The Dehydrogenation of All-*cis*-5, 11, 14-Eicosatrienoic Acid to Arachidonic Acid

By Toru TAKAGI

(Received March 5, 1965)

Early investigators have indicated, as a result of studying the feeding of linoleate concentrates to rats, that linoleate is transformed to arachidonate.[1] The transformation was confirmed by Mead and his co-workers using ^{14}C-labeled linoleate.[2] The transformations of all-*cis*-6,9,12-octadecatrienoic acid[3] and all-*cis*-8,11,14-eicosatrienoic acid[4] to arachidonic acid were also confirmed by Mead and his co-workers.

Recently the present author reported that the unique fatty acid of *Podocarpus nagi* seed oil may be characterized as all-*cis*-5,11,14-eicosatrienoic acid.[5] The all-*cis*-acid was also found in *Ginko biloba*[6] and pinewood and tall oil.[7] The dehydrogenation of this acid to arachidonic acid in animals could be expected, because the structure of the acid differed from that of arachidonic acid only in lacking the double bond in the 8-position. Therefore, the metabolism of the eicosatrienoate has been investigated in our laboratory. This paper will present the results of the feeding experiments; an analysis of the fatty acid composition will follow.

Results and Discussion

Twenty male rat weanlings (20 days old) of the Wister strain were kept on a basic fat-free-diet[8] obtained from the Nutrition Biochemical Co. After 100 days feeding, a gas chromatographic analysis of the fatty acids in the rat livers showed the presence of less than 1.1% linoleate and 2.0% arachidonate respectively of the total of the fatty acids. The rats were then divided into three groups of five rats in each. One gram portions of ethyl linoleate and ethyl all-*cis*-5,11,14-eicosatrienoate were fed daily intragastrically, by means of a syringe equipped with a round-end needle, to the first group and the second, respectivly. No fat was given to the third group. The fat-free-diet was fed to each group continuously. After 5 days, the rats were killed and the livers were quickly removed and immediately frozen. The fatty acid compositions of the liver lipids analyzed by the gas chromatographic method are given in Table I. A "shorthand designation" of the structure of each acid is included in the table; e.g., 18:2 is a C-18 acid with 2 double bonds, and 18:0 a saturated

1) L. C. A. Nunn and I. Smedley-MacLean, *Biochem. J.*, **32**, 2178 (1938); I. Smedley-MacLean and E. M. Hume, ibid., **35**, 990 (1941).
2) J. F. Mead, G. Steinberg and D. R. Howton, *J. Biol. Chem.*, **205**, 683 (1953).
3) J. F. Mead and D. R. Howton, ibid., **229**, 575 (1957).
4) D. R. Howton and J. F. Mead, ibid., **235**, 3380 (1960).
5) T. Takagi, *J. Am. Oil Chem. Soc.*, **41**, 516 (1964).
6) J. L. Gellerman and H. Shlenk, *Experientia*, **19** (19), 522 (1963).
7) T. Lehtinen, E. Elomaa and J. Alhojarvi, *Suomen. Kemistilehti B.*, **36** (7—8), 124 (1963); *Chem. Abstr.*, **57**, 9649 (1963).
8) J. G. Wooley and W. H. Sebrell, *J. Nut.*, **29**, 191 (1945).

TABLE I. FATTY ACID COMPOSITIONS OF LIVER LIPIDS

No. of rat group	I	II	III
Dietary ester	Linoleate	5,11,14-Eicosatrienoate	None
Fatty acids found in liver lipids			
16 : 0	22.7±2.0*	25.4±0.2	25.6±2.0
16 : 1	5.3±0.5	6.5±1.0	14.4±0.5
18 : 0	17.7±1.2	15.8±0.9	10.4±1.4
18 : 1	25.5±2.1	25.1±3.5	40.6±1.5
18 : 2	14.2±0.9	2.4±0.3	1.0±0.1
20 : 3	2.6±0.9	15.9±1.5	6.2±1.2
20 : 4	12.2±0.5	9.0±1.6	1.8±0.2

* Standard deviation

** Small peaks corresponding those of myristate and others were disregarded in the calculation of the compositions.

C-18 acid. Each acid shown in the table was identified by internal standards. The low level of linoleate (18:2) and arachidonate (20:4) and the high level of eicosatrienoate in the fatty acid from the fat-free-diet group were consistent with the results of metabolic studies of the fatty acids reported by Mead and his co-workers.[9] The linoleate percentage was affected only to a slight degree by the dietary 5,11,14-eicosatrienoate, while it was significantly elevated by the dietary linoleate. On the contrary, the eicosatrienoate percentage was significantly elevated by the dietary 5,11,14-eicosatrienoate, while it was slightly decreased by the dietary linoleate. In spite of such differences, the arachidonate percentages were elevated in both cases. It is plausible that the dehydrogenation of 5,11,14-eicosatrienoate at the 8-position directly produced arachidonic acid in this experiment, because there was probably no other source for arachidonic acid except such dehydrogenation.

As the arachidonic acid detected by gas chromatography was identified only by the retention time, a structural confirmation was sought by a spectrophotometric analysis of alkali-isomerized products and by a gas chromatographic analysis of the degraded products. The eicosatrienoate and the eicosatetraenoate were therefore isolated from the liver lipids of rats fed with 5,11,14-eicosatrienoate by elution chromatography using silver-nitrate impregnated silicic acid as the stationary phase. The specific extinction coefficents at the maxima in the spectrophotometric absorption of the alkali-isomerized products of the eicosatrienoic acid and the eicosatetraenoic acid shown in Table II. The accordance of the specific extinction coefficients of the alkali-isomerized eicosatetraenoic acid with those of the alkali-isomerized arachidonic acid indicates that the greater part of the double bond in the

9) A. J. Fulco and J. F. Mead, *J. Biol. Chem.*, **234**, 1411 (1959).

TABLE II. SPECTROPHOTOMETRIC ABSORPTION OF ALKALI-ISOMERIZED PRODUCTS OF THE ACIDS

Wavelength mμ	Specific extinction coefficient		
	Eicosatrienoic Found	Eicosatetraenoic	
		Found	Reported*
233	76.5	45.2	39.7
268	13.0	49.0	48.2
316	0	55.8	60.6

* Reported data for arachidonic acid in Ref. 14.

eicosatetraenoate belongs to the divinyl methylene system, for a double bond separated by more than 2 carbons can not be isomerized to the conjugated position with an adjacent double bond by alkali treatment. The oxidation of the eicosatrienoate by the periodate-permanganate reagent[10] gave mainly glutaric acid (96%) indicating the insertion of five carbon atoms between the carboxyl group and the first double bond in the eicosatetraenoate. The eicosatetraenoate fraction did not indicate the peak of a trans double bond in infrared spectrophotometric analysis. We may conclude, then, that the eicosatetraenoic acid from the liver lipids of rats of group II consists chiefly of arachidonic acid.

The data in Table II shows that more than 80% of the eicosatrienoate was changed to conjugated diene, not triene, by alkali isomerization; these data support the idea that the triene consisted chiefly unchanged 5,11,14-eicosatrienoate.

Further studies using the isotopic tracer technique are being planned in this laboratory in order to get direct evidence for the dehydrogenation and reliable knowledge concerning the effect of 5,11,14-eicosatrienoic acid as a new essential fatty acid.

Experimental

Materials.—*Ethyl All-cis-5,11,14-Eicosatrienoic Acid.*—The ether extract (1.4 kg.) from *Podocarpus nagi* seeds was saponified with 4 l. of 10% ethanolic potassium hydroxide, and the free acids were esterified by refluxing them with 4 l. of 1% sulfuric acid - ethanol for 1 hr. The ethyl ester (1362 g.) was fractionally distilled through a glass column packed with stainless steel helices (1.5 cm. in diameter, 1 m. long) and with a heated jacket under 0.5—1 mmHg. Each fraction was analyzed by gas chromatography; a C-20 fraction (251 g.) containing less than 0.5% C-18 acid was collected. Further fractionation at 0——20°C by the urea adduct method,[5] followed by distillation, yielded 70 g. of the eicosatrienoate, which was found to be 99.6% pure by gas chromatography.

Ethyl Linoleate. — Linoleate was prepared from safflower oil by the urea adduct method.[11] A gas

10) E. von Rudloff, *Can. J. Chem.*, **34**, 1413 (1956); C. G. Youngs, *J. Am. Oil Chem. Soc.*, **38**, 62 (1961).

11) J. G. Keppler, S. Sparreboom, J. B. A. Strorick and J. D. von Mikusch, *J. Am. Oil Chem. Soc.*, **36**, 38 (1959); A. R. Johnson and G. M. Ali, ibid., **38**, 453 (1961).

chromatographic analysis of the ester exhibited no peak other than that of linoleate.

The Analysis of Fatty Acids.—The livers were homogenized and extracted with chloroform-methanol (2:1 v/v) according to Folch's method.[12] The lipid extracted was then saponified by refluxing it for 1 hr. with 10% ethanolic potassium hydroxide. The fatty acids obtained from the saponified mixture were esterified by refluxing for 1 hr. with a 1% sulfuric acid - methanol solution. The esters obtained were then analyzed by gas chromatography using a stainless steel column, 6 min. in diameter and 2.25 m. long, packed with diethyleneglycol succinate-Celite 545 (60—80 mesh) (1 : 5 w/w) and with a hydrogen-flame-ionization detector. The remaining esters were separated according to column chromatography by the de Vries method.[13] The adsorbent was prepared by impregnating 100 g. of silicic acid with 200 ml. of 50% of an aqueous solution of silver nitrate. It was then filtered to remove the excess of the solution and activated at 120°C for 16 hr. Twenty grams of the adsorbent was packed into the glass tube using hexane and a hexane solution of the methyl ester (300 mg.) was added to the column. The sample was fractionated by step-by-step elution with 0—100% diethyl ether - hexane, and then the eicosatrienoate and the eicosatetraenoate were collected from the eluates. Their purities were found to be higher than 97% by gas chromatography. A portion was then isomerized by heating it with 11 g. of potassium hydroxide-ethyleneglycol for 15 min., after which the methanol solution of the products was subjected to spectrophotometric measurements according to the Herb method.[14] The other portion was oxidized by the periodate-permanganate method.[10] Ten milligrams of the sample in 20 ml. of *t*-butanol was added to 13.5 ml. of the periodate-permanganate reagent, which contained sodium periodate (284 mg.), potassium permanganate (1.10 mg.) and potassium carbonate (16.7 mg.), and the solution was refluxed for 1 hr. After the excess oxidizing reagent and *t*-butanol had been removed, the products were acidified and extracted with ether. The methyl esters were then analyzed by gas chromatography.

12) J. Folch, N. Lees and G. H. Sloane-Stanley, *J. Biol. Chem.*, **226**, 497 (1957).
13) B. De Vries, *Chem. & Ind.*, **1962**, 1049; *J. Am. Oil Chem. Soc.*, **40**, 184 (1963).
14) S. F. Herb and R. W. Riemenschneider, *Anal. Chem.*, **25**, 953 (1953).

Summary

Ethyl 5,11,14-eicosatrienoate has been fed to fat-deficient rats. The gas chromatographic analysis of the fatty acids in the liver lipids and the alkali isomerization and oxidation scission of the eicosatetraenoate separated from the fatty acid has given evidence for the transformation of all-*cis*-5,11,14-eicosatrienoate to arachidonate.

The author wishes to thank Professor Emeritus Yoshiyuki Toyama of this university; Mr. Yoshito Koyama of the Food Research Institute, Aichi Prefecture for providing *Podocarpus nagi* seed oil; the Ono Pharmaceutical Co. for its financial support and for the sending an experimental assistant, and Dr. Kazuo Fukuzumi for his discussions.

Department of Applied Chemistry
Faculty of Engineering
Nagoya University
Chikusa-ku, Nagoya