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Pteroic Acid Derivatives. II. Pteroyl-γ-glutamylglutamic Acid and Pteroyl-γ-glutamyl-γ-glutamylglutamic Acid

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In the course of its degradation the fermentation *L. casei* factor was shown to contain three molecules of glutamic acid in contrast to the liver *L. casei* factor which contained only one molecule of glutamic acid.[1] Because pteroyl-α-glutamylglutamic acid and pteroyl-α,γ-glutamyldiglutamic acid which are described in the preceding communication showed[2] very low activities when assayed biologically against *S. faecalis R* or *L. casei*, it was decided to prepare some glutamic acid peptides in which only the γ-carboxyl groups were involved in the peptide linkages. This communication deals with the preparation of the dipeptide and the tripeptide of this type, and the subsequent preparation of their pteroyl derivatives. The general method used in this synthesis was to start with α-ethyl carbobenzoxyglutamate[3] which could not be satisfactorily crystallized from ether as described by Neuberger. Therefore, the crude mono ester was converted to the acid chloride[4,5] and treated with diethyl glutamate to form the carbobenzoxy dipeptide which was then reduced to triethyl γ-glutamylglutamate and crystallized as the hydrochloride.

For the preparation of pteroyl-γ-glutamylglutamic acid this dipeptide was *p*-nitrobenzoylated by the Schotten–Baumann method which at the same time hydrolyzed the esters to form *p*-nitrobenzoyl-γ-glutamylglutamic acid. The nitro group was reduced and without isolation of the intermediate *p*-aminobenzoyl dipeptide, the product was condensed with α,β-dibromopropionaldehyde and 2,4,5-triamino-6-hydroxypyrimidine as described for pteroylglutamic acid.[6] The pteroyl-γ-glutamylglutamic acid was isolated from the crude reaction product and was found to be in the range of 60 to 70% as active as pteroylglutamic acid when assayed against *S. faecalis R*, or *L. casei*.

For the preparation of the pteroyl tripeptide, triethyl γ-glutamylglutamate was condensed with the acid chloride of α-ethyl carbobenzoxyglutamate to form tetraethyl carbobenzoxy-γ-glutamyl-γ-glutamylglutamate. This carbobenzoxy tripeptide was reduced to tetraethyl γ-glutamyl-γ-glutamylglutamate hydrochloride which was *p*-nitrobenzoylated in benzene in the presence of diethyl amine. The *p*-nitrobenzoyl tripeptide was reduced to the corresponding *p*-aminobenzoyl derivative and condensed with α,β-dibromopropionaldehyde and 2,4,5-triamino-6-hydroxypyrimidine to form the pteroyl derivative.

The amount of pteroyl-γ-glutamyl-γ-glutamylglutamic acid prepared by this method was too small to isolate and completely characterize. It was biologically assayed, however, and showed the same ratio of activity for the two test organisms as does the fermentation *L. casei* factor, *i. e.* a low activity for *S. faecalis R* and a high activity for *L. casei*.

This method of synthesis served to indicate the probable structure of the fermentation *L. casei* factor but was not a desirable method from a preparative standpoint. Therefore, relatively little further work was done on this procedure, and efforts were directed toward finding a method more feasible for large scale preparations. This work will be the subject of future communications.

Experimental[7]

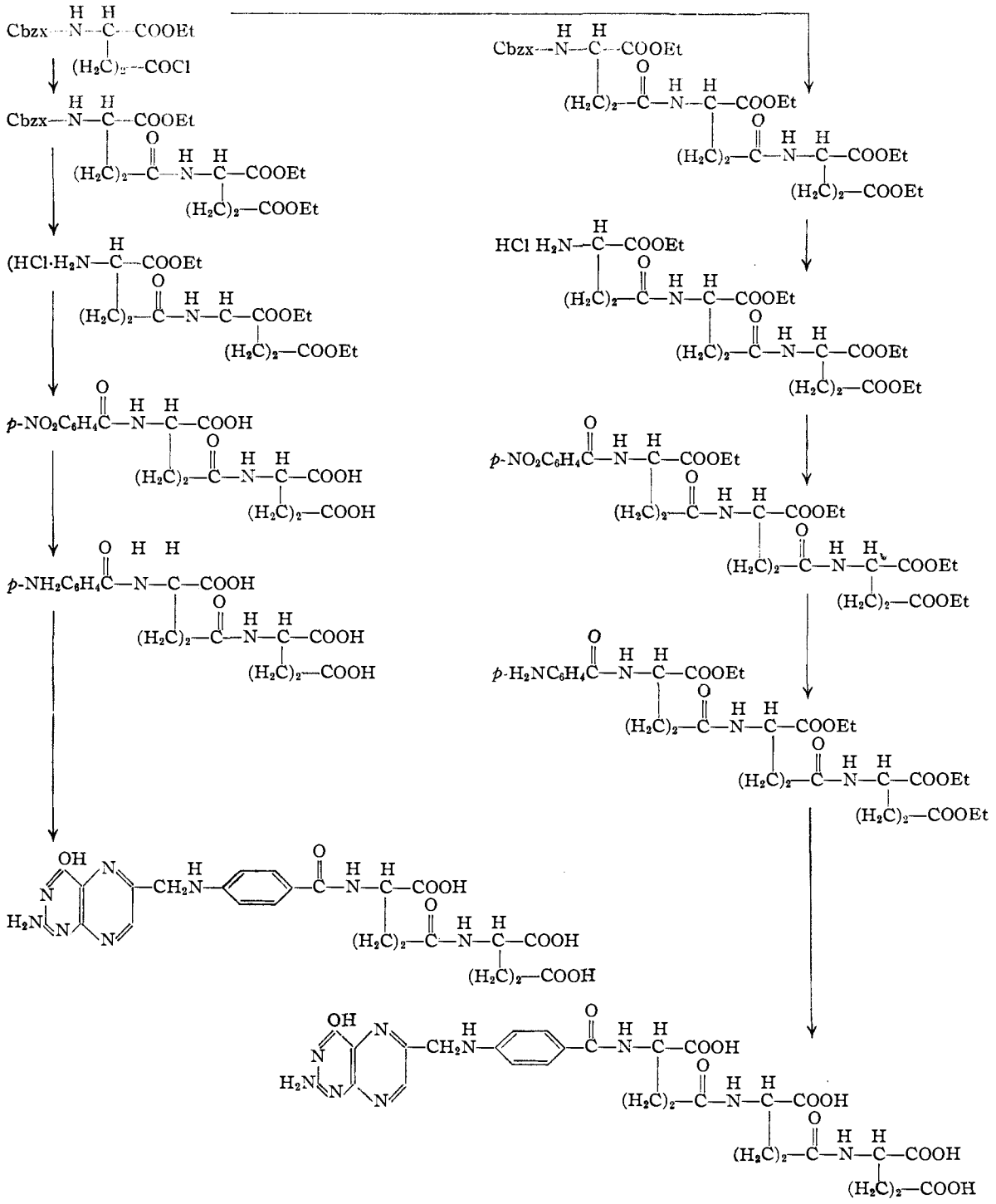
Triethyl Carbobenzoxy-γ-glutamylglutamate.—Seventy-eight grams of crude α-ethyl carbobenzoxyglutamate was dissolved in 250 cc. of anhydrous ether and cooled to −10°. With stirring and exclusion of moisture, 67.8 g. of phosphorus pentachloride was added in portions over a twenty minute period, not allowing the temperature to rise over −5°. The mixture was stirred thirty-five minutes more at −5 to 0°, filtered quickly and concentrated *in vacuo* to a residual sirup keeping the temperature below 0°. This residue was washed four times by decantation with cold, dry heptane, and then dissolved in 250 cc. of cold, dry chloroform and put in a Dry Ice-bath. It was then mixed with 114 g. of diethyl glutamate in 250 cc. of cold, dry chloroform and allowed to stand at room temperature two and one-half hours. The chloroform solution was then extracted several times with 0.2 *N* hydrochloric acid until the excess diethyl glutamate had been removed. It was then extracted twice with 0.2 *N* sodium bicarbonate solution and washed with water. This solution was dried over magnesium sulfate and the chloroform distilled off *in vacuo*. The oily residue changed to a hard wax-like material which was used directly for the next step; wt. 90.3 g. A small amount was purified by dissolving in hot ether and cooling. It tended to gel but by alternate warming and cooling it changed over to a white solid which was filtered off and dried; m. p. 91–92°.

Anal. Calcd. for $C_{24}H_{34}O_9N_2$: C, 58.2; H, 6.9; N, 5.66. Found: C, 59.0; H, 6.89; N, 5.54.

Triethyl γ-Glutamylglutamate Hydrochloride.—Sixty-five grams of crude triethyl carbobenzoxy-γ-glutamylglutamate was dissolved in 435 cc. of alcohol, treated with Norite and filtered. Two hundred seventeen cc. of water, 17 cc. of acetic acid and 2.6 g. of 10% palladium–charcoal catalyst were added. A stream of hydrogen was passed through with good stirring and carbon dioxide was evolved. After two hours the carbon dioxide evolution had almost ceased. Another 2.6 g. of catalyst was

(1) Hutchings, *et al.*, THIS JOURNAL, **70**, 10 (1948).
(2) Mowat, *et al.*, *ibid.*, **70**, 1096 (1948).
(3) Neuberger, *Biochem. J.*, **30**, 2085 (1936).
(4) du Vigneaud and Miller, *J. Biol. Chem.*, **116**, 469 (1936).
(5) Harrington and Mead, *Biochem. J.*, **29**, 1602 (1935).
(6) Waller, *et al.*, THIS JOURNAL, **70**, 19 (1948).

(7) All melting points are corrected and were taken by U. S. P. prescribed conditions.



added and the reduction was complete in another hour. Some runs of this reaction took much longer to reduce and required more catalyst, apparently due to catalyst poisons. The catalyst was filtered off and the filtrate was concentrated to complete dryness *in vacuo*. The residue was dissolved in 100 cc. of dry chloroform, hydrogen chloride was bubbled through for twenty minutes, and 650 cc. of anhydrous ether was added which precipitated an oil. This oil slowly crystallized and was filtered off and dried; wt. 36.5 g. Upon repeated crystallization from ethyl acetate or from chloroform and ether, a constant m. p. was reached at 132–133°, $[\alpha]^{26}D$ −5.6° (*c* 4 in water).

Anal. Calcd. for $C_{16}H_{28}O_7N_2 \cdot HCl$: C, 48.4; H, 7.31; N, 7.08; α-amino N, 3.54. Found: C, 48.7; H, 7.62; N, 7.26; α-amino N, 4.18.

***p*-Nitrobenzoyl-γ-glutamylglutamic Acid.**—Four hundred twenty milligrams of triethyl γ-glutamylglutamate hydrochloride was dissolved in 0.7 cc. of water and 1.8 cc. of 2 *N* sodium hydroxide. Three hundred sixty-

two milligrams of *p*-nitrobenzoyl chloride and 1.7 cc. of 2 *N* sodium hydroxide was added concurrently over a ten to fifteen minute period. The solution was stirred twenty minutes more and 0.425 cc. of concentrated hydrochloric acid was added. The precipitated *p*-nitrobenzoic acid was quickly filtered off and on cooling the filtrate, the product crystallized out rather slowly. After filtering and drying the product weighed 0.370 g. After repeated crystallization from water the m. p. was 194–195°.

Anal. Calcd. for $C_{17}H_{19}O_{10}N_3$: C, 48.0; H, 4.51; N, 9.88. Found: C, 48.28; H, 5.01; N, 9.97.

This reaction was also run in the same way on crude triethyl γ-glutamylglutamate before forming the hydrochloride. The same product was obtained in lower yield and the aqueous solution had to be concentrated after filtering off the *p*-nitrobenzoic acid.

Pteroyl-γ-glutamylglutamic Acid.—Eighteen grams of *p*-nitrobenzoyl-γ-glutamylglutamic acid was mixed with 720 cc. of water, 72 cc. of acetic acid, and 0.9 g. of platinum oxide. This mixture was shaken with hydrogen until 3.28 liters was absorbed, which required about two and one-half hours. The catalyst was removed by filtration and the filtrate was diluted to 1170 cc. A Bratton–Marshall amine test showed that the solution contained 14.0 g. (83.5% yield) of *p*-aminobenzoyl-γ-glutamylglutamic acid. Eighteen grams of 2,4,5-triamino-6-hydroxypyrimidine dihydrochloride was dissolved in the solution and while the *p*H was maintained at 4.0 by the addition of sodium hydroxide, 8.35 cc. of α,β-dibromopropionaldehyde in 600 cc. of alcohol was added slowly. After stirring one hour the precipitated product was filtered off and dried; yield 12.5 g. This crude material contained 2.4 g. of pteroyl-γ-glutamylglutamic acid by chemical assay.[8] The 12.5 g. of crude material was dissolved in 10 liters of 0.2 *N* sodium hydroxide, barium chloride was added to make 0.2 *N* and ethanol was added to the extent of 3%. The precipitate was discarded, the filtrate was diluted to 40 liters, and the barium ions were removed with sulfate ions. Zinc chloride was then added to *p*H 6.8 and the precipitate containing the desired material was filtered off and stirred with 65 liters of dilute sodium hydroxide containing 15% ethanol.

The *p*H was brought to 6.8 with zinc chloride, then heated to 90° and filtered, the active material remaining in the precipitate. This hot zinc salt precipitation was repeated twice and then the material was dissolved in 10 l. of 0.2 *N* sodium hydroxide and treated with barium chloride and alcohol as previously described. After removal of the barium ions the *p*H of the solution was brought to 3 and the precipitate was collected. It was then reprecipitated four times from 2 liters of hot water, by cooling and after filtering and drying weighed 0.522 g.

Anal. Calcd. for $C_{24}H_{26}O_9N_8$: C, 50.5; H, 4.56; N, 19.65. Found (corrected for 1.46% ash): C, 49.55; H, 4.96; N, 19.53. Microbiological assays: *S. faecalis R.*, 70.9%; *L. casei*, 62.9%. Both assays used pteroylglutamic acid as a standard.

Tetraethyl Carbobenzoxy-γ-glutamyl-γ-glutamylglutamate.—Twenty-five g. of triethyl γ-glutamylglutamate hydrochloride was dissolved in 100 cc. of chloroform. A solution of 1.448 g. of sodium in 50 cc. of ethanol was added to the chloroform solution which formed a colloidal precipitate of sodium chloride. The solution was evaporated to complete dryness *in vacuo*, taken up in 30 cc. of chloroform, and treated with a chloroform solution of the acid chloride of α-ethyl carbobenzoxyglutamate as already described for the dipeptide. The reaction mixture was worked up the same way and on concentrating the chloroform solution to dryness *in vacuo* a solid residue was obtained which was shaken with ether and filtered off; yield 9.5 g. A small portion was crystallized from chloroform and ether; m. p. 109–110°.

Anal. Calcd. for $C_{31}H_{45}O_{12}N_3$: C, 57.2; H, 6.91; N, 6.45. Found: C, 56.4; H, 6.57; N, 6.52.

(8) Hutchings, *et al.*, *J. Biol. Chem.*, **168**, 705 (1947).

Tetraethyl γ-Glutamyl-γ-glutamylglutamate Hydrochloride.—Tetraethyl carbobenzoxy-γ-glutamyl-γ-glutamylglutamate (11.32 g.) was reduced in 125 cc. of alcohol, 40 cc. of water, 4 cc. of acetic acid, and 0.4 g. of 10% palladium–charcoal catalyst as described above. It was necessary to add 0.4 g. more of catalyst after two hours and the total reduction time was five hours. The catalyst was filtered off and the filtrate was concentrated to 40 cc. and diluted with 75 cc. of water which precipitated a small amount of white flocculent material. This was removed by filtration and the filtrate was concentrated to dryness *in vacuo* and dried under vacuum over phosphoric anhydride. The residue was dissolved in 30 cc. of chloroform, hydrogen chloride was bubbled through, and the product was precipitated by adding 150 cc. of ether. The dried product weighed 7.5 g. A portion was precipitated twice from hot ethyl acetate by cooling but was apparently amorphous; m. p. 147–150°.

Anal. Calcd. for $C_{23}H_{39}O_{10}N_3$: C, 49.8; H, 7.07; N, 7.59. Found: C, 49.45; H, 7.8; N, 7.45.

Tetraethyl *p*-Nitrobenzoyl-γ-glutamyl-γ-glutamylglutamate.—A mixture of 5.32 g. of tetraethyl γ-glutamyl-γ-glutamylglutamate hydrochloride, 50 cc. of dry benzene, and 1.94 cc. of diethylamine were mixed well and the diethylamine hydrochloride was filtered off. To the filtrate was added a solution of 3.56 g. of *p*-nitrobenzoyl chloride in 15 cc. of ether and 50 cc. of benzene. The solution changed to a jelly-like solid in a few minutes and was then shaken well for thirty minutes. The mixture was then diluted with ether and the solid was removed by filtration and washed well with ether. After drying, the product was powdered, washed well with water, and again dried; yield 4.2 g. A small portion was crystallized twice from hot alcohol by cooling very slowly to prevent gelling: m. p. 173–174°,[9] $[\alpha]^{28}D$ −4.0° (*c* 2 in acetic acid).

Anal. Calcd. for $C_{30}H_{42}O_{13}N_4$: C, 54.0; H, 6.34; N, 8.40. Found: C, 54.0; H, 6.95; N, 8.60.

Tetraethyl *p*-Aminobenzoyl-γ-glutamyl-γ-glutamylglutamate.—A mixture of 3.0 g. of tetraethyl *p*-nitrobenzoyl-γ-glutamyl-γ-glutamylglutamate, 25 cc. of water, 50 cc. of ethanol, 1.5 g. of iron powder, and 1 cc. of concentrated hydrochloric acid was warmed to 35–40° and stirred vigorously for two and one-half hours during which time the solid all dissolved. Two cc. of 28% ammonium hydroxide was added and hydrogen sulfide was bubbled in for twenty minutes. The mixture was filtered and the precipitate was washed with ethanol. A Bratton–Marshall amine test on the filtrate indicated that it contained an 87% yield of the product. Eighty per cent. of the solution was adjusted to *p*H 7.5 and evaporated to a small volume *in vacuo*. Ethanol and several drops of ammonium hydroxide were added and the solution was evaporated to complete dryness. The residue was taken up in 35 cc. of ethanol, filtered to remove the ammonium chloride and evaporated to dryness. The product was taken up in 12 cc. of ethanol and 30–40 cc. of water was added which caused an oil to separate. This oil crystallized on standing overnight; yield 1.6 g. One gram of this material was dissolved in 10 cc. of ethanol, 60 cc. of water, and 0.3 cc. of concentrated hydrochloric acid and was filtered to remove traces of unreduced nitro compound. To the filtrate was added 0.6 cc. of pyridine which caused the product to oil out and later crystallize. A portion of this was recrystallized twice from alcohol and ether; m. p. 147–148°.

Anal. Calcd. for $C_{30}H_{44}O_{11}N_4$: C, 56.6; H, 6.90; N, 8.80. Found: C, 56.57; H, 6.56; N, 8.71.

Pteroyl-γ-glutamyl-γ-glutamylglutamic Acid.—One hundred milligrams of tetraethyl *p*-nitrobenzoyl-γ-glutamyl-γ-glutamylglutamate was reduced as described

(9) In some other preparations of this compound by the same method, it was obtained in a form melting at 158–159°. One form could not be changed to the other at will by seeding although this occasionally happened during recrystallization. *Anal.* C, 54.08; H, 5.48; N, 8.34. This compound was later prepared by other methods and the same m. p. behavior was noted.

above. After one and one-half hours the excess iron was filtered out and washed with 0.5 cc. of acetic acid and 3 cc. of water. The filtrate was diluted to 8 cc. and a Bratton–Marshall amine test showed that 97% of the theoretical amine was present. Sixty-five milligrams of 2,4,5-triamino-6-hydroxypyrimidine dihydrochloride was added to the reduced solution and the *p*H was adjusted to 3.8. Sixty-six milligrams of dibromopropionaldehyde in 1 cc. of acetic acid was added slowly with stirring and maintaining the *p*H at 3.5 to 4. The precipitate was rather sticky so the water was poured off and precipitate was washed with alcohol and ether; yield, 25 mg. Chemical assay: 23.4%. Microbiological assays after hydrolysis of the esters for twelve hours in 0.1 *N* sodium hydroxide: *S. faecalis R.*, 0.6%; *L. casei*, 16.5%. If these assay figures are corrected for the inert material present in the crude reaction mixture the values are then: *S. faecalis R.*, 2.57%; *L. casei*, 70.6%. These figures are very similar to those obtained for the fermentation of *L. casei* factor.

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Summary

Pteroyl-γ-glutamyl-glutamic acid has been synthesized and found to stimulate the growth of the two test organisms, *S. faecalis R.* and *L. casei*, to the extent of 60–70% as well as does pteroylglutamic acid.

Pteroyl-γ-glutamyl-γ-glutamylglutamic acid has been synthesized and found to show the same ratio of activity for the two test organisms as does the fermentation *L. casei* factor. This synthetic compound was not isolated from the reaction mixture and was only characterized to the extent of its microbiological activity.

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Configuration of Vaccenic Acid[1]

BY P. C. RAO AND B. F. DAUBERT

Recent interest in the purported growth-promotional activity for the rat of vaccenic acid, a fatty acid reportedly isolated from several animal fats by Bertram[2] in 1928, has prompted a reinvestigation of the presence of the fatty acid in animal fat and a confirmation of its configuration.

Several investigators,[3,4] including Bertram,[2] had observed that the iodine value of the solid fatty acids separated from the mixed fatty acids of an animal fat (tallow) by the Twitchell lead salt procedure invariably showed a higher iodine value than the solid acids separated from vegetable fat. This seemed to indicate the presence of a solid unsaturated fatty acid. It therefore seemed desirable to Bertram[2] to prove the presence of the fatty acid in beef tallow and, if present, to accomplish its isolation. As a result of his investigation, Bertram[2] reported the isolation of a solid unsaturated acid from beef tallow and furthermore, on the basis of a study of the fatty acid, concluded that it was an 11,12-octadecenoic acid of *trans* configuration (11,12-elaidic acid). He also reported its isolation from sheep fat and butterfat.

Subsequently other investigators[5,6,7] have confirmed the presence of vaccenic acid in animal fat but, to the authors' knowledge, the *trans* configuration of vaccenic acid as postulated by Bertram[2] has not been confirmed. However, the preparation of an 11,12-elaidic acid (vaccenic) has been reported by Böeseken and Hoagland[8] on partial hydrogenation of α-eleostearic acid.

In view of the alleged nutritional significance of vaccenic acid, the purpose of the current communication is to present further evidence concerning its configuration.

Experimental

Preparation of Vaccenic Acid.—The vaccenic acid was isolated from beef tallow essentially by the procedure described by Bertram.[2] However, a final lead salt separation of the acids from the liquid mercury salts was made in order to free the vaccenic acid of liquid unsaturated fatty acids. The vaccenic acid (10 g.) so obtained was esterified to the methyl ester, and fractionated in an efficient fractionating column. A highly purified methyl ester fraction (2.2 g.) was obtained of iodine value 84.0 (calcd. 85.6), and b. p. 172–173° at 3 mm. The vaccenic acid obtained after saponification of the methyl ester was crystallized several times from cold acetone, and had the constants as listed in Table I.

TABLE I

	This study	Bertram[2]
Iodine value, Wijs	87.6 (calcd. 89.9)	86.5
Saponification equivalent	282.1 (calcd. 282.4)	281.5
Melting point, °C.	42.5	39.0
Saturated acids	Trace	0.9%
Refractive index	1.4439[a] at 60°	1.44071 at 70°

[a] Assuming dn/dt to be 0.00037, the calculated value at 70° is 1.4402.

(1) The generous financial assistance of the Buhl Foundation in support of this investigation is gratefully acknowledged.

(2) Bertram, *Biochem. Z.*, **196**, 433 (1928).

(3) Twitchell, *J. Ind. Eng. Chem.*, **13**, 806 (1921).

(4) Hilditch, "Chemical Constitution of Fats," John Wiley and Sons, New York, N. Y., 1941.

(5) Boer, Jansen and Kentie, *J. Nutrition*, **33**, 339 (1947).

(6) Elvehjem, *et al.*, *J. Biol. Chem.*, **169**, 229 (1947).

(7) Grossfeld and Simmer, *Z. Untersuch Lebens.*, **59**, 237 (1930).

(8) Böeseken and Hoagland, *Rec. trav. chim.*, **46**, 632 (1927).