TABLE VII

Fatty Acid Composition of Rat Epididymal Fat

	C_{12}	C_{14}	C_{16}	$C_{16:1}$	C_{18}	$C_{18:1}$	$C_{18:2}$	% Glycerine
Basic diet	....	1.5	34.4	18.1	1.0	35.4	9.6	10.7
Lard diet	....	0.7	28.2	6.2	1.7	52.5	10.8	10.5
Drewmulse 125–14	....	0.4	31.5	13.5	0.5	41.5	12.5	10.8
Drewmulse 125–20	....	0.4	33.4	11.7	0.3	42.2	12.0	10.2
Drewmulse 137–75	....	1.3	20.6	13.2	1.1	40.9	22.9	10.8
Drewmulse 137–76	....	1.6	29.6	12.0	2.0	40.2	14.7	11.1
Drewmulse 137–71	0.3	1.6	30.2	10.6	1.0	42.7	13.7	11.4
Drewmulse 137–72	0.2	1.1	29.0	9.5	2.7	46.1	11.4	10.6
Drewmulse 137–68	....	0.5	31.0	9.2	0.6	41.1	17.2	10.6
Drewmulse 137–69	....	1.1	29.2	11.2	1.7	43.5	13.5	11.0
Drewmulse 137–79	....	0.8	33.2	7.9	1.6	43.1	13.5	10.8

some spots which had been contributed by the test materials. No spots were discernible which did not appear to correspond with spots on the control fecal plates or on the test material plates.

Autopsies and Histopathology. Throughout the experimental period all animals had a normal appearance. However, during the course of the ad libitum feeding period diarrhea was observed in those groups fed the test materials. Autopsies performed at the end of the experiment and the histological examination of the liver, kidney and ileum revealed no abnormalities attributed to the consumption of any of the test materials.

Character of Depot Fat. The GLC analysis of the fatty acids of the extracted lipids from the epididymal fat pads and the per cent glycerine analyzed from the polyol portion of the extracted lipids show in Table VII. Results are in the range of glycerine and not that of the various polyglycerols. The glycerine content of the extracted lipid suggested that only triglycerides were present or that at least no appreciable amounts of polyglycerols were deposited.

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[Received July 31, 1963—Accepted March 9, 1964]

The Metabolism of *Trans, Trans*-Octadecadienoic Acid. Incorporation of *Trans, Trans*-Octadecadienoic Acid into the C_{20} Polyunsaturated Acids of the Rat[1]

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Abstract

Trans,trans-9,12-octadecadienoic acid-1-C^{14} was fed to adult rats. After four hr the animals were killed and the fatty acids isolated from their organ lipids. The 20-carbon fatty acids were isolated and degraded stepwise.

Radioactivity of the degradation products indicated that the fed acid was incorporated into the isolated C_{20} acids, mainly eicosatetraenoic acid probably with two *trans*-double bonds, while radioactivity throughout the chain gave evidence for a synthesis of eicosatrienoic and eicosatetraenoic acid from acetate derived from the fed material.

In a separate experiment, unlabeled *trans,trans*-9,12-octadecadienoic acid was fed to weaning rats for 14 days. Isolation of their fatty acids also gave evidence for the incorporation of the fed acid into eicosatrienoic and eicosatetraenoic acids containing *trans* double bonds.

[1] Supported in part by Contract AT(04-1)GEN-12 between Atomic Energy Commission and University of California, by lipid training grant USPHS TI HE-5306 and by a grant from the Nutrition Foundation.
[2] Supported in part by Public Health Service Research Career Award No. GM-K6-19, 177 from the Div. of General Medical Sciences, National Institutes of Health.

Introduction

PRIOR TO THE advent of isotopic tracer methods, the alkaline isomerization technique provided some insight into the transformations of the polyunsaturated acids. For example, an increase in a tetraenoic acid following the feeding of linoleate was taken as presumptive evidence that linoleate was transformed into arachidonate in the animal body (1). This idea was later confirmed by experiments involving the feeding of linoleic-1-C^{14} acid to rats, followed by the isolation and stepwise degradation of arachidonic acid formed in their organs (2).

Evidence obtained by the alkaline isomerization method has also indicated that some of the *trans* isomers of polyunsaturated acids may undergo similar transformation in the animal body. Thus, Holman (3) reported that feeding of *trans,trans*-octadecadienoate to fat-deficient rats resulted in some increase in both tetraene and hexaene content of the body fat, although deficiency symptoms were not alleviated.

The present experiments represent an attempt to interpret these results. While these studies were in progress, Blank and Privett (4,5) reported that dur-

TABLE I

Amounts and radioactivities of Fatty Acids from Fraction IV (soluble at −70C), Separated by Gas Chromatography

Fatty acids (chain length)	Amt (mg)	Specific activity (dis/min/mg x 10^{-3})
First experiment		
1) C_{14}-group (mainly tetradecanoic acid)	20.8	1.51
2) C_{16}-group (mainly hexadecenoic acid)	81.6	0.71
3) C_{18}-group (mainly octadecadienoic and octadecenoic acid)	437.8	8.66
4) C_{20}-group (mainly eicosatetraenoic acid)	125.0	0.84
5) C_{22}-group (mainly docosapentaenoic and docosahexaenoic acid)	99.7	1.49
Fractions 1) and 5) were contaminated with octadecadienoic acid to some extent, fraction 2) contained only a trace of octadecadienoic acid.		
Second experiment		
1) C_{12}-group	15.2	0.61
2) C_{14}-group	17.8	0.56
3) C_{16}-group	65.1	0.70
4) C_{18}-group	407.7	2.26
5) C_{20}-group	102.0	0.94
6) C_{22}-group	90.5	0.82

ing 17-day feeding experiments involving fat-deficient rats, some *cis,trans*-octadecadienoate was transformed into a tetraenoic acid containing *trans* double bonds, but that *trans,trans*-octadecadienoate did not undergo such a transformation.

Experimental

Preparation of trans,trans-*9,12-octadecadienoic acid.* Methyl linoleate (23.0 g) isolated from safflower oil, was heated 5.5 hr at 210C with 0.2 g Se powder. At the end of this period, the methyl ester was distilled at 134C and 250 μ pressure to give 20.5 g almost colorless oil. Following saponification of the methyl ester with methanolic KOH, the free acid, obtained in the usual manner, was crystallized four times from acetone at −40C and twice at −20C to give 5.8 g (25% yield) pure product of mp 28.5–29.0C. (The melting point reported by Cass and Burr (6) was 28–29C.) The UV absorption spectrum revealed no diene conjugation while the IR spectrum had a symmetrical *trans* peak at 10.35 μ and gave no indication of any *cis* double bond. Estimation of the amount of *trans* double bonds from the IR spectrum indicated about 230%, using pure methyl elaidate as a calibration standard. As additional proofs of purity, GLC performed with a

TABLE II

Specific Activities of Degradation Products of the Mixed C_{20}-acids (first experiment) and of Eicosatri- and -tetraenoic Acid from the Second Experiment

Fraction	Specific activity	
	dis/min/mg	dis/min/m*M*
First experiment		
Benzoic acid (carbon-1 of arachidic acid)	1.78×10^2	2.17×10^4
Nonadecanoic acid (carbons 2–20 of arachidic acid)	1.13×10^2	3.37×10^4
Benzoic acid (carbon-2 of arachidic acid)	4.22	5.15×10^2
Stearic acid (carbons 3–20 of arachidic acid)	1.23×10^2	3.49×10^4
Benzoic acid (carbon-3 of arachidic acid corrected for dilution of stearic acid)	2.09×10^2	2.55×10^4
Margaric acid (carbons 4–20 of arachidic acid, corrected for dilution of stearic acid)	3.46×10^1	9.28×10^3
Second experiment		
Arachidic acid (from hydrogenation of eicosatrienoic acid)	1.47×10^4	4.60×10^6
Benzoic acid (carbon-1 of arachidic acid)	7.75×10^1	9.46×10^3
Benzoic acid (corrected for dilution of starting acid)	2.33×10^3	2.84×10^5
Nonadecanoic acid (carbons 2–20 of arachidic acid)	4.42×10^2	1.32×10^5
Nonadecanoic acid (corrected for dilution of starting acid)	1.33×10^4	3.95×10^6
Arachidic acid (from hydrogenation of eicosatetraenoic acid)	5.77×10^2	1.81×10^5
Benzoic acid (carbon-1 of arachidic acid)	7.84×10^1	9.57×10^3
Benzoic acid (corrected for dilution of starting acid)	6.26×10^2	7.66×10^4
Nonadecanoic acid (carbons 2–20 of arachidic acid)	3.55×10^1	1.05×10^4
Nonadecanoic acid (corrected for dilution of starting acid)	2.84×10^2	8.44×10^4

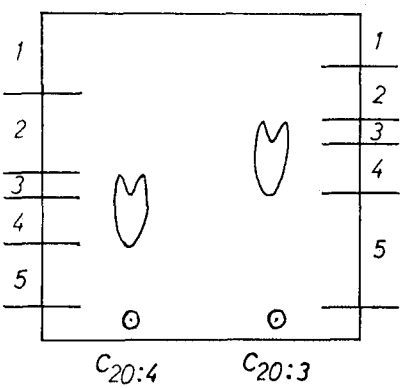

FIG. 1. Scheme for dividing the Si/$AgNO_3$ thin-layer plates into zones.

Barber-Colman Model 10 apparatus with a 3 ft x 0.25 in. column of ethylene glycol succinate, 13% on siliconized firebrick revealed a single peak in the correct position of C_{18} dienoic acid and reductive ozonolytic cleavage gave only caproic aldehyde (from the terminal end) thus ruling out the possibility of positional isomers.

Preparation of trans,trans-*9,12-octadecadienoic acid-1-C^{14}.* Introduction of carbon-14 into the carboxyl group of the dienoic acid was accomplished essentially as described by Howton and Nevenzel (7) for oleic acid with a few modifications. The tetrabromostearic acid derived by bromination in the usual manner, was decarboxylated using the method of Cristol and Firth (8) and debrominated to yield 1.65 g (29.5% yield) 1-bromo-*trans,trans*-8,11-heptadecadiene, purified by distillation. This was converted to the desired carboxyl-labeled product (0.28 g, purity 93.6%) in the manner described by Howton and Nevenzel (7).

Feeding experiments. The labeled acid (230 mg) was diluted with 770 mg unlabeled acid to give 1.0 g *trans,trans*-9,12-octadecadienoic acid with a specific activity of 6.4×10^5 dis/min/mg. A total of 250 mg of this mixture was fed intragastrically to each of 3 rats, weighing about 325 g each, by using a bent 18-gauge blunt-end needle on a tuberculin syringe. After 4 hr the rats were killed and their livers, hearts, kidneys, spleens and some mesenteric fat were pooled and frozen immediately. After lyophilization, the organs were extracted with chloroform:methanol (2:1) and the lipid, freed from solvent at room temp under nitrogen, was saponified under these same conditions with 10% methanolic KOH. The fatty acids (6.3 g), obtained in the usual manner from the saponification mixture, were separated by low-temp crystallization from acetone into fraction I (2.0 g) insoluble at −20C, fraction II (1.8 g) insoluble at −40C, fraction III (1.5 g) insoluble at −70C and fraction IV (1.0 g), soluble at −70C. Fraction IV, after purification on a silicic acid column to remove nonacidic substances, was found by GLC to contain about 16% of C_{20} acids. These were isolated by preparative gas-chromatography of their methyl esters on a Wilkens Instrument Co. A-100 Aerograph apparatus with a 0.5 in. x 5 ft column of SE-30 silicone stationary phase, 10% on chromosorb W support. The amounts and radioactivities are shown in Table I.

In a second experiment, 125 mg *trans,trans*-9,12-octadecadienoic acid-1-C^{14} of the same specific activity was administered to each of two rats weighing ca. 250 g each. The same procedure for isolation was followed to yield 102 mg of C_{20} unsaturated acids. These were separated by reversed phase chromatogra-

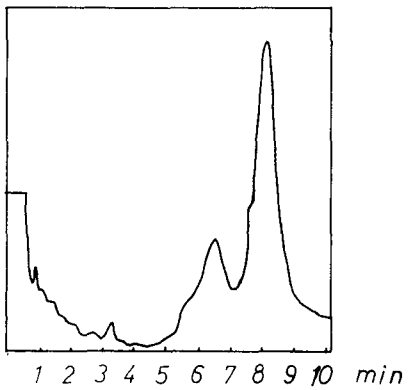

FIG. 2a. GLC analyses of the isomerized methyl arachidonate, containing some methyl eicosatrienoate:

All-*cis*-trienoate at 6.4; isomerized trienoate at 6.6.
All-*cis*-tetraenoate at 7.4; isomerized tetraenoate at 8.2.

phy as previously described (11) into diene, triene, tetraene and pentaene fractions. The amt and radioactivities of these fractions also show in Table I.

The unsaturated C_{20} acids obtained in the first experiment (80 mg) were hydrogenated over Pd/charcoal and diluted with inactive arachidic acid (290 mg) to give 370 mg product with a specific activity of 180 dis/min/mg. This acid was degraded stepwise by the method of Dauben et al. (9,10) to three successive samples of benzoic acid, the carboxyl carbons of which represented carbon atoms 1,2 and 3, respectively, and heptadecanoic acid representing carbons 4–20 of the original arachidic acid. In the second experiment, the triene and tetraene fractions were degraded to benzoic and nonadecanoic acids.

In the feeding experiment with unlabeled linelaidic acid eight weanling rats (21 days, average wt, 50 g) were maintained on a commercial diet but were given, daily by stomach tube, 100 mg each of a mixture of *trans,trans*-9,12-octadecadienoic acid and methyl oleate (1:1). After 11 days, the dose was increased to 200 mg for 3 more days; thus each rat received 850 mg *trans,trans*-9,12-octadecadienoic acid during the feeding period. One day following the last feeding, the rats (averaged wt, 94 g) were killed and their livers, kidneys, hearts, spleens and mesenteric adipose tissue were pooled and extracted as described above to yield 6.5 g crude lipid extract.

Saponification of the total lipids gave 4.8 g fatty acids following acidification. Low-temp crystallization gave 0.93 g of a fraction that did not crystallize at −70C from an 8% solution in acetone. This fraction, which contained the C_{20} acids, was methylated with diazomethane and separated by preparative GLC to give, after rechromatography, 84 mg eicosatrienoic and eicosatetraenoic acids. Reversed-phase chromatographic (10) separation of this fraction resulted in 43 mg tetraenoic fraction and 31 mg trienoic fraction, each fraction containing small amounts of the other.

Results

Table II lists the specific activity of products of the stepwise degradation of the C_{20} acids from the first experiment and of the decarboxylation of the eicosatrienoic and eicosatetraenoic acids from the second experiment. Stepwise degradation of the mixed C_{20} fatty acids of the first experiment revealed the following distribution of activity:

	$CH_3-(CH_2)_{16}-$	CH_2-	CH_2-	COOH
Per cent of total activity	16	45	1	38

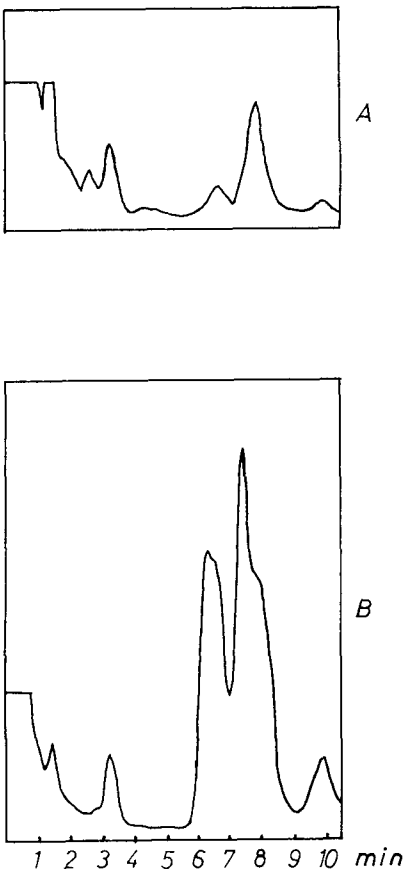

FIG. 2b. GLC analyses of the extracted methyl esters from scrapings from TLC:

A) Zone I of TLC
B) Zone II of TLC.
All-*cis*-trienoate at 6,3; *trans*-double bond-containing trienoate at 6.7.
All-*cis*-tetraenoate at 7,4; *trans*-double bond-containing tetraenoate at 7.8.

The decarboxylation of the eicosatrienoic acid from the second experiment gave the following:

	$CH_3-(CH_2)_{18}-$	COOH
Per cent of total activity	93	7

The eicosatetraenoic acid gave the following:

	$CH_3-(CH_2)_{18}-$	COOH
Per cent of total activity	52	48

In the case of the feeding experiments with unlabeled acids, evidence was sought that the isolated tetraenoic and, hopefully, trienoic acids actually contained *trans* double bonds. An attempt was therefore made to separate the *cis* and *trans* isomers by TLC on silver nitrate-silica gel plates following the procedure of Privett, Blank and Romanus (15). Development with ether:petroleum ether (4:6, v/v), followed by visualization with dichlorofluorescein, revealed the expected spots for the *cis*-tetraenoic acids. Since no spots were visible in the expected position of the *trans* compounds (above the corresponding *cis* acids), the plates were divided into zones (Fig. 1) which were scraped off separately. The visible spots were also divided into front and remaining sections, which were also scraped off. All scrapings were extracted with pentane and purified by chromatography on silicic acid.

As a standard for GLC identification, a sample of methyl arachidonate (containing a small amt of eicosatrienoate) was isomerized with Se. The all-*cis* esters

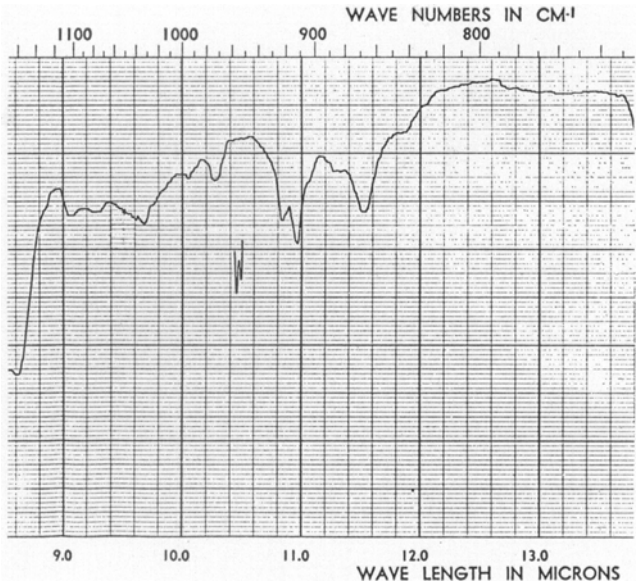

FIG. 3. IR spectrum of the combined and purified extracts from Zones I and II from TLC.

had retention times of 6.4 min (for trienoate) and 7.4 min (for tetraenoate). After isomerization, the major peaks had retention times of 6.6 and 8.2 min under identical conditions. Apparently the change from *cis* to *trans* bonds in these esters was accompanied by an increase in retention times (Fig. 2a).

The visible spots from the thin-layer plates, when analyzed by GLC, gave the expected retention times for the all-*cis* esters. From the areas near the solvent front, extracts gave GLC peaks with T_R's of 7.8 min, midway between the all-*cis* and all-*trans* isomers (Fig. 2b). The same shift toward longer T_R's was seen in the eicosatrienoate sections of the plates.

For further confirmation that the faster-running areas were indeed *trans* isomers, they were extracted as before and purified chromatographically and by saponification and acidification to remove some nonpolar impurities probably resulting from the reversed-phase chromatogram. Ca. 1 mg purified material was finally obtained which gave a peak at 10.3 μ, corresponding to a *trans* double bond (Fig. 3).

Discussion

From the results of the first experiment, it is evident that *trans,trans*-9,12-octadecadienoic acid was incorporated into an eicosatetraenoic acid. The high proportion of radioactivity in carbon-3 of the C_{20} polyunsaturated acids (mainly tetraenoic) could only have been derived from carbon-1 of the fed octadecadienoic acid, while the radioactivity in carbon-1 of the eicosatetraenoic acid was derived from acetate formed by degradation of the fed acid. The ratio of activities of carbon-1:carbon-3 (0.9) when compared with a value of 3 for the eicosatetraenoic acid derived from *cis,cis*-9,12,-octadecadienoic acid (linoleic) (2) may indicate that the conversion of the *trans,trans* isomer is at least as efficient as that of the true linoleic acid. If this were not so, carbon-1 would have a much higher relative radioactivity.

The amt of activity beyond carbon-3 was not readily explained on the basis of incorporation of a carboxyl-labeled 18-carbon acid *in toto* into a 20-carbon acid. The second experiment was therefore performed to ascertain whether the C_{20}-polyenoic acids might contain some species formed by total synthesis. It can be seen that while the true eicosatetraenoic acid may have a ratio of radioactivity of carbon-1:carbon-3 not too different from that of the C_{20} polyenoic acid from the first experiment, this ratio is quite different for the eicosatrienoic acid. This acid, the major isomer of which may be formed from oleic (or palmitoleic) acid (12) would be expected to have more activity (from acetate) throughout the chain than would the eicosatetraenoic acid. This appears to be the case in the present experiments and it seems safe to say, in view of the high radioactivity of the eicosatrienoic acid, that a great deal of the carbon-14 incorporated into the C_{20} polyenoic acid fraction came from total synthesis rather than direct incorporation of the starting acid. Nevertheless, it is evident that the *trans,trans* isomer of linoleic acid is incorporated into a C_{20} tetraenoic acid.

The attempt to identify this product in the experiment in which larger amt of unlabeled acid were fed reveals the difficulty of identifying a small amt tetraenoic acid containing *trans* double bonds in admixture with a large proportion of its all-*cis* isomer. Not only was it present as only a small fraction of a mixture containing components with almost identical properties but the means of identification did not easily distinguish it from them. Thus, the location of *trans* tetraene on the thin-layer plate was made difficult by the similarity of its R_f to those of *cis* and *trans* trienes. The T_R of the desired acid was very close to that of the corresponding all-*cis* tetraenoic acid, and the 10.3 μ peak in the IR spectrum was nearly obscured by the presence of impurities, the removal of which resulted in large losses. Nevertheless, the thin-layer and the IR evidence combined with a GLC retention time corresponding to that which would be calculated leaves little doubt that the fed *trans,trans*-9,12-octadecadienoic acid was incorporated into a C_{20} tetraenoic acid, presumably *cis,cis*-5,8-*trans,trans*-11,14-eicosatetraenoic acid.

A rough idea of the efficiency of this incorporation may be gained by a comparison of the ratio of the specific activity of the C_{20} polyenoic acid isolated in the first experiment to the total activity of the fed acid/100 g of rat. Such an estimation reveals that this measure of incorporation is of the same order of magnitude as is that for linoleate itself (approx 2×10^{-5} for both cases). It may be recalled, however, that the eicosatetraenoic acid formed from the *trans* dienes does not function as an essential fatty acid (1,13,14). Thus, the requirements for EFA activity are not met by an eicosatetraenoic acid containing *trans* bonds even though it is formed in amounts which would be adequate for the all-*cis* isomer.

ACKNOWLEDGMENT

IR spectra determined by George Alexander.

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[Received December 11, 1963—Accepted March 3, 1964]