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Synthesis of Pteroylglutamic Acid (Liver *L. casei* Factor) and Pteroic Acid. I

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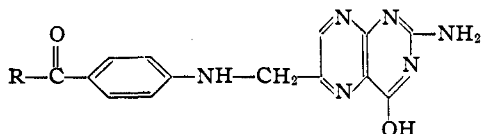
In a preliminary note[4] it was announced that the total synthesis of the liver *L. casei* factor had been accomplished. In a second note[5] the structure was given and the method of synthesis was outlined. The purpose of this communication is to describe in detail this method of synthesis of the liver *L. casei* factor.

The present status of the nomenclature of the *L. casei* factors and related compounds is extremely ambiguous. Mitchell, Snell and Williams[6] used the term "Folic Acid" to represent a product obtained from spinach and defined folic acid "as the material responsible for growth stimulation of *Streptococcus lactis* R on a given medium." Folic acid has become popular as a term to represent any material with such activity without regard for differences in chemical nature; this usage makes it unsuited to represent a chemical entity. The complexity of these compounds precludes the general usage of their chemical names. Therefore, it would be desirable to have a short, pseudochemical name for the basic compound from which the name of any related compound can be derived. The name suggested for the liver *L. casei* factor[5] and its structure is as represented by I. The basic compound whose synthesis is reported in detail herein is represented by II.



Compound	R—	Name
I	HOOC—CH—NH— CH₂ HOOC—CH₂	Liver *L. casei* factor, pteroylglutamic acid, N-[4-{[(2-amino-4-hydroxy-6-pteridyl)-methyl]-amino}-benzoyl]-glutamic acid
II	HO—	Pteroic acid, 4-{[(2-amino-4-hydroxy-6-pteridyl)-methyl]-amino}-benzoic acid

In Table I the compounds which have been obtained in a pure state are summarized according to their biological activities and the number of glutamic acid residues in each molecule. The number of glutamic acid residues ranges from none for pteroic acid to seven for pteroylhexaglutamylglutamic acid with one and three for pteroylglutamic acid and the fermentation *L. casei* factor, respectively. Pteroic acid has the basic structure found in all of these compounds. The exact name of the fermentation *L. casei* factor will be given when the nature of the peptide linkage is reported.

TABLE I

	No. of glutamic acid residues	Activities in micrograms per ml. for half maximum growth *S. faecalis* R	*L. casei*	Active for chicks	Refs.
Pteroic acid	0	0.0008	0.07	No	5
Pteroylglutamic acid (liver *L. casei* factor)	1	.0003	.00007	Yes	4, 5, 7, 8, 9
Fermentation *L. casei* factor	3	.0042	.000061	Yes	5, 7, 8, 12
Pteroylhexaglutamylglutamic acid (B_c conjugate)	7	Slight[a]	Slight[a]	Yes	10, 11

[a] One microgram is equivalent to 0.003–0.006 microgram of vitamin B_c when measured with *L. casei* and 0.002 microgram when using *S. faecalis*.

In a previous publication[9] the pteridines were synthesized from 2,4,5-triamino-6-hydroxypyrimidine and a compound in which adjacent carbons contained functional groups capable of reacting with amines. By analogy a reaction should occur between the 2,4,5-triamino-6-hydroxypyrimidine and a three-carbon compound in which the *p*-aminobenzoylglutamic acid is attached to a terminal carbon atom and functional groups such as bromine or oxygen are on each of the other two carbons to give pteroylglutamic acid.

The first reaction attempted in this synthesis was to combine α,β-dibromopropionaldehyde and diethyl *p*-aminobenzoylglutamate and then to treat the product with 2,4,5-triamino-6-hydroxypyrimidine. When diethyl *p*-aminobenzoylglutamate reacted with the dibromopropionaldehyde, a crude, hygroscopic, non-crystalline product (III) was obtained. This material appeared to contain an anil structure because it hydrolyzed readily to diethyl *p*-aminobenzoylglutamate and a tar. Upon condensation of III with 2,4,5-triamino-6-hydroxypyrimidine, a very low yield of biologically active material was obtained.

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(4) Angier, *et al.*, *Science*, **102**, 227 (1945).
(5) Angier, *et al.*, *ibid.*, **103**, 667 (1946).
(6) Mitchell, Snell and Williams, THIS JOURNAL, **66**, 267 (1944).
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(8) Hutchings, *et al.*, *ibid.*, **70**, 1, 10 (1948).
(9) Mowat, *et al.*, *ibid.*, **70**, 14 (1948).
(10) Pfiffner, *et al.*, *Science*, **102**, 228 (1945).
(11) Pfiffner, *et al.*, THIS JOURNAL, **68**, 1392 (1946).
(12) Hutchings, *et al.*, *Science*, **99**, 371 (1944).

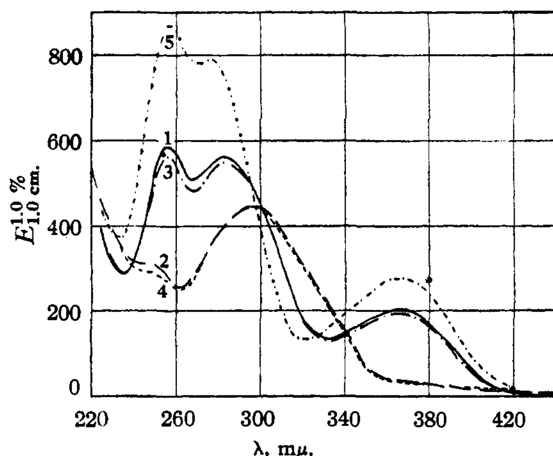

Fig. 1.—Ultraviolet absorption spectra: (1) synthetic liver *L. casei* factor (pteroylglutamic acid[a]) in 0.1 *N* sodium hydroxide; (2) synthetic liver *L. casei* factor (pteroylglutamic acid[a]) in 0.1 *N* hydrochloric acid; (3) natural liver *L. casei* factor in 0.1 *N* sodium hydroxide; (4) natural liver *L. casei* factor in 0.1 *N* hydrochloric acid; (5) pteroic acid in 0.1 *N* sodium hydroxide.

[a] *Anal.* Calcd. for $C_{19}H_{19}O_6N_7 \cdot H_2O$: C, 49.6; H, 4.15; N, 21.4. Found: C, 49.5, 49.5: H, 4.36, 4.16; N, 21.40, 21.5.

In view of the failure of the first reaction to give appreciable yields of pteroylglutamic acid, the dibromopropionaldehyde was then treated with the 2,4,5-triamino-6-hydroxypyrimidine in an attempt to obtain a 6-bromomethylpteridine or a 6-hydroxymethylpteridine. From this condensation a crude product was obtained and separated into two fractions. One fraction was identified as 2-amino-4-hydroxy-7-methylpteridine. The other fraction (IV) could be characterized only to the extent that it was a 6-substituted-pteridine since it could be oxidized with alkaline permanganate to 2-amino-4-hydroxypteridine-6-carboxylic acid.

In a final attempt to obtain pteroylglutamic acid by the use of dibromopropionaldehyde, equal molecular amounts of 2,4,5-triamino-6-hydroxypyrimidine and the *p*-aminobenzoylglutamic acid were dissolved in water and treated with the dibromopropionaldehyde which was dissolved in an organic solvent. The yields were 30–50% of crude material which was shown to contain 10–25% of pteroylglutamic acid. A series of experiments at various acidities showed that the best yields were obtained when the *p*H was maintained at 4. The addition of the *p*-aminobenzoylglutamic acid to a reaction mixture of the 2,4,5-triamino-6-hydroxypyrimidine and the dibromopropionaldehyde gave much less pteroylglutamic acid than when all reactants were mixed together.

The synthesis of pteroic acid, which is reported herein, was accomplished by substituting *p*-aminobenzoic acid for *p*-aminobenzoylglutamic acid in the above reaction.

Pteroylglutamic and pteroic acids were purified in the same general way. The crude materials were dissolved in alkali, brought to *p*H 7, filtered and brought to *p*H 3 to precipitate the active compounds. The products were purified further by repeated treatments with charcoal in alkaline solution and recrystallizations from water at *p*H 3.

A comparison of pteroylglutamic acid with the liver *L. casei* factor indicated that the two compounds are identical.[13]

The crystalline structure of pteroylglutamic acid and the liver compound are identical. The ultraviolet absorption spectra for pteroic acid, pteroylglutamic acid and the liver *L. casei* factor are plotted in Fig. 1. The infrared absorption spectra for pteroylglutamic acid, the liver *L. casei* factor, racemic pteroylglutamic acid, racemic liver *L. casei* factor from the anaerobic alkaline hydrolysis of the fermentation *L. casei* factor and pteroic acid are given in Fig. 2. The infrared absorption spectrum of the liver *L. casei* factor is identical with that of pteroylglutamic acid and this spectrum of the racemic liver *L. casei* factor is identical with that of the racemic pteroylglutamic acid.

Degradation of pteroylglutamic acid and pteroic acid with alkali and oxygen gave 2-amino-4-hydroxypteridine-6-carboxylic acid which was the same acid obtained from the natural liver and fermentation *L. casei* factors.

Experimental

Materials.—The 2,4,5-triamino-6-hydroxypyrimidine was prepared by nitrosation and reduction of 2,4-diamino-6-hydroxypyrimidine.[14] The *p*-aminobenzoyl-*l*(+)-glutamic acid and the *p*-aminobenzoyl-*d,l*-glutamic acid were prepared by nitrobenzoylation and reduction of *l*(+)-glutamic acid and *d,l*-glutamic acid according to the method of Van Der Scheer and Landsteiner.[15] The dihalopropionaldehydes were prepared by direct halogenation of acrolein in carbon disulfide[16] or in carbon tetrachloride.[17] The dihalopropionaldehydes were purified by repeated fractionation; the fractions boiling at 53 to 54° at 2.5 mm. pressure and 46 to 49° at 12 to 14 mm. pressure for the dibromopropionaldehyde and dichloropropionaldehyde, respectively, were used.

Diethyl *p*-Aminobenzoyl-*l*(+)-glutamate.—The *p*-aminobenzoyl-*l*(+)-glutamic acid (25 g.) was slurried in 200 ml. of 95% ethanol which had previously been saturated with hydrogen chloride at 0°. This mixture was allowed to stand for two days during which time the *p*-aminobenzoylglutamic acid gradually dissolved. The excess hydrogen chloride and part of the ethanol were removed by evaporation under reduced pressure at room temperature. The residual solution was diluted to 1 liter with cold water and neutralized with ammonia to about *p*H 8. A slightly colored crystalline product was filtered off at once, washed and dried; yield 24 g. (75%); m. p. 139–141°. This crude product is satisfactory for most purposes. For analytical sample it was recrystallized twice from ethanol after decolorizing the solution with charcoal; yield 16 g.; m. p. 143–144°.

(13) Pfiffner[11] found that pteroylglutamic acid and vitamin B_c were identical.

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

(15) Van Der Scheer and Landsteiner, *J. Immunology*, **29**, 373 (1935).

(16) Fischer and Tafel, *Ber.*, **20**, 3389 (1887).

(17) Moureu and Boismenu, *Ann. Chim.*, [9] **15**, 209 (1921).

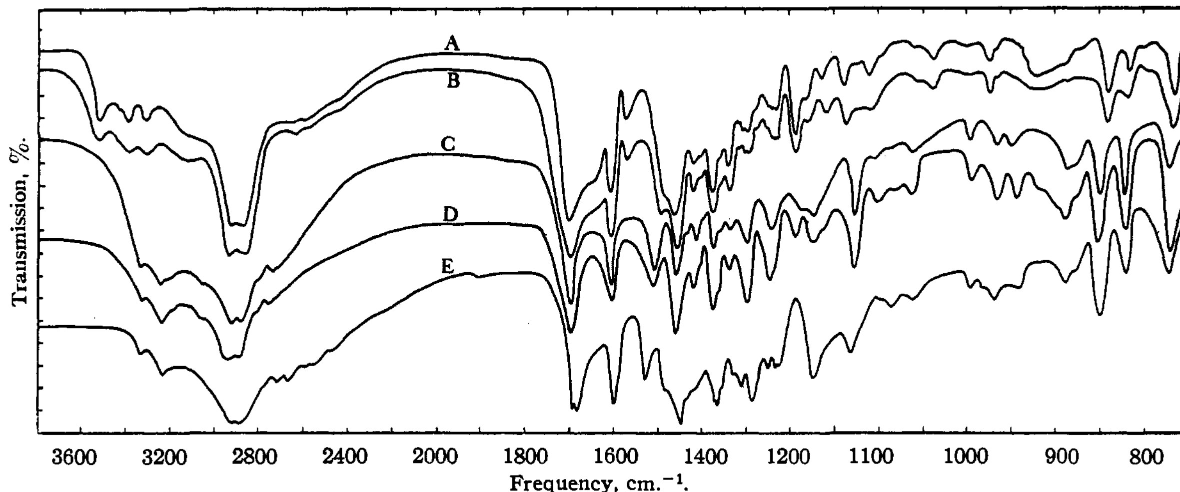

Fig. 2.—Infrared absorption spectra: A, natural liver *L. casei* factor; B, synthetic liver *L. casei* factor (pteroylglutamic acid; C, synthetic racemic liver *L. casei* factor (racemic pteroylglutamic acid); D, natural racemic liver *L. casei* factor; E, pteroic acid.

Anal. Calcd. for $C_{16}H_{22}O_5N_2$: C, 59.61; H, 6.89; N, 8.69. Found: C, 59.2; H, 6.7; N, 8.84.

Preparation of Anil (III).—A solution of 5 g. of diethyl *p*-aminobenzoyl-*l*(+)-glutamate and 3.35 g. of α,β-dibromopropionaldehyde in 200 ml. of diethyl ether was refluxed for four hours. After standing overnight the red oil was separated from the ether solution, dissolved in 15 ml. of ethanol and precipitated with 500 ml. of ether. The resulting oily product was dried to a powder.

A sample of the above anil and an equal weight of sodium bisulfite when heated in water for twenty minutes deposited a tar. This tar was collected and discarded. The solution deposited a crystalline material when cooled which melted at 138 to 140°. It was diethyl *p*-aminobenzoylglutamate.

Diethyl Pteroylglutamate from Anil (III).—To a solution of 0.5 g. of 2,4,5-triamino-6-hydroxypyrimidine and 0.88 g. of sodium acetate in 50 ml. of water was added a solution of 2.3 g. of anil (III) in 50 ml. of ethanol. After standing overnight a very small amount of precipitate was collected, washed and dried. After alkaline hydrolysis this crude product was shown to contain 1.9% of biologically active material calculated as pteroylglutamic acid.

Preparation of (IV).—To a solution of 3 g. of 2,4,5-triamino-6-hydroxypyrimidine in 300 ml. of 10% sodium acetate solution was added dropwise with stirring 4.7 g. of α,β-dibromopropionaldehyde dissolved in 150 ml. of ethanol. Fifteen minutes after completion of the addition 2 g. of the precipitated product was collected. When the acidity of this reaction was maintained at *p*H 4 by the gradual addition of sodium hydroxide solution instead of using the acetate buffer, a comparable yield of the same product was obtained.

Attempted purification and identification of this material was unsuccessful; however, when a sample was oxidized with hot alkaline permanganate solution 2-amino-4-hydroxypteridine-6-carboxylic acid[9] was obtained.

2-Amino-4-hydroxy-7-methylpteridine and (IV).—To a solution of 30 g. of 2,4,5-triamino-6-hydroxypyrimidine dihydrochloride in 300 ml. of 1 *N* hydrochloric acid was added a solution of 30 g. of α,β-dibromopropionaldehyde in 300 ml. of ethanol. After standing for one hour, 6 g. of the precipitated product was collected and identified as 2-amino-4-hydroxy-7-methylpteridine.[9] The filtrate after buffering with a slight excess of sodium acetate deposited 4 g. more of the above 7-methylpteridine.

The above filtrate on standing with the excess acetate buffer for two hours deposited 1.7 g. of material which had the properties of compound IV.

Pteroylglutamic Acid: A.—Ten grams of 2,4,5-triamino-6-hydroxypyrimidine and 19 g. of *p*-aminobenzoyl-*l*(+)-glutamic acid were dissolved in 1 liter of 10% sodium acetate solution. To this solution was added slowly with stirring a solution of 15.3 g. of α,β-dibromopropionaldehyde in 100 ml. of ethanol. After stirring for ninety minutes 4.8 g. of the precipitated product was collected. This crude product was shown to contain 10% of pteroylglutamic acid by bioassaying with *S. faecalis* R.

A reaction was run as above except the *p*-aminobenzoylglutamic acid was added five minutes after the addition of the dibromopropionaldehyde was complete. The resulting crude was found to contain only 4% of pteroylglutamic acid.

B.—Fifty grams (0.355 mole) of 2,4,5-triamino-6-hydroxypyrimidine and 95 g. (0.355 mole) of *p*-aminobenzoylglutamic acid were dissolved in 7.5 liters of water. The acidity of this solution was adjusted and maintained at *p*H 4 throughout the reaction. To this solution was added slowly with stirring over a ninety-minute period a solution of 77 g. (0.355 mole) of α,β-dibromopropionaldehyde in 7.5 liters of ethanol. The resulting mixture was then stirred for two hours more. The precipitated product was collected, washed with water and alcohol and dried. The yield of crude material was 50 g. which was shown to contain 20.8% of pteroylglutamic acid by the bioassay.

This synthesis was repeated using 0.007 mole each of 2,4,5-triamino-6-hydroxypyrimidine, *p*-aminobenzoylglutamic acid and α,β-dichloropropionaldehyde. The crude product weighed 0.8 g. and contained 14.5% pteroylglutamic acid. Further repetition of this synthesis using benzene, chloroform, carbon tetrachloride, acetic acid or acetone as solvents for the dibromopropionaldehyde instead of ethanol gave yields of the crude material ranging from 0.8 g. to 1.5 g. using 0.007 mole of each of the reactants. The pteroylglutamic acid in these crudes was about 20%.

A series of condensations was run at various acidities using 0.0035 mole of each of 2,4,5-triamino-6-hydroxypyrimidine-α,β-dibromopropionaldehyde and *p*-aminobenzoylglutamic acid. At *p*H 2, 3, 4 and 5 the yields of the crude products were 0.24, 0.30, 0.35 and 0.33 g.; and were shown by bioassay to contain 13, 24, 35 and 14%, respectively, of pteroylglutamic acid. At *p*H 6, 7, 8 and 10 there were no appreciable amounts of precipitates formed; however, pteroylglutamic acid was found by bioassay to be present in the reaction mixtures to the extent of 20 to 30% as much as the condensation usually yielded at *p*H 4.

Racemic Pteroylglutamic Acid.—This compound was prepared by the method given above for pteroylglutamic acid (method B) except that the *p*-aminobenzoyl-*l*(+)-glutamic acid was replaced by *p*-aminobenzoyl-*d,l*-glutamic acid.

Purification of Pteroylglutamic Acid.—A sample of crude material (5.85 g.) containing 0.755 g. of pteroylglutamic acid was dissolved in 3500 ml. of hot water at *p*H 10–12. This alkaline solution was brought to *p*H 7, cooled and filtered. The filtrate was adjusted to *p*H 3 and the active material was collected. This partially purified material was dissolved in 200 ml. of water at *p*H 10–12, clarified with 1 g. of activated charcoal and diluted with 800 ml. of hot water containing enough acetic acid to bring the acidity to *p*H 3–3.5. After cooling 0.61 g. of product was obtained. A bioassay showed the presence of 76.8% pteroylglutamic acid giving a 62% recovery.

Two hundred milligrams of the above 76.8% material was dissolved in 100 ml. of water at *p*H 10–12, clarified with 0.4 g. of charcoal, diluted to 700 ml. with boiling water containing enough acetic acid to bring to *p*H 3–3.5, filtered and cooled. The active material crystallized as rosets. This was collected and dried; yield, 166.2 mg.; bioassay, 92% pteroylglutamic acid. Recrystallization from water gave needle-like crystals.

Anal. As magnesium salt, calcd. for $C_{19}H_{15}O_6N_7Mg_{1.5}\cdot 2H_2O$: C, 44.8; H, 3.74; N, 19.4; Mg, 7.06. Found: C, 44.8; H, 3.8; N, 19.3; Mg, 6.6.

Degradation of Synthetic Pteroylglutamic Acid.—A 0.275-g. sample of purified synthetic pteroylglutamic acid when hydrolyzed for eight hours at 100° with 10 ml. of 1 *N* solution of sodium hydroxide in a current of oxygen as described[4] for the natural *L. casei* factor, gave 0.058 g. (46%) of pure 2-amino-4-hydroxypteridine-6-carboxylic acid. No 2-amino-4-hydroxypteridine-7-carboxylic acid was detected in the hydrolysate.

Pteroylglutamic acid (0.5 g.) was cleaved with sulfite as described for the fermentation *L. casei* factor.[5] The solution when freed of sulfur dioxide and treated with phenylhydrazine gave 0.206 g. of the red phenylhydrazone. Analytical figures agree with those given for the hydrazone from the fermentation compound.

Pteroic Acid.—A vigorously stirred solution of 1 g. each of 2,4,5-triamino-6-hydroxypyrimidine and *p*-aminobenzoic acid in 150 ml. of water was adjusted and maintained at *p*H 4 throughout the reaction. A solution of 1.53 g. of α,β-dibromopropionaldehyde in 150 ml. of ethanol was then added dropwise. The resulting mixture was stirred for ninety minutes after the addition of the aldehyde was complete. The precipitated product was collected, washed with water and alcohol and dried. A yield of 0.59 g. of crude containing 19.2% of pteroic acid was obtained.

This synthesis using the same amounts of the reactants and substituting benzene for ethanol as the solvent for the dibromopropionaldehyde gave 0.9 g. of crude product containing 13.5% of pteroic acid as shown by bioassay with *S. faecalis* R.

Synthesis of Ethyl Pteroate.—A vigorously stirred solution of 1 g. of 2,4,5-triamino-6-hydroxypyrimidine and 1.2 g. of ethyl *p*-aminobenzoate in a mixture of 150 ml. of water and 100 ml. of ethanol was adjusted and maintained at *p*H 4 while 1.53 g. of α,β-dibromopropionaldehyde in 50 ml. of ethanol was added over a twenty-minute period. The mixture was stirred for an additional ninety minutes and filtered. A yield of 0.38 g. of crude was obtained. This crude after hydrolyzing with sodium hydroxide solution in the cold was shown to contain 14.3% pteroic acid.

Purification of Pteroic Acid.—The crude material containing 15% of pteroic acid was dissolved in 0.01 *N* sodium hydroxide solution at a concentration of 1 mg. of crude per ml., brought to *p*H 7, filtered and brought to *p*H 3. The precipitated material at *p*H 3 was collected and shown by bioassay to contain 60% pteroic acid. This represented a recovery of 11%. The precipitate at *p*H 7 contained the bulk of the active material which can be largely recovered by repeating the procedure several times.

Final purification was accomplished by the following procedure: The partially purified pteroic acid was clarified in 0.2 *N* sodium hydroxide solution with charcoal at a concentration of 1 mg. per ml. The resulting alkaline filtrate was diluted to 0.1 mg. per ml., heated to boiling and neutralized slowly with dilute hydrochloric acid to *p*H 3. The crystals of pteroic acid formed in the hot solution. After cooling this material was collected and recrystallized three times by dissolving in hot sodium hydroxide solution at a concentration of 0.1 mg. per ml. and bringing to *p*H 3 with dilute hydrochloric acid.

Anal. Calcd. for $C_{14}H_{12}O_3N_6$: C, 53.85; H, 3.85. Found: C, 53.3; H, 4.15.

Degradation of Pteroic Acid.—When 0.1 g. of purified pteroic acid was hydrolyzed with 5 ml. of 1 *N* solution of sodium hydroxide in the presence of oxygen at 100° for six hours, 0.0225 g. (37.5%) of pure 2-amino-4-hydroxypteridine-6-carboxylic acid was obtained. No 2-amino-4-hydroxypteridine-7-carboxylic acid was detected in the hydrolysate.

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Summary

The preparation of pteroic acid and pteroylglutamic acid is given. This method of preparation illustrates a general procedure for the synthesis of pteroyl derivatives.

Physical, chemical and biological data on pteroylglutamic acid and the liver *L. casei* factor appear to show that the two compounds are identical.

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