9. Dymock, W., C. J. H. Warden, and D. Hooper, "Pharmacographia Indica," Vol. 2, Trübner & Co., London, p. 241 (1891).
10. Kesava-Menon, A., J. Soc. Chem. Ind., *29*, 1428 (1910).
11. Chopra, R. N., "Indigenous Drugs of India," The Art Press, Calcutta, p. 409 (1933).
12. Chopra, R. N., N. N. Ghosh, and A. K. Mukerji, Indian J. Med. Research, *22*, 183 (1934).
13. Majumdar, D. N., Indian J. Pharm., *5*, 61 (1943).
14. Nadkarni, K. M., "The Indian Materia Medica," Bombay, p. 883 (1927).
15. Caius, J. F., and K. S. Mhaskar, Indian J. Med. Res., *11*, 353 (1923).
16. Kirtikar, K. R., and B. D. Basu, "Indian Medicinal Plants," Indian Press, Allahabad, Part I, p. 670; Part III, Plate No. 515A (1918); also 2nd ed., p. 1325 (1933).
17. Bhaduri, K., Proc. Chem. Soc. (London), *28*, 53 (1912).
18. Vidyarthi, N. L., Proc. Ind. Sci. Congress, Sect. III, 79 (1940).
19. Vidyarthi, N. L., and M. V. Mallya, J. Indian Chem. Soc., *16*, 479 (1939).
20. Vidyarthi, N. L., Patna Univ. J., *1*, 51 (1945).
21. Gunstone, F. D., J. Chem. Soc., 1611 (1954).
22. Smith, C. R., Jr., K. F. Koch, and I. A. Wolff, JAOCS, *36*, 219 (1959).
23. Morris, L. J., "The Absolute Configuration of *cis*-12:13-Epoxyoleic Acid from Vernonia Oil," Meeting of the A.O.C.S., St. Louis, Mo., May 1–3, 1961.
24. Morris, L. J., H. Hayes, and R. T. Holman, JAOCS, *38*, 316 (1961).
25. Earle, F. R., I. A. Wolff, and Q. Jones, *Ibid.*, *37*, 254 (1960).
26. Van Etten, C. H., R. W. Miller, I. A. Wolff, and Q. Jones, J. Agr. & Food Chem., *9*, 79 (1961).
27. Miwa, T. K., F. R. Earle, G. C. Miwa, and I. A. Wolff, "Biosynthesis of Epoxyoleic Acid in Maturing *Vernonia anthelmintica* Seeds. Dihydroxyoleic Acid—A Precursor." Meeting of American Chemical Society, St. Louis, Mo., March 21–30, 1961.
28. Riser, G. R., J. R. Hunter, J. S. Ard, and L. P. Witnauer, JAOCS (to be published).
29. Bharucha, K. E., and F. D. Gunstone, J. Sci. Food Agr., *7*, 606 (1956).
30. Chisholm, M. J., and C. Y. Hopkins, Can. J. Chem., *35*, 358 (1957).
31. Hopkins, C. Y. and M. J. Chisholm, JAOCS, *35*, 96 (1959).
32. Hopkins, C. Y., and M. J. Chisholm, *Ibid.*, *37*, 682 (1960).
33. Smith, C. R., Jr., M. O. Bagby, R. L. Lohmar, C. A. Glass, and I. A. Wolff, J. Org. Chem., *25*, 218 (1960).
34. Vioque, E., L. J. Morris, and R. T. Holman, JAOCS, *38*, 489 (1961).
35. Hopkins, C. Y., and H. J. Bernstein, Can. J. Chem., *37*, 775 (1959).
36. Gunstone, F. D., and P. J. Sykes, J. Sci. Food and Agr., *12*, 115 (1961).
37. Smith, C. R., Jr., K. F. Koch, and I. A. Wolff, Chem. and Ind., 259 (1959).
38. Morris, L. J., R. T. Holman, and K. Fontell, J. Lipid Res., *2*, 68 (1961).
39. Swern, D., and G. B. Dickel, J. Am. Chem. Soc., *76*, 1957 (1954).
40. Pigulevskii, G. V., and I. N. Naidenova, Zhur. Obshchei. Khim., *28*, 234 (1958).
41. Osbond, J. M., Proc. Chem. Soc., 221 (1960) and 5270 (1961).
42. Tulloch, A. P., B. M. Craig, and G. A. Ledingham, Can J. Microbiology, *5*, 485 (1959).
43. Tulloch, A. P., Can. J. Chem., *38*, 204 (1940).
44. Chisholm, M. J., and C. Y. Hopkins, Chem. and Ind., 1154 (1959).
45. Gunstone, F. D., and L. J. Morris, J. Chem. Soc., 2127 (1959).
46. Earle, F. R., E. H. Melvin, L. H. Mason, C. H. Van Etten, and I. A. Wolff, JAOCS, *36*, 304 (1959).
47. Morris, L. J., and R. T. Holman, J. Lipid Res., *2*, 77 (1961).
48. Durbetaki, A. J., Anal. Chem., *28*, 2000 (1956).
49. Scott, W. E., C. F. Krewson, and R. W. Riemenschneider, "*Vernonia anthelmintica* (L.) Willd. Enzyme Activity in the Seed," Meeting of the A.O.C.S., Chicago, Ill., Oct. 30–Nov. 1, 1961.
50. Radlove, S. B., R. V. Madrigal, and R. Slutkin, JAOCS, *37*, 570 (1960).
51. Daubert, B. F., and E. S. Lutton, J. Am. Chem. Soc., *69*, 1449 (1947).

[Received February 1, 1962]

The Nature of the C_{18} Polyethenoic Fatty Acids of Butter Fat[1,2]

K. SAMBASIVARAO and J. B. BROWN, Department of Physiological Chemistry, The Ohio State University, Columbus, Ohio

Abstract

Methyl C_{18} polyenoate concentrates were prepared from two samples of butter fat by low temperature crystallization and fractional distillation. The concentrates were fractionated on a silicic acid column and the resulting fractions were analyzed by ultraviolet and infrared spectrophotometric methods. About 42 and 30%, respectively, of the non-conjugated dienoate in the two samples were shown to have the *cis,trans* configuration. Fractions rich in dienoate and trienoate were prepared from the C_{18} polyene concentrates by silicic acid chromatography and the nature of these acids was studied by bromination, lipoxidase enzyme methods, and by alkali isomerization for varying periods of time. About 65 and 73%, respectively, of the non-conjugated dienoate in the two samples investigated were found to consist of linoleic acid while 79 and 71% of the trienoate were linolenic acid. Linoleic and linolenic acids were identified by preparing the characteristic tetra- and hexabromostearic acids. A *trans,trans* isomer of linoleic acid does not seem to be present in butter fat. A major proportion of the non-conjugated dienoic acid other than linoleic acid was found to have widely separated double bonds with *cis,trans* configuration. Occurrences of a C_{15} saturated acid, a branched-chain C_{17} saturated acid, and a heptadecenoic acid were indicated by gas chromatography.

[1] From a dissertation submitted by K. Sambasivarao to the Ohio State University in partial fulfillment of the requirements for the Ph.D. degree, June, 1960.

[2] This work was supported in part by a grant from the Ohio State University Development Fund to the Institute of Nutrition and Food Technology and by several teaching assistantships from the Department of Physiological Chemistry.

Introduction

BUTTER FAT is one the most complex of all animal fats, more than 25 fatty acids having been identified. However, the nature of the C_{18} polyethenoic acids, particularly the dienoic, has not been fully characterized. Eckstein (1) reported small amounts of linoleic and linolenic acids. Many workers were unable to find any linoleic acid (2–8), because of failure to isolate either a petroleum ether-insoluble tetrabromide or the tetrahydroxystearic acids, which are characteristic derivatives of linoleic acid. Green and Hilditch (4) and Brown (9) suggested that the octadecadienoic acids of butter fat are principally composed of geometric (*cis,trans* or *trans,cis*) isomers of linoleic acid. Later White and Brown (10) reported the isolation of petroleum ether-insoluble tetrabromides from C_{18} polyenoic acid concentrates of butter fat. On the basis of tetrabromide yield they reported that 66 and 75% of the octadecadienoic acids were linoleic acid. Shorland (8) reported that, while the octadecatrienoic acid is composed entirely of linolenic acid, the octadecadienoic acids from the New Zealand butter fat are principally composed of isomers of linoleic acid. In view of these divergent reports, it seemed important to reinvestigate the nature of the C_{18} polyethenoic acids of butter fat.

Experimental

Materials. The two specimens of summer butter fat used in the present investigation were kindly supplied by the Pickaway Dairy Co-operative Association Inc., Circleville, Ohio and the Department of Dairy Technology of the Ohio State University. One of these was received in the summer of 1956 (this will hereafter be referred to as SB-56) while the

TABLE I

Results of Spectrophotometric Analysis of Methyl Esters and C_{18} Polyene Concentrates of Butter Fats

Sample	I.V.	Preconj. diene, %	Non-conjugated, %		
			Diene	Triene	Tetraene
Methyl esters (SB-56)	38.9	1.3	1.4	0.7	0.6
C_{18} Polyene conc. (C)	165.8	5.6	28.3	21.3	0.4
Methyl esters (SB-58)	35.3	0.9	1.3	0.6	0.5
C_{18} Polyene conc. (C')	153.2	4.6	27.8	15.8	0.2

other was procured in the summer of 1958 (designated as SB-58). Immediately on receiving the specimens, they were melted, most of the water siphoned out, dehydrated under reduced pressure and filtered. They were stored at −20C until used.

Analytical Methods. Iodine values, saponification equivalents and U.V. analyses before and after alkali isomerization were carried out according to the Official Methods of the A.O.C.S. Linoleic and linolenic acids of selected samples were estimated by the bromination procedures of White and Brown (11,12). All-*cis* single methylene-interrupted polyunsaturated fatty acids of certain samples were determined by an enzymatic procedure (13). The *trans* acids were determined by an I.R. spectrophotometric method (14). Extinction coefficients of methyl esters used as reference compounds in the calculation of *trans* acids are as follows: stearate, 0.027; oleate, 0.038; elaidate, 0.428; linoleate, 0.060; linolenate, 0.065. *Trans* acid contents were calculated using the following equation which was developed by Shreve *et al.* (14).

Trans component, weight % = 100 $(K_{ob} - K_cY - K_sZ)/(K_t - K_c)$ where K_{ob} = observed extinction coefficient for the mixture =

$$\frac{\text{O.D. at } 10.36\ \mu}{(\text{concn. of solute g/l})(\text{cell thickness, cm})}$$

K_c = extinction coefficient of methyl oleate
K_t = extinction coefficient of methyl elaidate
K_s = extinction coefficient of methyl stearate
Y = total weight fraction of octadecenoate
Z = total weight fraction of stearate

The above equation was modified in certain cases where there were no saturated esters present. In fractions composed of octadecenoate and octadecadienoate, K_c and K_s are the extinction coefficients of linoleate and oleate, respectively, while Y and Z are the total weight fractions of octadecadienoate and octadecenoate. In fractions composed of octadecadienoate and octadecatrienoate, K_c and K_s are the E.C. of linoleate and linolenate, respectively, and Y and Z are the total weight fractions of octadecadienoate and octadecatrienoate.

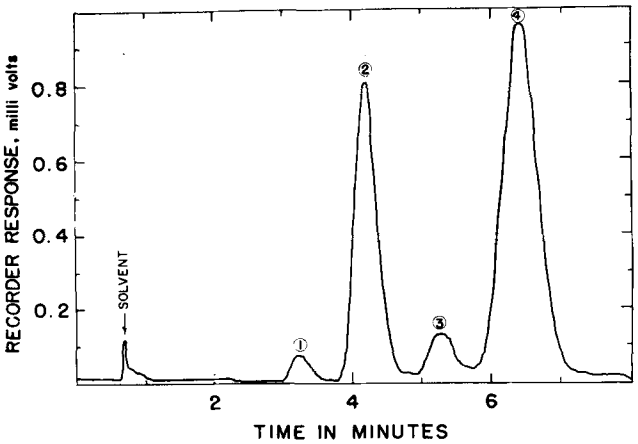

FIG. 1. Gas chromatographic separation of fraction 1 (Table II). Peak identification: 1. C_{15} ester, 2. C_{17} branched-chain ester, 3. heptadecenoate, 4. octadecenoate. Column 10 ft by ¼ inch packed with ethylene glycol succinate supported on Chromosorb W (Wilkens Instrument & Research Inc., California); column temperature 206C, helium pressure 15 psig, sample size 0.3 μl.

Preparation of Concentrates of C_{18} Polyene Esters from Butter Fats. Methyl esters were prepared from the two samples of butter fat (SB-56 and SB-58) by transesterification with methanol using hydrogen chloride gas as catalyst. Unwanted saturated esters that are present in large quantities in the methyl esters were first removed by a preliminary crystallization of methyl esters at −35C using methanol as the solvent. The esters from the filtrates were then fractionally distilled at low pressure to obtain the C_{18} esters. These C_{18} esters were then successively crystallized from methanol at −40 and −65C. Over 90% of the C_{18} polyene esters was concentrated into the final −65C filtrate fractions. The results of U.V. spectrophotometric analysis of the concentrates as well as the original esters are given in Table I.

Chromatographic Separation of C_{18} Polyene Concentrates and Analysis of the Different Fractions. The C_{18} polyene concentrates (C and C') were fractionated on a silicic acid column following the procedure of Riemenschneider *et al.* (15). The adsorbent mixture, which consisted of 4 parts of silicic acid and 1 part filter aid, was heated to 75C for 4 hr prior to packing in the column. The column (3.4 cm x 50 cm) was packed with 150 g of the adsorbent mixture in the form of a slurry in redistilled petroleum ether (bp

TABLE II

Analytical Results of the Various Fractions Obtained by Silicic Acid Chromatography of C' Esters

Fraction No.	Eluate vol. l	Wt. fraction, g	I.V. (Wij's)	I.V. (corrected)[a]	Monoene,%[b]	Preconj. diene, %	Non-conj. diene, % (U.V.)	Non-conj. diene (calcd.)[b]		Non-conj. triene, % (U.V.)	$K_{10.36\mu}$	*Trans*, %	*Trans* monoene, %[c]	*Trans* diene		Non-conj. diene as *trans*, %
								%	Wt, g					%	Wt. g.	
1[d]	2.0	0.98	59.5	59.8	68.9	0.4	0.1	0.1	0.00	0.0	0.052	4.6	4.6	0.0	0.00	0.0
2	1.15	1.01	102.3	103.6	79.3	1.5	11.2	19.2	0.19	0.0	0.110	18.5	5.3	13.2	0.13	68.8
3	1.0	0.73	118.1	120.7	59.6	3.0	23.9	37.4	0.27	0.0	0.117	19.0	4.0	15.0	0.11	40.1
4	1.6	0.92	139.8	143.9	32.8	4.7	38.0	62.5	0.58	0.0	0.128	20.7	2.2	18.5	0.17	29.6
5	2.0	0.75	152.6	158.2	15.2	6.4	50.6	78.4	0.59	0.0	0.136	21.5	1.0	20.5	0.15	26.1
6	3.0	0.71	154.5	160.6	13.6	7.0	54.1	79.4	0.56	0.0	0.123	17.9	0.9	17.0	0.12	21.4
7	5.2	0.86	158.1	164.5	9.1	7.4	50.2	83.5	0.72	0.0	0.121	17.1	0.6	16.5	0.14	19.8
8	1.5[e]	1.03	183.4	189.5	0.2	7.0	47.0	73.2	0.75	19.6	0.131	19.0	0.0	19.0	0.20	26.0
9[f]	1.1[e]	2.78	208.0	212.9	3.3	5.6	25.7	42.4	1.18	47.8	0.121	15.8	0.2	15.6	0.43	36.8
		9.77							4.84						1.45	

[a] Conjugated dienoic acids absorb only half the theoretical amounts of halogen (16) and the I.V. were corrected accordingly.
[b] Composition of fractions 2-7 was calculated from I.V. as a mixture of monoene and diene and fractions 8 and 9 as a mixture of monoene, diene, and triene.
[c] 6.7% of the monoene. For explanation see text.
[d] Contained 30.6% saturated esters.
[e] Fractions 8 and 9 were eluted with petroleum ether containing 1 and 10% ethyl ether, respectively.
[f] Contained 0.9% tetraene.

30–60C). The column was operated under nitrogen pressure (18 cm mercury). About 10 g of the concentrate dissolved in 100 ml P.E. was added to the column. Elutions were carried out first with P.E. and later with P.E. to which ethyl ether was added to hasten the elution of esters. The eluted fractions were analyzed by U.V. and I.R. spectrophotometric methods and the results obtained with one of the concentrates (C′) are described in Table II.

The progressive increment in the iodine values of the different fractions in Table II reflect the excellent fractionation achieved. Triene was absent from fractions 2–7 which have a high concentration of dienoate. Three conclusions drawn from the analytical results are discussed below.

The first conclusion concerned fraction 1 which contained 30.6% saturated components; this amounted to 3% of the concentrate (0.1% of the butter fat). The concentrate C′ is the fraction soluble at −65C and the saturated components are unlikely to be stearate or palmitate since both these esters are extremely insoluble at this temperature (17). In order to identify the saturated components, fraction 1 was further resolved by gas chromatography. The instrument used was an Aerograph A-90-C gas chromatograph (Wilkens Instrument & Research, Inc., Calif.) and the results are shown in Figure 1.

The peaks in Figure 1 were identified on the basis of relative retention times. Peak 1 with a retention time between those of myristate and palmitate was believed to be a C_{15} saturated ester which, however, comprised only a small proportion of the total saturated components. Shorland *et al.* (18) have reported the isolation of pentadecanoic acid from butter fat. Peak 2 eluted slightly earlier than that would be expected for a C_{17} saturated ester. Based on the observation that branched-chain esters elute before the corresponding straight-chain esters (19), peak 2 was believed to be a C_{17} branched-chain ester. Hansen and Shorland (20) reported the presence of two isomeric C_{17} branched-chain acids in New Zealand butter fat. Peak 3 corresponded to a C_{17} ester with one double bond which to our knowledge has not been previously reported. (Since writing this report Hansen *et al.* [Biochem. J. *77*, 64 (1960)] reported the isolation of a *cis*,9-heptadecenoic acid from butter fat.) Further identification of these esters was not attempted due to lack of reference compounds.

The second conclusion concerned fractions 2 through 7 which did not contain triene; hence a reasonable calculation of the composition of these fractions could be made from the iodine values as a mixture of monoene and diene esters. Considerable difference was found between the calculated percentages of non-conjugated dienoate and those obtained by alkali isomerization. For example, by I.V. fraction 7 contained 83.5% total non-conjugated dienoate, whereas by alkali isomerization only 50.2% was found. Thus about 33.3% was not conjugated by the usual conditions of isomerization. Only a part of this diene that is not conjugated represents a *cis,trans* isomer of linoleic acid, since fraction 7 contained only 16.5% *trans* diene and since a part of the *trans* diene will be conjugated under the conditions of isomerization employed (21). The remainder should therefore consist of positional isomers whose double bonds are separated by more than one methylene group. These have not been reported previously.

The last conclusion is concerned with the *trans* diene esters. The *trans* components in the various fractions in Table II were due to monoene, diene, and also possibly triene esters. The I.R. spectrophotometric method (14) does not distinguish between these acids, but estimates them together. The *trans* diene percentages, therefore, could be ascertained only after making several assumptions. Thus, a *trans,trans* isomer of linoleic acid was assumed to be absent in butter fat, an assumption which was in part validated by later work. It was further assumed that the preconjugated dienoate had the same I.R. extinction coefficient as linoleate. Fraction 1 contained only traces of diene and, therefore, all the *trans* content in this fraction must have been contributed by monoene only. Thus the *trans* monoene in fraction 1 amounts to 6.7% of the monoene. Consequently, it was assumed that the *trans* monoene in the rest of the fractions amounted to not more than 6.7% of the monoene. Actually there would probably be less since Scott *et al.* (22) have observed that *trans* components will be concentrated in the earlier fractions during chromatography. A similar observation was made with respect to *trans* diene as evidenced in the last column of Table II. It was further assumed in fractions 8 and 9 that the trienoate had an all-*cis* configuration and did not contribute to the total *trans* content. This assumption was not entirely valid as indicated in fractions 8 and 9 by the increasing percentages of non-conjugated dienoate that has *trans* configuration. As also found by Scott *et al.* (22), *trans* diene was concentrated in the earlier fractions and the percentage of non-conjugated diene having *trans* configuration decreased progressively. However, the percentages suddenly increased as triene began to elute which indicated that there may be some *trans* triene.

The percentage of *trans* diene in each fraction was

TABLE III

Preparation of Dienoate and Trienoate Concentrates by Silicic Acid Cromatography

Fraction No.	Eluate vol, l	Wt fraction, g	I.V. (Wij's)	I.V. (corrected)	Esters, weight %[b]						
					Saturated	Monoene	Preconj. diene	Non-conj. diene (U.V.)	Non-conj. diene (calc.)	Non-conj. triene	Non-conj. tetraene
Original C 10.0 g											
C-1	3.8	1.2	54.9	55.7	37.7	59.6	0.9	1.8	1.8	0.0	0.0
C-2	16.2	4.3	147.4	152.4	0.0	23.1	5.8	41.7	71.0	0.0	0.0
C-3	1.7[a]	4.3	208.3	213.2	0.0	4.6	5.7	30.6	44.2	44.7	0.8
		9.8									
Original C′ 10.0 g											
C′-1	2.4	1.4	60.2	60.3	29.7	70.2	0.1	0.0	0.0	0.0	0.0
C′-2	14.2	4.7	141.6	146.0	0.0	30.4	5.1	41.1	64.5	0.0	0.0
C′-3	1.4[a]	3.7	200.6	205.5	0.0	4.7	5.7	33.9	47.8	41.1	0.7
		9.8									

[a] Eluted with petroleum ether containing 10% ethyl ether.

[b] The compositions of the different fractions were calculated from their corrected I.V. after making the following assumptions. In fractions C-1 and C′-1, it was assumed that all the dienoate was estimated by the alkali isomerization method. The compositions of C-2 and C′-2 were calculated from the I.V. as a mixture of monoene and diene esters. In C-3 and C′-3, it was assumed that all the trienoate and tetraenoate were estimated by the alkali isomerization method.

TABLE IV
Estimation of Linoleic and Linolenic Acids by Bromination

Acids from	%			
	Linoleic by bromination	Non-conj. dienoic	Linolenic by bromination	Non-conj. trienoic (U.V.)
C-2	37	71	....	0.0
C'-2	38	65	....	0.0
C-3	....	....	35	45
C'-3	....	....	29	41

obtained by subtracting the percentage *trans* monoene from the total *trans*. When the *trans* dienoate in all the fractions was added, it amounted to 15% in C'. Thus about 30% of the non-conjugated dienoate had the *cis,trans* configuration. Again, it is to be stated that this value was obtained only after making several assumptions. Nevertheless, a considerable proportion (70%) of the non-conjugated dienoate had an all-*cis* configuration.

Similar results were obtained with the concentrate (C) from SB-56. The saturated components amounted to 0.2% of the butter fat. About 42% of the non-conjugated dienoate had the *trans* configuration.

Preparation of Dienoate and Trienoate Concentrates by Chromatography and Their Characterization by Various Methods. In Table II trienoate was absent from the fractions (2–7) containing a high concentration of dienoate, suggesting that concentrates of dienoate free of trienoate could be prepared. About 10 g each of the concentrates (C and C') were separated into three fractions on a similar column as shown in Table III.

Fractions C-2 and C'-2 contained a high concentration of dienoate while C-3 and C'-3 were rich in trienoate. The nature of the dienoic and trienoic acids in these fractions was studied by bromination, lipoxidase enzyme methods, and by alkali isomerization for varying periods.

Bromination Studies. White and Brown (11,12) described empirical methods for the estimation of linoleic and linolenic acids. The yields of P.E.-insoluble tetrabromides and ether-insoluble hexabromides were related to the percentages of linoleic and linolenic acids, respectively. Portions of the dienoate (C-2 and C'-2) and trienoate (C-3 and C'-3) concentrates of Table III were converted into fatty acids and brominated according to the procedures of White and Brown (11, 12) and the results are given in Table IV.

Contrary to a previous report (8) which stated that all the trienoate was linolenic acid, only 79 and 71%, respectively, of the trienoate from C and C' was linolenic acid, the remainder probably consisting of geometrical isomers. The ether-insoluble hexabromides were separately crystallized 6 times from xylene at room temperature when a crystalline product with a mp of 180–181C was obtained. The mp was not depressed when mixed with synthetic hexabromide. This is conclusive evidence for the presence of linolenic acid in butter fat. Likewise, the P.E.-insoluble tetrabromides were crystallized 4 times from pentane-ether (1:1 vol) at −20C to give a white crystalline product with a mp of 115–116C. No change in mp was observed when synthetic tetrabromide was added. This is considered as an absolute proof for the presence of linoleic acid.

Lipoxidase Enzyme Studies. MacGee (13) described an enzymatic procedure for the estimation of all-*cis* polyunsaturated fatty acids (PFA) in which the double bonds are separated by one methylene group. Any *trans* material is not a substrate for the enzyme

TABLE V
Results of Lipoxidase Enzyme Studies

Fraction	%		
	All-*cis* polyunsaturated fatty acid	Linoleic	Linolenic
C-2	39	39	0
C'-2	41	41	0
C-3	73	37[a]	35
C'-3	73	43[a]	29

[a] % PFA—% linolenic (Table IV)—% tetraene (Table III).

lipoxidase and this method thus affords a specific means for estimating linoleic and linolenic acids in the presence of their geometric isomers. This procedure does not distinguish between the different polyunsaturated fatty acids, but estimates them together. However, the only all-*cis* polyunsaturated fatty acid that is likely to be present in the dienoate concentrates (C-2 and C'-2) is linoleic acid. Both linoleic and linolenic acids are present in C-3 and C'-3. It is conceivable that positional isomers of linoleic acid with an all-*cis* configuration (e.g., 12,15-octadecadienoic) will be also estimated by this method, but acids of this type have not been reported in butter fat. Also the tetrabromides from these acids should be expected to be different from those obtained from linoleic acid. Since the mp of the tetrabromides obtained from butter fat did not change when mixed with synthetic tetrabromides (prepared from vegetable oils), it can be reasonably assumed that isomers of this type are not present in butter fat.

The dienoate and trienoate concentrates of Table III were analyzeed following MacGee's procedure and the results are shown in Table V.

The linoleic acid content of C-2 and C'-2 as determined by this method agreed (±3%) with those obtained by bromination method. It should be borne in mind that both these methods are only empirical. The percentages of linoleic acid in C-3 and C'-3 were obtained by subtracting the percentage linolenic (determined by bromination) acid and the small amount of tetraene present from the total polyunsaturated fatty acid. If the linoleic acid in both dienoate and trienoate concentrates is added, it amounted to 65% of the non-conjugated dienoate in C and 73% in the case of C', the remainder (35 and 27%) consisting of geometrical isomers. The later values are comparable to those obtained by I.R. method of analysis (42 and 30%) in which several assumptions were made.

Alkali Isomerization for Varying Periods. Jackson *et al.* (21) reported that the geometrical isomers of linoleic acid (*cis,trans* or *trans,cis* and *trans,trans*) are isomerized to conjugated acids at different rates than linoleic acid. These authors studied the isomerization rates of pure esters and developed three simultaneous equations for their estimation. In the absence of a *trans,trans* isomer, these equations are simplified into two equations which are as follows:

$$K_1 = 87.2x + 57.4y$$
$$K_2 = 88.7x + 80.4y$$

solving for x and y

$$x = 0.0419K_1 - 0.0299K_2$$
$$y = 0.0454K_2 - 0.0462K_1$$

TABLE VI
Alkali Isomerization of C-2 and C'-2 for Varying Periods

Fraction	Non-conj. diene, %	K_{232}			Linoleic, %	*Cis,trans* or *trans,cis*, %
		25 min	60 min	4 hr		
C-2	71	38.7	40.9	40.8	40	7
C'-2	65	39.6	41.1	41.4	43	4

TABLE VII

Approximate Composition of Octadecadiene in the Concentrates

	Percentage of total diene	
	C	C'
Preconjugated diene	10	9
Non-conjugated diene	90	91
Linoleic	59	66
Isomeric linoleic[a]	31	25
Total *trans* diene	38	27

[a] Includes both *cis,trans* or *trans,cis* isomer of linoleic acid and geometric isomers with widely separated double bonds.

where K_1 and K_2 are the observed specific extinction coefficients of the sample at 232 mμ following alkali isomerization for 25 and 60 min, respectively (after correcting for conjugation originally present); x and y are the weight fractions of linoleate and *cis,trans* or *trans,cis* isomer, respectively.

Fractions C-2 and C'-2 were isomerized for 25 min, 60 min, and 4 hr with 6.5% KOH in ethylene glycol following the procedure of Jackson *et al.* (21) and the results appear in Table VI.

As mentioned by Jackson *et al.* (21), the results are not quantitative. Nevertheless, two conclusions could be drawn from the results. A *trans,trans* isomer of linoleic acid is not present in amounts detectable by this method since the K_{232} values at 4 hr are essentially the same as at 60 min. Also contrary to previous reports (4,9), only a small amount of the isomers in these fractions consisted of *cis,trans* isomers of linoleic acid. The remainder of the isomers must consist of non-conjugated acids whose double bonds having *cis,trans* configuration are separated by more than one methylene group, since the specific extinction coefficient did not increase even after long periods of isomerization. The trienoate concentrates (C-3 and C'-3) could not be examined by this method since linolenic acid undergoes destruction during the long periods of isomerization (23–25).

Further Comments on the Nature of Octadecadienoic Acids. The presence of appreciable amounts of linoleic acid in butter fat has been reported only once (10) and has not since then been confirmed. The present work offers conclusive evidence for the presence of linoleic acid. On the basis of the results, the approximate composition of octadecadiene in the concentrates was calculated and is given in Table VII.

In explanation, the calculation of the composition of one of the fractions (C') may be described as follows: fraction C' contained 4.6% preconjugated diene (Table I) and 48% non-conjugated diene (obtained by adding the non-conjugated diene in all the fractions in Table II). These values were converted to percentage of total diene. About 73% of the non-conjugated diene was linoleic acid (from bromination and lipoxidase enzyme methods), the remainder of the non-conjugated diene being isomers of linoleic acid. The *trans* diene percentage represented 30% of the non-conjugated diene.

The *trans* diene percentages were slightly higher than those of isomeric linoleic acid. This was probably caused by a combination of two factors. The *trans* diene values are undoubtedly subject to error since several assumptions were made in their calculation. A major error is probably caused by assuming that all the *trans* content in fractions 8 and 9 was due to diene whereas in fact some triene seemed to have *trans* configuration. The percentages of linoleic acid were obtained by bromination and lipoxidase enzyme methods which are semi-quantitative. Actually the difference was amplified by expressing them as percentage of total diene. For example, in C' the total *trans* diene amounted to 15% whereas the percentage of isomeric linoleic acid could be calculated to be 13.

The isomeric linoleic acid is composed of *cis,trans* or *trans,cis* isomers of linoleic acid and geometrical isomers with widely separated double bonds, the former amounting to only a minor proportion of the total isomers. Isomers with widely separated double bonds should have a *cis,trans* type of configuration since the *cis,trans* isomer of linoleic acid as estimated by the procedure of Jackson *et al.* did not account for all the *trans* diene. It is possible, however, for one half the positional isomers to have a *trans,trans* configuration, the other half having an all-*cis* configuration.

Since a considerable proportion of the non-conjugated diene of butter fat consists of positional isomers of linoleic acid with widely separated double bonds, further work should be done to isolate these acids, to determine the position of the double bonds, and to study the nutritional significance, if any, of these acids.

Acknowledgments

The authors wish to thank J. I. Watters of the Department of Chemistry for the use of a Beckman IR-2 spectrophotometer. We also appreciate the help given by L. A. Horrocks in interpreting the results of gas chromatographic analysis.

REFERENCES

1. Eckstein, H. G., J. Biol. Chem., *103*, 135 (1933).
2. Bosworth, A. W. and J. B. Brown, *Ibid.*, *103*, 115 (1933).
3. Bosworth, A. W., and E. W. Sisson, *Ibid.*, *107*, 489 (1934).
4. Green, T. G., and T. P. Hilditch, Biochem. J., *29*, 1564 (1935).
5. Hilditch, T. P., and H. E. Longenecker, J. Biol. Chem., *122*, 497 (1938).
6. Hilditch, T. P., and H. Jasperson, J. Soc. Chem. Ind. (London), *58*, 241 (1939).
7. *Ibid.*, *60*, 305 (1941).
8. Shorland, F. B., Nature, *166*, 745 (1950).
9. Brown, J. B., Oil & Soap, *15*, 102 (1938).
10. White, M. F., and J. B. Brown, JAOCS, *26*, 385 (1949).
11. *Ibid.*, *29*, 292 (1952).
12. *Ibid.*, *26*, 133 (1949).
13. MacGee, J., Anal. Chem., *31*, 298 (1959).
14. Shreve, O. D., M. R. Heether, H. B. Knight, and D. Swern, Anal. Chem., *22*, 1261 (1950).
15. Riemenschneider, R. W., S. F. Herb, and P. L. Nichols, Jr., JAOCS, *26*, 371 (1949).
16. Markley, K. S., "Fatty Acids," Interscience Publishers Inc., p. 337, New York, 1947.
17. Sedgwick, R. S., C. W. Hoerr, and H. J. Harwood, J. Org. Chem., *17*, 327 (1952).
18. Shorland, F. B., T. Gerson, and R. P. Hansen, Biochem. J., *59*, 350 (1955).
19. Woodford, F. P., and C. M. van Gent, J. Lipid Research, *1*, 188 (1960).
20. Hansen, R. P., and F. B. Shorland, Biochem. J., *50*, 207 (1951).
21. Jackson, J. E., R. F. Paschke, W. Tolberg, H. W. Boyd, and D. H. Wheeler, JAOCS, *29*, 229 (1952).
22. Scott, W. E., S. F. Herb, P. Magidman, and R. W. Riemenschneider, J. Agr. Food Chem., *7*, 125 (1959).
23. Cornwell, D. G., R. H. Backderf, C. L. Wilson, and J. B. Brown, Arch. Biochem. Biophys., *46*, 364 (1953).
24. Mitchell, J. H., H. R. Kraybill, and F. P. Zscheile, Ind. Eng. Chem., Anal. Ed., *15*, 1 (1943).
25. Bradley, T. F., and D. Richardson, Ind. Eng. Chem., *34*, 237 (1942).

[Received December 22, 1961]

• *Erratum*

JAOCS, *39*, 278 (1962), MORREN ET AL.: CONTINUOUS ONE-STEP REFINING-WATER WASHING OF CRUDE COCONUT OIL. Delete line six of the introduction, first column. The first paragraph, second sentence should read:

"... Commercial installations have demonstrated good performance of these machines in continuous processes for degumming, alkali refining, and water washing of a variety of oils (2,3)."