L. casei* factor have been described in a preliminary communication.[5] The synthetic liver *L. casei* factor has been reported to be the same as vitamin B_c[6] which had previously been isolated from liver and yeast.[7,8]

The relationship of the liver *L. casei* factor to folic acid[9] has not been definitely established although available evidence indicates they are the same.[10]

The liver *L. casei* factor also appears to be the same as the factor designated as the norite eluate factor.[11]

This communication deals with the degradation of the fermentation compound by alkaline hydrolysis under aerobic and anaerobic conditions and by acid hydrolysis.

In experiments on the degradation of the *L. casei* factor from liver and fermentation sources, it was soon observed that hydrolysis with acid or alkali led to the formation of a diazotizable aromatic amine which could be estimated by the method of Bratton and Marshall.[12] The liberation of amine was most rapid in alkaline solutions and it was found that oxygen had a marked effect on the course of the reaction. When the fermentation *L. casei* factor was heated with 1.0 *N* sodium hydroxide at 100° in the presence of oxygen there was a marked change in the absorption spectrum, a diazotizable aromatic amine was produced, a fluorescent pigment was formed and rapid biological inactivation occurred. The *L. casei* factor before hydrolysis has three absorption maxima in 0.1 *N* sodium hydroxide at 257, 282 and 365 mμ. After aerobic alkaline hydrolysis it has two maxima at 263 and 365 mμ.

When the hydrolysis was carried out anaerobically with 1.0 *N* sodium hydroxide at 100° there

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(4) Hutchings, Stokstad and SubbaRow, THIS JOURNAL, **70**, 1 (1948).

(5) Angier, Boothe, Hutchings, Mowat, Semb, Stokstad, SubbaRow, Waller, Cosulich, Fahrenbach, Hultquist, Kuh, Northey, Seeger, Sickels and Smith, Jr., *Science*, **103**, 667 (1946).

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(8) Binkley, Bird, Bloom, Brown, Calkins, Campbell, Emmett and Pfiffner, *ibid.*, **100**, 36 (1944).

(9) Mitchell, Snell and Williams, THIS JOURNAL, **63**, 2284 (1941).

(10) Johnson, *J. Biol. Chem.*, **163**, 255 (1946).

(11) Snell and Peterson, *J. Bact.*, **39**, 273 (1940).

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

was no change in the absorption spectrum and very little aromatic amine or fluorescent pigment was produced. The activity for *L. casei* was only slightly decreased during anaerobic hydrolysis while the activity for *S. faecalis* R was greatly increased. After prolonged anaerobic alkaline hydrolysis the ratio of activities for *L. casei* and *S. faecalis* R was approximately the same as that for the liver *L. casei* factor. A summary of results of anaerobic and aerobic alkaline cleavage appear in Table I. It should be noted here that

TABLE I

EFFECT OF OXYGEN ON ALKALINE HYDROLYSIS OF *L. casei* FACTOR

	Temperature and time of heating Hr.	°C.	Bio act. for *L. casei*, units per mg.	*S. faecalis* R act. coef.[a]	Aromatic amine liberated; expressed as PABA, %	Pteridine liberated,[b] %	α-Amino acid nitrogen in % of total N
	None		710	0.075	0.14	2.3	4.3
No O_2	10	100	750	.66	0.90	3.0	27.0
	20	100	730	.70	1.05	3.25	28.0
	40	100	720	.52	1.02	3.25	27.0
	10	120	780	.61	1.14	2.8	29.0
O_2	0.5	100	lost	.07	3.9	5.0	8.6
	1.0	100	550	.09	9.0	13.8	9.7
	2.0	100	170	.08	14.4	25.0	9.2
	3.0	100	83	.15	15.9	27.2	10.2
	4.0	100	0	...	16.9	29.5	13.5

[a] S. faecalis activity coefficient = *S. faecalis* activity of 1.0 *L. casei* unit of unknown / *S. faecalis* activity of 1.0 *L. casei* unit of liver *L. casei* factor

[b] Results of pteridine analysis expressed as 2-amino-4-hydroxy-6-pteridine carboxylic acid.

while *p*-aminobenzoic acid (PABA) was used as the standard in the Bratton and Marshall determination, the analytical data are reported in terms of PABA as the arbitrary standard; and that the diazotizable amine was not PABA. The aromatic amine was not extractable from aqueous solutions at *p*H 3.0 with ether, while PABA under the same conditions was extracted readily.

The α-amino acid which was formed during anaerobic hydrolysis had been cleaved from the rest of the molecule because it could be separated from the biologically active fragment. This alpha amino acid could be precipitated by barium hydroxide and 75% ethanol which indicated a dicarboxylic α-amino acid.[13]

The biologically active compound obtained by anaerobic hydrolysis with 1.0 *N* sodium hydroxide at 120° for ten hours was isolated and crystallized as the magnesium salt. It could not be crystallized as the free acid by the method previously employed to yield the crystalline liver *L. casei* factor. The ultraviolet absorption spectrum of this compound was identical with that of the liver *L. casei* factor. However, it was only 57% as active biologically as the liver compound by *S. faecalis* R assay and 58% by *L. casei* assay. The low microbiological activity suggested a racemic product.

(13) Dakin, *J. Biol. Chem.*, **44**, 499 (1920).

The identification of this compound as racemic liver *L. casei* factor was accomplished later by comparison of its infrared absorption spectrum with that of a synthetic specimen of racemic liver *L. casei* factor. The infrared absorption spectra of the racemic and natural isomers, which were significantly different, appear in a subsequent paper of this series. It is probable that racemization occurred during the extended alkaline hydrolysis.

The fluorescent pigment which was produced by aerobic alkaline hydrolysis was isolated by the following procedure. It was precipitated by acidifying the hydrolysate to *p*H 3.0 and crystallized as the sodium salt from 2.0 *N* sodium hydroxide. It could not be crystallized from weakly alkaline solutions. The free acid prepared by precipitation at *p*H 3.0 appeared amorphous but did show a micro-crystalline structure by X-ray diffraction. The elementary analysis suggested the empirical formula $C_7H_5N_5O_3$.

The presence of a guanidine group in the fluorescent pigment was observed. Oxidation with chlorine water and subsequent hydrolysis with 0.1 *N* hydrochloric acid at 140° or three hours yielded guanidine which was estimated colorimetrically. The Weber test[14] which is given by guanidine and methyl guanidine was positive. The Sullivan test[15] which is given by guanidine but not by methylguanidine was positive. The Sakaguchi test[16] which is given by methylguanidine but not by guanidine was negative. These colorimetric tests indicated the presence of a non-substituted guanidine in the hydrolyzed chlorine oxidation product. The formation of guanidine by chlorine oxidation constitutes evidence for a pyrinidine ring with an amino group in the 2-position.[17]

A titration curve of the sodium salt showed the presence of two acid groups with *pKa* values of 3.9 and 7.7. The presence of a carboxylic acid group was demonstrated by decarboxylation at 300° for three hours with the liberation of 0.8 mole of carbon dioxide. From this decarboxylated mixture a new fluorescent monobasic compound with a *pKa* of 8.0 was obtained. This decarboxylation of a dibasic acid to a monobasic acid with a *pKa* of 8.0 showed that the original compound contained a carboxylic and an enolic group.

This decarboxylated fraction had an ultraviolet absorption spectrum in 0.1 *N* sodium hydroxide with absorption maxima at 252 and 365 mμ while the original dibasic acid had maxima at 262 and 365 mμ. The absorption spectra of these two compounds in alkaline and acid solutions are shown in Figs. 1 and 2, respectively.

The empirical formula of $C_5H_7N_5O_3$, based on elementary analysis and titration data, and the formation of guanidine suggested a 2-aminopurine or 2-aminopteridine. The absorption spectra,

(14) Andes and Meyer, *ibid.*, **118**, 137 (1937).

(15) Sullivan, *Proc. Soc. Exptl. Biol. Med.*, **33**, 106 (1935).

(16) Dubnoff, *J. Biol. Chem.*, **141**, 711 (1941).

(17) Wieland, Metzger, Schopf and Bulow, *Ann.*, **507**, 226 (1933).

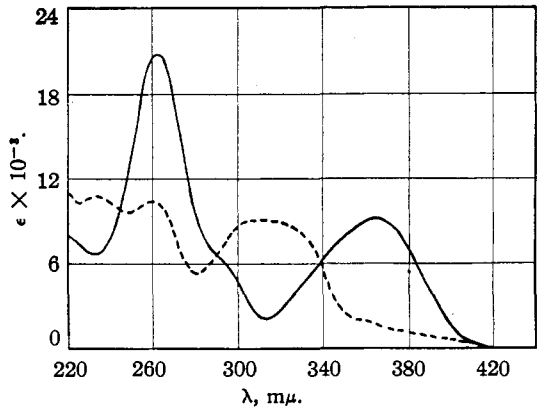

Fig. 1.—Ultraviolet absorption spectra of 2-amino-4-hydroxypteridine-6-carboxylic acid: ——, 0.1 *N* sodium hydroxide; – – – –, 0.1 *N* hydrochloric acid.

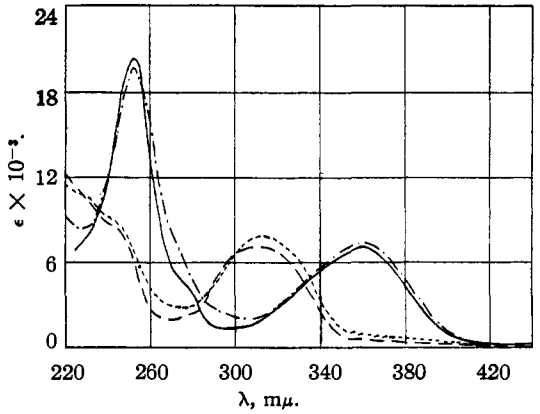

Fig. 2.—Ultraviolet absorption spectra of 2-amino-4-hydroxypteridine, synthetic: ———, in 0.1 *N* sodium hydroxide; — — —, in 0.1 *N* hydrochloric acid. Product obtained from decarboxylation of natural 2-amino-4-hydroxypteridine-6-carboxylic acid; — · — · — in 0.1 *N* sodium hydroxide; – – – – – – in 0.1 *N* hydrochloric acid. The absorption spectra of the decarboxylated product were determined on an aliquot of a solution of an unweighed sample and the values are arbitrarily plotted on the same scale to show similarity.

however, eliminated the possibility of a purine because purines do not have absorption maxima above 300 mμ. Thus the available evidence pointed toward 2-aminopteridine with an enolic and a carboxy group. Attempts were made to synthesize pteridines having these functional groups. With this objective, 2-amino-4-hydroxy-6-pteridinecarboxylic acid was synthesized and found to be identical with the fluorescent dibasic pigment. Identity was established by comparison of ultraviolet absorption spectra Fig. 1 and X-ray diffraction patterns. The synthesis and proof of structure of this compound will be given in a subsequent paper. The monobasic fluorescent pigment produced by decarboxylation was identified as 2-amino-4-hydroxypteridine.

Hydrolysis of fermentation *L. casei* factor with 1.0 *N* sulfuric acid anaerobically for eight hours at 100° yielded a fluorescent monobasic acid which was extracted from the hydrolysate with *n*-butanol at *p*H 7.0. This procedure left the dibasic pteridine in the aqueous phase. The monobasic compound was crystallized as the sodium salt from 10.0 *N* sodium hydroxide and then converted to the free acid. This was identified as 2-amino-4-hydroxy-6-methylpteridine by comparison of the ultraviolet and infrared absorption spectra of the natural and synthetic compounds. The synthesis and proof of structure of this compound will be given in a later paper.

The aromatic amine fraction was obtained by first hydrolyzing the fermentation compound anaerobically with alkali to give the racemic liver *L. caesi* factor and then further hydrolyzing aerobically to liberate the aromatic amine. After removal of pteridines the dried amine fraction was extracted as the free acid with ethanol. The aromatic amine obtained from racemic liver *L. casei* factor contained 2.1 atoms of total nitrogen for each atom of aromatic amine nitrogen as measured by the Bratton and Marshall method. On hydrolysis with 2.0 *N* sulfuric acid for sixteen hours at 100°, 45% of the total nitrogen appeared as alpha amino acid nitrogen. *p*-Aminobenzoic acid was isolated from this hydrolysate by extraction with ethyl acetate, crystallized from water and identified by mixed melting points and microbiological assay.

This evidence indicated that the aromatic amine from the racemic liver *L. casei* factor is a dipeptide of *p*-aminobenzoic acid and an α-amino acid. This peptide linkage must involve the carboxyl group of the *p*-aminobenzoic acid, as the aromatic amine group must be free in order to react in the Bratton and Marshall test.

Some evidence regarding the mode of linkage is furnished by the results of aerobic alkaline hydrolysis. The absence of fluorescence and of free aromatic amine in the original *L. casei* factor, and the simultaneous appearance of these two during aerobic alkaline hydrolysis suggests that the pteridine is linked to the aromatic amine nitrogen. As hydrolysis proceeds, the liberation of pteridine and amine, and biological inactivation proceed at approximately the same rate. No method of cleavage has been found which will liberate the aromatic amine without forming a pteridine. A linkage which can be cleaved only by oxidative hydrolysis is also demanded. This excludes the possibility of a peptide linkage between the carboxy group of the pteridine and the aromatic amine nitrogen. It also eliminates the possibility of the aromatic amine nitrogen being attached to the 4 position of the pteridine. Cleavage of such a bond to give a 4-hydroxypteridine would be simple hydrolysis and would not require oxidation.

Experimental

Effect of Oxygen on Alkaline Hydrolysis.—A solution of 17.4 mg. of fermentation *L. casei* factor in 0.5 ml. of 1.0 *N*

sodium hydroxide was hydrolyzed in a sealed 8-mm. test-tube. The tube was evacuated for anaerobic hydrolysis and filled with oxygen for aerobic hydrolysis. After hydrolysis for the times and temperatures indicated in Table I, the solutions were assayed microbiologically with *L. casei* and *S. faecalis* R, using the method of Tepley and Elvehjem.[18] α-Amino acid nitrogen was determined by the method of Van Slyke and aromatic amine by the method of Bratton and Marshall,[12] using *p*-aminobenzoic acid as the standard. The ultraviolet absorption spectra were measured in a Beckman spectrophotometer, and the fluorescence was measured in 0.05 *M* borate buffer with a fluorophotometer. The results were later recalculated in terms of 2-amino-4-hydroxypteridine-6-carboxylic acid.

Isolation of Racemic Liver *L. casei* Factor.—Fermentation *L. casei* factor, 170 mg. in 6 ml. of 1.0 *N* sodium hydroxide, was hydrolyzed for ten hours at 120° anaerobically in an evacuated sealed tube. The hydrolysate contained 39 mg. of liver *L. casei* factor. The biologically active material was precipitated twice with zinc acetate at *p*H 6.7 at 80°. The final zinc precipitate was dissolved in alkali, the zinc hydroxide removed, and the *L. casei* factor precipitated at *p*H 3.0. The precipitate was dissolved in 25 ml. of 0.025 *N* sodium hydroxide, the solution was treated with 100 mg. of activated charcoal (Darco) and the *L. casei* factor precipitated with barium chloride and 3 volumes of methanol.

The barium salts were extracted with hot water, and the extracts heated to 100° and acetic acid added to *p*H 3.0. The active material precipitated on cooling and was reprecipitated twice in a similar manner. These amorphous precipitates of the free acid, 18 mg., were converted into the crystalline magnesium salts by boiling with 5.0 mg. of magnesium oxide and 3.0 ml. of water, and separating off the excess magnesium oxide. On cooling the hot extract the magnesium salt crystallized. This was recrystallized from water. The final magnesium salt, when converted into the free acid by precipitation from a hot solution at *p*H 3, yielded spherulites which exhibited birefringence but which did not form the typical spear-shaped crystals characteristic of the natural isomer of the liver *L. casei* factor; yield of free acid, 5.0 mg. $E^{1\%}_{1\,cm.}$ for free acid of racemic liver *L. casei* factor as determined in 0.1 *N* sodium hydroxide, 257 mμ, 591; 285 mμ, 575; 365 mμ, 207. Biological activity compared with natural isomer of liver *L. casei* factor: *L. casei* assay, 58.0%; *S. faecalis* R assay, 57.0%. The infrared absorption spectrum of the free acid of the isolated racemic liver *L. casei* factor matched exactly that of the synthetic racemic liver *L. casei* factor.

Isolation of Fluorescent Dibasic Pigment.—Two hundred mg. of fermentation *L. casei* factor in 10.0 ml. 1.0 *N* sodium hydroxide was hydrolyzed six hours at 100° with a stream of oxygen bubbling through the solution. The hot hydrolysate was centrifuged to remove some brown pigment. On cooling the filtrate to 2° a crystalline precipitate (10 mg.) formed which later proved to be the disodium salt of 2-amino-4-hydroxypteridine-6-carboxylic acid. The greater part of the pteridine remained in the filtrate. This filtrate was adjusted to *p*H 5.0 with sulfuric acid and diluted to 20 ml., heated to 70° and the hot solution centrifuged to remove insoluble impurities. The filtrate containing 48 mg. of pteridine was adjusted to *p*H 2.3. The pteridine which precipitated as the free acid at this *p*H was redissolved in dilute alkali and reprecipitated at *p*H 2.3. This free acid (44 mg.) was dissolved at *p*H 11 in 2.0 ml. of water plus sodium hydroxide. After standing at 2° for sixteen hours, impurities were removed by centrifuging and the filtrate brought to 1.0 *N* with sodium hydroxide. On cooling to 2° for sixteen hours the crude sodium salt crystallized. This was recrystallized three times from 1.0 *N* sodium hydroxide.

At this point the products of five such hydrolyses (1.0 g. total fermentation *L. casei* factor) were combined and precipitated twice as the free acid at *p*H 2. The final product was washed with water, methanol and finally ether and dried two hours at 60° in high vacuum over phosphorus pentoxide; yield was 69 mg.

Anal. Found: C, 40.11, 41.03, av., 40.57; H, 1.75, 3.23, av., 2.50; N Kjeldahl, 32.2, 31.8; N Dumas, 32.4, 32.75, av., 32.3. Calcd. for $C_7H_5N_5O_3$: C, 40.58; H, 2.42; N, 32.3.

Formation of Guanidine by Chlorine Oxidation.—A fine suspension of 5 mg. of the fluorescent dibasic acid was treated with 10 ml. of 0.15 *N* chlorine water at 40° for fifteen minutes. The solution was evaporated to dryness, water added, and the solution again evaporated to remove excess chlorine. The residue was dissolved in 4.0 ml. of 0.1 *N* hydrochloric acid and heated three hours at 140°. Guanidine was estimated qualitatively by the method of Sullivan[15] and quantitatively by the method of Weber.[14] Under these conditions of oxidation and hydrolysis the fluorescent dibasic acid yielded 0.24 mole of guanidine, while xanthopterin gave 0.09 mole and leucopterin gave 0.27 mole of guanidine.

Decarboxylation.—The fluorescent dibasic acid (2.562 mg.) was heated at 250–300° for three hours in a stream of nitrogen and the carbon dioxide collected in barium hydroxide solution. The barium carbonate which formed was converted into barium sulfate and weighed. The results were as follows

Wt. of sample, mg.	2.562
Loss on heating, mg.	0.448
Wt. barium sulfate, mg.	1.852
Moles carbon dioxide (based on weight loss on heating and assuming mol. wt. 207)	0.82
Moles carbon dioxide based on barium sulfate found	.64

An electrometric titration of this heated product indicated that complete decarboxylation had not occurred.

A larger sample of 6.63 mg. was decarboxylated and fractionated as follows: It was dissolved in excess alkali, adjusted to *p*H 5.0 and diluted to 10.0 ml. The solution was heated to 90° and centrifuged hot. The filtrate was chilled to 2°. The precipitate which formed had a *pKa* of 8.0 and showed no evidence of an acid group with a *pKa* of 3.7. The absorption spectrum in 0.1 *N* sodium hydroxide was determined on an aliquot of this solution and the new decarboxylated pteridine was found to have maxima at 252 and 365 mμ. As insufficient material was present to be weighed, the absorption spectrum was based on an unknown weight of material. This absorption spectrum was found to be very similar to that of 2-amino-4-hydroxypteridine. The absorption curves of these two compounds appear in Fig. 2.

Isolation of Monobasic Fluorescent Pigment.—Fermentation *L. casei* factor (227 mg.) was dissolved in 25 ml. of 1.0 *N* sulfuric acid and hydrolysed anaerobically for eight hours at 100°. A large amount of black precipitate formed which was centrifuged off. The supernatant was adjusted to *p*H 7.0 and extracted five times with 10 volumes of butanol, using one portion of water to wash each butanol extract. The butanol extract, which contained *ca.* 17.0 mg. of pteridine by spectrophotometric analysis, was evaporated to 9.0 ml. and 3.0 ml. of ether added. The precipitated pteridine was centrifuged, dried and redissolved in 10.0 ml. of water and reextracted three times with 10 volumes of butanol. The combined butanol extracts were concentrated to 5.0 ml., 5.0 ml. ether added and the solution chilled to 0°. The precipitate, containing *ca.* 8.7 mg. pteridine was dried, dissolved in 5.0 ml. of hot water, centrifuged while hot and the supernatant chilled to 0°. The pteridine, which was soluble in hot water, precipitated on cooling. The precipitate, containing *ca.* 6.7 mg. of pteridine was dissolved in 6.0 ml. of dilute sodium hydroxide and treated with 30 mg. of activated charcoal (Darco). The filtrate (3.4 mg. pteridine) was adjusted to *p*H 5.0. On cooling to 0° the pteridine precipitated out. The precipitate was dried, and crystallized two times from 0.5 ml. of 10.0 *N* sodium hydroxide. Crystallization proceeded rapidly from 10.0 *N* sodium hydroxide but not from 5.0 *N*. The final crystalline product was dissolved in

(18) Tepley and Elvehjem, *J. Biol. Chem.*, **157**, 303 (1945).

0.55 ml. water. The amount of material present was so small, *ca.* 0.57 mg. by spectrophometric analysis at 365 mμ, that an aliquot of the solution was used to determine the complete absorption spectrum. The absorption spectrum matched with that of 2-amino-6-hydroxy-6-methyl pteridine. The remainder was converted to the free acid, dried and its infrared absorption spectrum determined. This was found to be identical with that of a synthetic specimen of 2-amino-4-hydroxy-6-methylpteridine. The ultraviolet and infrared absorption spectra of this compound will be given in the following paper of this series.

Isolation of Amine Fractions.—Fermentation *L. casei* factor (162 mg.) was dissolved in 9.5 ml. of 1.0 *N* sodium hydroxide and hydrolyzed anaerobically for ten hours at 120°. The racemic liver *L. casei* which formed was precipitated with silver nitrate at *p*H 3.0 and the precipitate freed of silver with hydrochloric acid. The silver precipitate fraction contained 92 mg. of racemic liver *L. casei* factor on the basis of a spectrophotometric determination.

This racemic liver *L. casei* factor was then hydrolyzed aerobically for six hours in 11 ml. of 1.0 *N* sodium hydroxide at 100°. The pteridine was removed by precipitation with silver sulfate at *p*H 5.0. The filtrate, after being freed of silver with hydrochloric acid, was adjusted to *p*H 3.0 and evaporated to dryness. The residue was extracted twice with a total of 75 ml. of hot absolute ethanol to extract this amine. The ethanol extracts were evaporated to dryness and extracted twice with 25-ml. portions of hot isopropanol. This extracted the amine without removing appreciable amounts of inorganic salts. This isopropanol solution was evaporated to dryness and the residue dissolved in 10 ml. of water. This solution analysed as follows:

Aromatic amine calcd. as PABA, mg.	19.0
Aromatic amine nitrogen, mg.	1.94
Alpha amino acid nitrogen, mg.	0.42
Total nitrogen, mg.	4.50
Distribution coefficient for aromatic amine at *p*H 3.0; ethyl acetate to water	0.20

This amine could be hydrolyzed by either 2.0 *N* sulfuric acid at 100° for sixteen hours or by 5.0 *N* sodium hydroxide for thirteen hours at 120°. After hydrolysis with acid, the distribution coefficient at *p*H 3.0 for ethyl acetate to water was 8.1; after alkaline hydrolysis, it was 11.0, while that for PABA under the same conditions was 11.0. *p*-Aminobenzoic acid was isolated by hydrolyzing 7.8 mg. of the amine fraction (calcd. as PABA) in 5.0 ml. of 2.0 *N* sulfuric acid for sixteen hours at 100°. The hydrolysate contained 6.7 mg. of amine (as PABA). The solution was diluted to 50 ml. and freed of sulfuric acid with barium carbonate, the filtrate concentrated to 10 ml. and adjusted to *p*H 8.0. Extraction at this *p*H with two 10-ml. portions of ethyl acetate removed no amine. The solution was adjusted to *p*H 3.0 and again extracted with ethyl acetate in the same way. The two *p*H 3.0 ethyl acetate extracts were each washed successively with one 5-ml. portion of water.

The ethyl acetate extract and the water phase analyzed as follows:

Ethyl acetate extract.	
Aromatic amine (as PABA), mg.	4.5
Aromatic amine nitrogen, mg.	0.47
Total nitrogen, mg.	.42
Aqueous residue plus washings.	
Aromatic amine (as PABA), mg.	0.10
Aromatic amine nitrogen, mg.	.01
Total nitrogen, mg.	.70

On evaporating the ethyl acetate extract to dryness, a crystalline product was obtained which was recrystallized from 0.3 ml. of hot water: yield was 0.8 mg.; m. p. unknown (hot stage), 183.5–184.5° uncor.; m. p. *p*-aminobenzoic acid (hot stage), 185.5–186.5° uncor.; mixed melting point (hot stage), 185.5–186.5° uncor.

Microbiological assays for *p*-aminobenzoic acid were made by the methods using *Clostridium acetobutylicum*[19] and *Acetobacter suboxydans*[20] which gave values of 95 and 81%, respectively.

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Summary

1. Anaerobic alkaline hydrolysis of the fermentation *L. casei* factor gives racemic liver *L. casei* factor and 2 moles of a dicarboxylic alpha amino-acid.

2. Aerobic alkaline hydrolysis gives 2-amino-4-hydroxypteridine-6-carboxylic acid and an aromatic amine.

3. The aromatic amine obtained by aerobic alkaline hydrolysis of the racemic liver *L. casei* factor consists of a dipeptide of *p-aminobenzoic* acid and an α-amino acid.

4. Anaerobic acid hydrolysis of the fermentation *L. casei* factor yields 2-amino-4-hydroxy-6-methylpteridine.

5. Linkage of the pterin to the amino group of *p*-aminobenzoic acid is indicated by the rates of liberation of pteridine and amine during aerobic alkaline hydrolysis.

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