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.

Acknowledgments.—The authors wish to acknowledge the able assistance of Miss Eleanora Boggiano in doing microbiological assays and Mrs. Anna deGrunigen in doing chemical assays. The chemical analyses were performed by Mr. Louis Brancone and co-workers. We are also indebted to Mr. Willard McEwen and Mr. William Kinley for the preparation of intermediates used in this synthesis.

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.

PEARL RIVER, NEW YORK RECEIVED OCTOBER 14, 1947

[CONTRIBUTION NO. 657 FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF PITTSBURGH]

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).

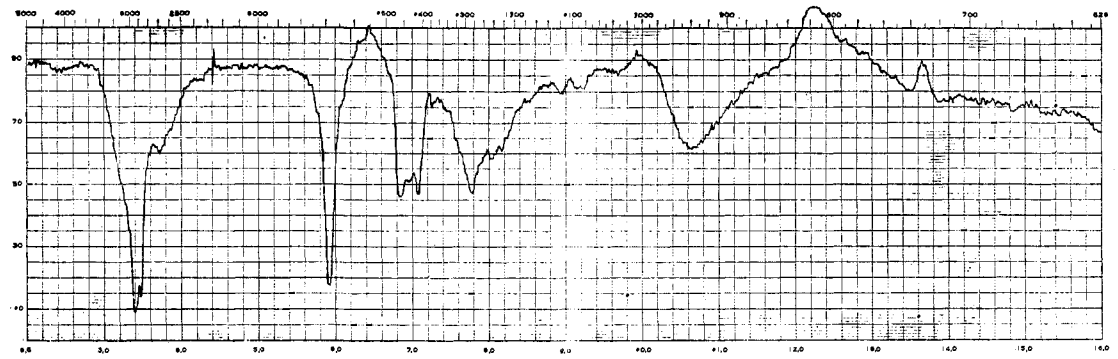

Fig. 1.—Infrared absorption curve for oleic acid.

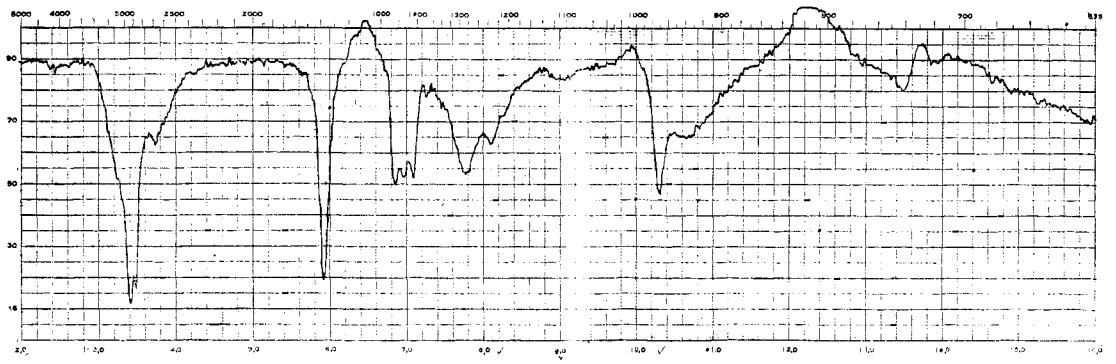

Fig. 2.—Infrared absorption curve for vaccenic acid.

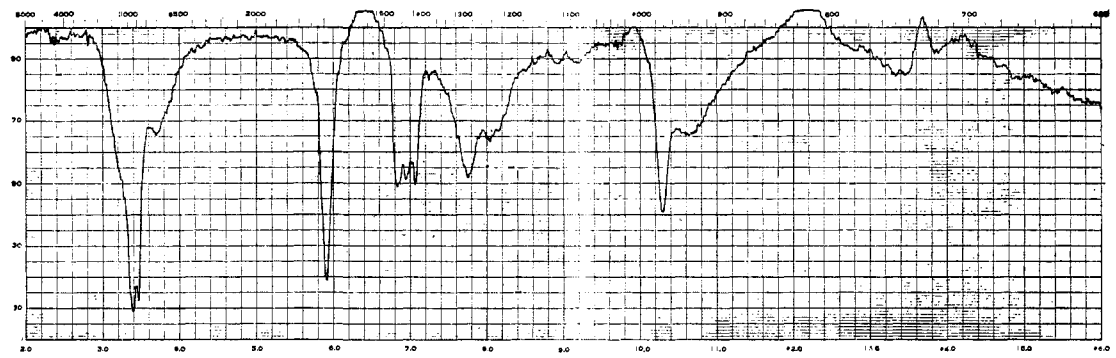

Fig. 3.—Infrared absorption curve for elaidic acid.

Preparation of Oleic and Elaidic Acids.—The oleic acid was prepared by fractional distillation and low-temperature crystallization of the methyl esters of the fatty acids of olive oil. Subsequent saponification of the highly purified methyl oleate and fractional distillation of the oleic acid gave a product of iodine value 89.8 (calcd. 89.9) containing a trace of dienoic acid. The purity of the oleic acid was comparable to the oleic acid prepared for use in the synthesis of glycerides as previously reported.[9]

Elaidic acid, m. p. 44.5°, I. V. 89.7, was prepared from the oleic acid by elaidinization according to the method of Lyutenberg.[10]

Infrared Spectra.—Infrared absorption curves for the vaccenic, oleic and elaidic acids were obtained using a Baird infrared spectrophotometer.[11] Solution of the respective fatty acids (10%) in carbon tetrachloride using 0.01-cm. cells was found satisfactory for measurement of the infrared absorption.

Discussion of Results

Essentially all of the evidence presented by Bertram in support of his conclusion that vaccenic acid is of *trans* configuration was based on a comparison of its freezing point and the constant K of van der Steur with those of oleic and elaidic acids, and the fact that on elaidinization the original vaccenic acid was recovered unchanged. Al-

(9) Daubert, Fricke and Longenecker, THIS JOURNAL, **65**, 2142 (1943).

(10) Lyutenberg, *Fettchem. Umschau*, **42**, 89 (1935).

(11) The coöperation of Dr. Harold Klug and Dr. A. L. Marston of the Mellon Institute for their assistance in obtaining the infrared absorption curves is gratefully acknowledged.

though all of the above evidence certainly seemed to support the conclusions of Bertram,[2] it was felt that additional proof of the configuration of vaccenic acid could be obtained by infrared absorption measurements in comparison with oleic and elaidic acids. The infrared absorption curves for oleic, elaidic and vaccenic acids, respectively, are given in Figs. 1, 2, and 3. On the assumption that oleic acid is *cis* and elaidic acid *trans*, it may be seen from the general similarity of the infrared patterns of elaidic and vaccenic acids that the latter is probably of *trans* configuration. Comparison of the curves for the three fatty acids in the region of wave length 3 to 7.5 μ shows a striking similarity. The minimum at 10.25 μ in the pattern for elaidic acid is also present in the vaccenic acid pattern, but absent for oleic acid. However, the doublets in the region of 8.8 to 9.2 μ present in the oleic acid curves are absent in the infrared pattern for vaccenic acid. Perhaps this may be attributed to the difference in the position of the double bond. Because of light scattering, no particular significance is attributed to small differences in the region 13 to 16 μ.

Although the vaccenic acid is probably of greater purity than the acid isolated by Bertram, the magnitude of the melting point in comparison with that reported by Bertram is somewhat surprising.

X-Ray diffraction analysis of the vaccenic acid is in progress and the results of this study will be reported in a separate communication.

In view of the pronounced similarity in infrared absorption of elaidic and vaccenic acids, additional evidence is therefore presented to confirm the *trans* configuration of vaccenic acid.

Infrared absorption measurements of highly purified vaccenic acid are reported in comparison with values for oleic and elaidic acids.

Acknowledgment.—The technical assistance of Miss Leatrice Klein in the preparation of the vaccenic acid is gratefully acknowledged.

Summary

Comparison of the infrared patterns of the three fatty acids seems to confirm the *trans* configuration of vaccenic acid.

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[CONTRIBUTION NO. 637 FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF PITTSBURGH]

Kinetics of the Fumarase System[1]

BY E. M. SCOTT AND RUTH POWELL

Dakin[2] in 1922 showed that an enzyme, fumarase, catalyzed the equilibrium reaction

$$\text{fumarate} + H_2O \rightleftharpoons \text{l-malate}$$

Clutterbuck[2a] found that phosphate affected both the solubility of the enzyme and the rate of the reaction. Borsook and Schott[3] studied the equilibrium and calculated the heat of formation of *l*-malic acid. It was reported by Ionescu, *et al.*,[4] that the *p*H of optimum activity of the enzyme with *l*-malate as substrate was more alkaline than that with fumarate. Jacobsohn[5] found that while the reaction with both substrates appeared to follow the first-order course, the well-known relation of the first-order reaction constants to the equilibrium constant

$$K_1/K_2 = K_{eq.} \qquad (1)$$

did not apply.

The present investigation was designed to test Jacobsohn's observations and to determine why equation (1) did not hold. In contrast to the experiments cited above, highly purified enzyme prepared according to the crystallization procedure of Laki and Laki[6] was used in our investigations.

A titrimetric determination of fumarate similar to the method of Straub[6a] was found to be much more convenient and somewhat more accurate than the polarimetric methods used in earlier kinetic studies.[2a,4,5]

Experimental

Eastman Kodak Co. fumaric acid was recrystallized from water, dissolved and neutralized as one substrate; Eastman *l*-malic acid was dissolved and neutralized to provide the other. Purity of the substrates for present purposes was established by the following evidence: (1) Both acids gave correct neutral equivalents; (2) *l*-malic acid gave no reaction with permanganate under the conditions stated below; (3) both substrates gave the same equilibrium concentration of fumarate; and (4) no evidence of inhibition by either substrate was found (*vide infra*, "Effect of Substrates on Stability").

The enzyme used was an amorphous fraction, obtained after crystallization of the protein described by Laki and Laki. This preparation had about three times as much activity per unit protein N as did the crystals. The initial rate of hydration of fumarate at *p*H 7.29 and 30° by this enzyme was 0.015 mole/sec. g. of protein N.

Unless otherwise indicated, the enzyme tests were run in 10 ml. of a solution containing phosphate buffer (*p*H 7.29, ionic strength 0.2), 0.1 *M* sodium fumarate or *l*-malate, and enzyme to give a protein N concentration of 4.2 p. p. m. One ml. samples were removed, added to 10 ml. of water containing 0.5 ml. of concentrated

(1) Aided by grants of the National Institute of Health and the Buhl Foundation. Presented at the 111th Meeting of the American Chemical Society, April 14–18, 1947.

(2) Dakin, *J. Biol. Chem.*, **52**, 183 (1922).

(2a) Clutterbuck, *Biochem. J.*, **22**, 1193 (1928).

(3) Borsook and Schott, *J. Biol. Chem.*, **92**, 559 (1931).

(4) Ionescu, Stanciu and Radalescu, *Ber.*, **72B**, 1949 (1939).

(5) Jacobsohn, *Biochem. Z.*, **254**, 112 (1932); **274**, 167 (1934).

(6) Laki and Laki, *Enzymologia*, **9**, 139 (1941).

(6a) Straub, *Z. physiol. Chem.*, **236**, 43 (1935).