

DATE: January 6, 2000

TO: Dockets Management Branch (HFA-305)
Food and Drug Administration
5630 Fishers Lane, Rm. 1061
Rockville, MD 20852

3357 '00 DEC 19 P1:26

FROM: Fred A. Kummerow
University of Illinois
Burnsides Research Laboratory
1208 W. Pennsylvania Ave. #205
Urbana, IL 61801

RE: Submission of Comments for docket number 94P-0036

In my opinion docket number 94P-0036 proposed by the Food and Drug Administration (FDA) to modify its regulations on nutrition labeling to require the amount of trans fatty acids present in a food and or a dietary supplement should be included on the Nutrition Facts panel. This information would be beneficial to all residents in the USA.

At present the trans fat in a food product are "hidden calories" which have added to obesity. The October 27, 1999 issue of the Journal of the American Medical Association (JAMA vol. 282, No. 16) devoted the entire issue to the growing obesity among us without considering the role of trans fat in obesity. Gram per gram, trans fatty acids contribute as many calories to the diet as do saturated fatty acids. Furthermore, trans fat increases the plasma cholesterol concentration more significantly than does a saturated fat. Although the amount of total fat as well as saturated fat is provided on the label, the consumer may assume that only saturated fatty acids should be of concern.

We have recently shown that endothelial arterial cells cultured in a medium containing a saturated or trans fatty acids incorporated the fatty acid into its cell membrane (JACN;70:832-8, 1999). When these cultured cells were pulsed with radioactive calcium, the influx of calcium was greater in cells containing trans acids in its cell membrane than cells containing oleic or stearic acid. As the influx of calcium into arterial cells is responsible for the "hardening" of arteries, trans fat may have a significant role in this process.

From 5-17% trans fatty acids are present in the milk of women eating foods containing trans fat and is, therefore, available for incorporation into endothelial arterial cells of their infants. A recent study of two year old autopsied infants showed that 100% already had fatty streaks (the first stage in the development of coronary heart disease) (JAMA, Vol. 281, No. 8, February 24, 1999). By young adulthood up to 20% calcium was present in the arteries. Whether the trans fatty acids in human milk are responsible for fatty streaks must still be determined. The influence of trans fat is dependent on its concentration; therefore knowing the amount present in a food product can be helpful to the consumer.

94P-0036

C 2189

Endothelial Lipase: A New Member of the Family

Lipases are key enzymes in the hydrolysis of triglycerides, phospholipids, and cholesteryl esters, including those of dietary origin. Their actions are essential to maintain lipid homeostasis and cardiovascular health. This report describes the finding and characterization of a new lipase of endothelial origin, one that may play an important role in plasma high-density lipoprotein metabolism.

Triglycerides, esterified cholesterol, and phospholipids are the major fat components of the diet. These dietary lipids cannot be directly absorbed by the enterocyte. Rather, they are emulsified in the jejunum and hydrolyzed by specific lipases into unesterified fatty acids, monoacylglycerol, free cholesterol, and lysophospholipid forms that the enterocyte can absorb from the intestinal lumen. Once inside the enterocyte, these lipids are reesterified and packaged into large, triglyceride-rich lipoprotein (TRL) particles known as chylomicrons that are then secreted into the mesenteric lymph and transported into the thoracic duct to enter the blood circulation through the subclavian vein. Nascent chylomicrons, which consist primarily of dietary lipids and apolipoproteins B-48, apoC-II, and apoA-IV, are rapidly metabolized into chylomicron remnants. These remnant particles are removed from the circulation, primarily by the liver, via receptor-mediated pathways.

Liver cells secrete TRL particles known as very-low-density lipoproteins (VLDL), which consist of endogenous lipids and apolipoproteins B-100, E, C-II, and C-III. These particles are metabolized first into intermediate-density lipoproteins (IDL) and then to low-density lipoproteins (LDL) through mechanisms similar to those acting on chylomicrons. Lipoprotein lipase (LPL) is the key enzyme in the hydrolysis of triglycerides into chylomicrons and VLDL and the subsequent conversion into their respective remnant particles.^{1,2} LPL is found primarily in skeletal muscle, adipose tissue, and heart. Additionally, arterial wall macrophages produce LPL. In adipose tissue and muscle, LPL is synthesized in the parenchymal cells and translocated to the luminal side of the capillary endothe-

lium, where it is bound to heparan sulfate proteoglycan chains. From this site, it hydrolyzes triglycerides from TRL and releases fatty acids for uptake by the tissues. In addition to the endothelium-bound LPL, there is some LPL circulating in the blood. The following roles have been suggested for this fraction: for transfer to tissues where LPL is not synthesized, for association with lipoproteins to target them to cell-surface proteoglycans or to specific receptors, and for the transport of LPL for degradation in the liver. Although the site of action of LPL is the luminal surface of vascular endothelium, no lipase expression has been demonstrated in these cells. Hepatic lipase (HL), another member of this family of lipases, preferentially hydrolyzes phospholipids but also hydrolyzes triglycerides in HDL₂ (large HDL) and participates in its conversion to HDL₃ (small HDL). Therefore, HL is involved in the reverse transport of cholesterol from peripheral tissues to the liver.³

A report by Jaye et al.⁴ describes the finding and characterization of a new lipase by using a human placenta cDNA library to isolate a clone containing an open reading frame encoding 500 amino acids. This lipase was identified as a new member of the triglyceride lipase family and was named endothelial lipase (EL). The corresponding gene locus was named LIPG in accordance with the nomenclature used for this gene family (Table 1). Using a series of careful and elegant experiments, these authors clearly described the steps used to accomplish the following: find the relevant cDNA, isolate and characterize the gene product, characterize the specificity of its enzymatic activity, generate antibodies to examine the expression of the protein, determine the sites of *in vivo* production, and examine its physiologic role *in vivo*.

To find the cDNA for the new lipase (EL), Jaye et al. exposed differentiated THP-1 cells (a human monocyte cell line from a male with acute monocytic leukemia) to oxidized LDL or control media and isolated mRNAs from both conditions. They then synthesized cDNAs and subjected them to analysis by differential display. A polymerase chain reaction (PCR) product from a cDNA that was preferentially expressed in those cells incubated with the oxidized LDL was gel-isolated, purified, and cloned. This clone was used to screen a human placental cDNA library to isolate a full-length cDNA clone for EL. The sequence of the clone revealed a 45% homology with LPL, 40% with HL, and 27% with pancreatic lipase (PL). These findings strongly suggested that this cDNA was coded

This review was prepared by Jose M. Ordovas, Ph.D., Lipid Metabolism Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging, Tufts University, Boston, MA 02111, USA.

Effect of *trans* fatty acids on calcium influx into human arterial endothelial cells¹⁻³

Fred A Kummerow, Qi Zhou, and Mohamedain M Mahfouz

ABSTRACT

Background: A recent task force of The American Society for Clinical Nutrition and American Society for Nutritional Sciences recommended in a position paper on *trans* fatty acids that models be developed to assess the effects of changes in fat intake on disease risk.

Objective: The objective was to investigate, using human arterial endothelial cells as a model, the influence of *trans* fatty acids and magnesium on cell membrane composition and on calcium influx into arterial cells, a hallmark of atherosclerosis.

Design: Endothelial cells were cultured for 3 d in media with high (adequate) or low (inadequate) amounts of magnesium plus various concentrations of *trans,trans* linoelaidic; *cis,cis* linoleic; *trans* elaidic; oleic; or stearic acids. The cells were then harvested and the fatty acid composition and the amount of ⁴⁵Ca²⁺ incorporated into the cell was determined.

Results: The percentage of fatty acids incorporated into the endothelial cells was proportional to the amount added to the culture medium. Adequate magnesium was crucial in preventing calcium influx into endothelial cells. Without an adequate amount of magnesium in the culture medium, linoelaidic and elaidic acids, even at low concentrations, increased the incorporation of ⁴⁵Ca²⁺ into the cells, whereas stearic acid and oleic acid did not ($P < 0.05$).

Conclusion: Our model indicated that a diet inadequate in magnesium combined with *trans* fat may increase the risk of calcification of endothelial cells. *Am J Clin Nutr* 1999;70:832-8.

KEY WORDS *trans* Fatty acids, calcium infiltration, endothelial cells, atherosclerosis, umbilical cord, magnesium

INTRODUCTION

The Task Force on *Trans* Fatty Acids of the American Society for Clinical Nutrition and the American Society for Nutritional Sciences (1) stated "Concerns about possible adverse effects of *trans* fatty acids on health center on the question of whether the intake of *trans* fatty acids is associated with the development and/or acceleration of disease, thereby increasing morbidity and mortality." Epidemiologic (2-7) and clinical (8-13) studies have addressed this issue. Additional overviews and commentaries have emphasized the importance of continued research of this issue (14-19).

The percentage of *trans* fat in a food item is not provided on the label, so one can only surmise the percentage from the published literature (20). Margarine and the shortenings that are used

in baked products contain from 6.8% to 41.0% and cooking oils and coating fat on candies contain from 1.4% to 79.0% *trans* fatty acids (21, 22). The major *trans* isomeric fatty acid in hydrogenated fat is *trans* elaidic acid (*t*-18:1n-9). *t,t*-Linoelaidic acid (*t,t*-18:2) is present in partially hydrogenated vegetable oils and *cis,cis* linoleic acid (*c,c*-18:2n-6) is present in unhydrogenated vegetable oil. *trans* Fatty acids consumed as part of the US diet are metabolized and are deposited in human tissue, as are other dietary fatty acids. We found 4-14% *trans* fatty acids in the heart and 4.6-9.3% in arterial tissue (23). Human milk contains from 4.7% (24) to as much as 17.0% (25) *trans* fatty acids.

Many cellular functions are affected when the composition of unsaturated fatty acids in the cell membrane (26, 27) or the polar head group of the cell membrane (28) is modified. *trans* Unsaturated fatty acids inhibit phosphatidylcholine-sterol *O*-acyltransferase and alter its positional specificity (29). Engelhard et al (30) found that the addition of a *trans* fatty acid to a culture medium resulted in a large increase in the fluidity of choline-supplemented membranes. Tongai et al (31) showed that erythrocyte plasma membrane from magnesium-deficient rats was more fluid than that of control rats. We found that a medium deficient in magnesium changed the polyunsaturated fatty acid content of the cell membrane (32), changed the percentage of cellular phospholipid in the cell membrane, and increased free cytosolic calcium concentrations in the cell compared with cells cultured in a sufficient amount of magnesium (33). Because stenosis is due to atherosclerosis of the coronary arteries (34), characterized by lipid and calcium deposits in the arterial wall (35-37), we chose to study the effects of *trans* fatty acids on arterial cell membrane composition and on calcium influx into cultured arterial endothelial cells. In previous studies (38, 39), we found that a decrease in the magnesium concentration of culture medium from 0.95 mmol/L [normal content in Eagle's minimum essential medium (MEM)] to <0.57 mmol/L affected cell

¹From the University of Illinois, Burnside Research Laboratory, Urbana, and The HE Moore Heart Research Foundation, Champaign, IL.

²Supported by The Wallace Research Foundation, Cedar Rapids, IA, and the Verna L and John R Hildebrand Foundation, Denver.

³Address reprint requests to FA Kummerow, Burnside Research Laboratory, 1208 West Pennsylvania Avenue, Urbana, IL 61801. E-mail: fkummerow@uiuc.edu.

Received September 15, 1998

Accepted for publication March 26, 1999.

growth and functions. We chose magnesium concentrations of 0.95 and 0.57 mmol/L and a 3-d incubation period to test the effect of *trans* fatty acids on cell fatty acid composition and on $^{45}\text{Ca}^{2+}$ influx into endothelial cells.

MATERIALS AND METHODS

$^{45}\text{Ca}^{2+}$ was purchased from Moravak (Brea, CA) and New England Nuclear (Boston); the fatty acids were purchased from NuCheck Prep Inc (Elysian, MN). The endothelial cells were isolated from the arteries of human umbilical cords obtained by cesarean delivery (Carle Foundation Hospital, Urbana, IL) and were cultured to 60% confluence in Eagle's MEM containing 10% calf bovine serum. These cells were then cultured in MEM containing 5% calf bovine serum and various combinations and concentrations of *t,t*-18:2, *c,c*-18:2n-6, *t*-18:1n-9, oleic acid (18:1n-9), or stearic acid (18:0) and either a high (0.95 mmol/L) or low (0.57 mmol/L) amount of magnesium. After 3 d of incubation, the cells were harvested and lipids were extracted with 10 mL chloroform:methanol (2:1, by vol). The lipids then were saponified and acidified and the fatty acids converted to methyl esters. The composition of fatty acids in each sample was determined in a model 428 gas chromatograph equipped with an all-glass injection splitter and flame ionization detector (Hewlett-Packard, Palo Alto, CA). Retention time, peak areas, and relative peak area percentages were determined electronically with a Hewlett-Packard model 3390A Reporting Integrator. Relative retention times were compared with authentic standards to identify methyl esters of fatty acids (32). Butylated hydroxytoluene was added as an antioxidant at a concentration of 0.005% (wt:vol) to all solvents, and all procedures were carried out under nitrogen. We also determined the fatty acid composition of both the freshly isolated arterial cells from the umbilical cords and those cultured in MEM, which were used as experimental models.

Because calcification of arterial cells is a common hallmark of atherosclerosis (34-37), we tested the influx of calcium ($^{45}\text{Ca}^{2+}$) into the cells. After the cells had been cultured for 3 d in MEM

containing the various combinations and concentrations of fatty acids and either 0.95 or 0.57 mmol Mg/L, the cells were pulsed with $^{45}\text{Ca}^{2+}$ (37 MBq/L, or 1 μCi) in 1 mL MEM for 1 h. The monolayers then were washed and digested. The amount of radioactivity incorporated into the cells was counted in a Beckman (Fullerton, CA) scintillation counter (39).

To test the efflux of $^{45}\text{Ca}^{2+}$, we exposed the fatty acid-treated cells to $^{45}\text{Ca}^{2+}$ (74 MBq/L, or 2 $\mu\text{Ci}/\text{mL}$) for 1 h. We then washed $^{45}\text{Ca}^{2+}$ -pulsed cells with phosphate-buffered saline and cultured them for 1 h in 1 mL fresh MEM. Aliquots (100 mL) were collected at 5, 15, and 60 min, and the amount of $^{45}\text{Ca}^{2+}$ in the MEM was measured at each time interval.

The data were analyzed by analysis of variance and with a Scheffe test (40). A *P* value <0.05 was considered significant. All data are presented as means \pm SEs.

RESULTS

The percentage of *c,c*-18:2n-6 in the endothelial cells increased from 1.2% in the cells freshly isolated from the umbilical cord to 2.8% in the cells cultured in MEM containing 0.95 mmol Mg/L (Table 1). The percentages of 18:0 and 18:1n-9 increased whereas the percentages of palmitoleic (16:1n-9) and arachidonic (20:4n-6) acids decreased significantly after being cultured for 3 d in MEM. Endothelial cells cultured for 3 d in 0.95 mmol Mg/L containing 12 μmol *t*-18:1n-9/L incorporated 0.9% *t*-18:1n-9 and more 18:2n-6 than did cells cultured in MEM. Cells cultured in a medium containing 18:0 contained more 18:0 and less Δ^7 -octadecenoic acid (18:1n-7) than did cells cultured in *t*-18:1n-9. There was no significant difference in fatty acid composition between endothelial cells cultured with 12 μmol *t*-18:1n-9/L and endothelial cells cultured with 12 μmol 18:0/L.

The percentage of *t,t*-18:2 and *c,c*-18:2n-6 incorporated into endothelial cells depended on the ratio of *t,t*-18:2 to *c,c*-18:2n-6 added to the media (Table 2). After endothelial cells were cultured for 3 d in MEM containing 100 μmol *c,c*-18:2n-6/L and 0.95 mmol Mg/L, the *c,c*-18:2n-6 content in the endothelial

TABLE 1
Percentage of fatty acids and the double bond index (DBI) in endothelial cells from freshly isolated umbilical cords and in cells cultured for 3 d in Eagle's minimum essential medium (MEM) containing either stearic (18:0) or *trans* elaidic (*t*-18:1n-9) acid¹

Fatty acid (%)	Freshly isolated	Cultured in MEM	Cultured with 12 μmol 18:0/L	Cultured with 12 μmol <i>t</i> -18:1n-9/L
14:0	1.7 \pm 0.1 ^{a,b}	1.6 \pm 0.0 ^a	2.0 \pm 0.1 ^{a,b}	1.8 \pm 0.0 ^b
16:0	26.7 \pm 0.9	27.7 \pm 2.1	26.8 \pm 0.5	28.3 \pm 1.0
16:1n-9	3.3 \pm 0.1 ^a	0.1 \pm 0.0 ^b	0.2 \pm 0.0 ^b	0.3 \pm 0.0 ^b
18:0	17.0 \pm 0.3 ^a	28.4 \pm 2.5 ^{b,c}	33.3 \pm 1.1 ^b	26.2 \pm 0.5 ^c
18:1n-9	15.4 \pm 0.4 ^a	23.2 \pm 2.6 ^b	24.8 \pm 2.3 ^b	27.1 \pm 0.9 ^b
18:1n-7	3.9 \pm 0.2 ^{a,b}	2.4 \pm 0.5 ^{a,b}	3.4 \pm 0.1 ^a	4.9 \pm 0.0 ^b
<i>t</i> -18:1	—	0.1 \pm 0.0 ^a	0.2 \pm 0.0 ^{a,b}	0.9 \pm 0.1 ^b
18:2n-6	1.2 \pm 0.1 ^a	2.8 \pm 0.1 ^b	3.6 \pm 0.2 ^{b,c}	4.6 \pm 0.1 ^c
20:3n-6	1.6 \pm 0.1 ^{a,b}	2.1 \pm 0.1 ^a	1.6 \pm 0.1 ^{a,b}	1.5 \pm 0.1 ^b
20:4n-6	12.8 \pm 0.5 ^a	3.6 \pm 0.3 ^b	3.0 \pm 0.1 ^b	2.7 \pm 0.2 ^b
22:4n-6	1.8 \pm 0.2 ^a	0.8 \pm 0.0 ^b	0.5 \pm 0.1 ^b	0.4 \pm 0.1 ^b
22:5n-6	2.9 \pm 0.1 ^a	0.2 \pm 0.0 ^b	0.3 \pm 0.0 ^b	0.2 \pm 0.0 ^b
22:5n-3	3.0 \pm 0.1	—	—	—
22:6n-3	4.1 \pm 0.2 ^a	0.2 \pm 0.0 ^b	0.3 \pm 0.0 ^b	1.0 \pm 0.1 ^b
DBI	142.3	54.7	57.7	65.4

¹ $\bar{x} \pm$ SE. Values in the same row with different superscript letters are significantly different, *P* < 0.05.

TABLE 2

Percentage of fatty acids and the double bond index (DBI) in endothelial cells cultured for 3 d in Eagle's minimum essential medium containing various concentrations of *trans,trans* linoelaidic (*t,t*-18:2) and *cis,cis* linoleic (*c,c*-18:2n-6) acids¹

	100 $\mu\text{mol } t,t\text{-}18:2 +$ 0 $\mu\text{mol } c,c\text{-}18:n-6$	75 $\mu\text{mol } t,t\text{-}18:2 +$ 25 $\mu\text{mol } c,c\text{-}18:n-6$	50 $\mu\text{mol } t,t\text{-}18:2 +$ 50 $\mu\text{mol } c,c\text{-}18:n-6$	25 $\mu\text{mol } t,t\text{-}18:2 +$ 75 $\mu\text{mol } c,c\text{-}18:n-6$	0 $\mu\text{mol } t,t\text{-}18:2 +$ 100 $\mu\text{mol } c,c\text{-}18:n-6$
Fatty acid (%)					
14:0	1.03 \pm 0.3 ^a	1.10 \pm 0.1 ^a	1.37 \pm 0.2 ^a	1.30 \pm 0.2 ^a	2.13 \pm 0.2 ^b
14:1	0.30 \pm 0 ^a	0.33 \pm 0.1 ^{a,b}	0.43 \pm 0.1 ^b	0.40 \pm 0.1 ^{a,b}	0.47 \pm 0.1 ^{a,b}
16:0	19.1 \pm 0.3 ^a	17.5 \pm 0.4 ^b	18.1 \pm 1.1 ^{a,b}	18.5 \pm 1.2 ^{a,b}	24.5 \pm 0.7 ^c
16:1n-7	0.78 \pm 0.2	0.80 \pm 0.2	0.75 \pm 0.1	0.55 \pm 0.1	0.63 \pm 0.1
16:1n-5	1.55 \pm 0.2 ^a	0.80 \pm 0.1 ^b	1.40 \pm 0.3 ^{a,b}	3.23 \pm 1.1 ^a	0.60 \pm 0.1 ^b
18:0	12.1 \pm 1.3 ^{a,b}	11.8 \pm 0.5 ^a	13.5 \pm 2.0 ^{a,c}	14.7 \pm 0.6 ^{b,c}	17.4 \pm 1.6 ^c
18:1n-9	9.50 \pm 1.7 ^{a,b}	10.3 \pm 0.6 ^b	8.40 \pm 0.7 ^{a,b}	9.70 \pm 1.2 ^{a,b}	7.98 \pm 0.6 ^a
18:1n-7	1.45 \pm 0.2 ^a	1.68 \pm 0.1 ^b	1.45 \pm 0.2 ^b	1.63 \pm 1.1 ^b	0.80 \pm 0.2 ^c
<i>t,t</i> -18:2	29.5 \pm 2.3 ^a	24.0 \pm 0.7 ^a	17.1 \pm 1.1 ^b	9.10 \pm 1.0 ^c	—
18:2n-6	5.35 \pm 0.2 ^a	10.3 \pm 0.4 ^b	13.4 \pm 0.9 ^c	15.8 \pm 0.8 ^d	15.5 \pm 1.0 ^{c,d}
18:3n-6	—	—	0.40 \pm 0.0	—	1.20 \pm 0.1
20:2n-6	0.20 \pm 0.0 ^{a,b,c}	0.45 \pm 0.1 ^a	0.95 \pm 0.1 ^b	1.53 \pm 0.1 ^c	1.88 \pm 0.4 ^{b,c}
20:3n-6	0.93 \pm 0.1 ^a	1.35 \pm 0.2 ^{a,b}	1.40 \pm 0.1 ^b	2.35 \pm 0.2 ^c	6.75 \pm 0.4 ^d
20:4n-6	6.30 \pm 0.2	7.60 \pm 0.6	7.40 \pm 0.7	7.80 \pm 0.7	6.80 \pm 0.6
22:2n-6	1.75 \pm 0.3 ^{a,c}	0.87 \pm 0.2 ^d	1.08 \pm 0.3 ^{a,d}	0.92 \pm 0.2 ^{b,d}	1.95 \pm 0.1 ^c
22:4n-6	2.30 \pm 0.2 ^a	3.13 \pm 0.2 ^b	3.60 \pm 0.3 ^b	3.95 \pm 0.5 ^b	3.08 \pm 0.2 ^b
22:5n-6	1.35 \pm 0.2	1.60 \pm 0.1	1.68 \pm 0.2	1.50 \pm 0.2	1.38 \pm 0.4
22:6n-3	0.80 \pm 0.0	1.28 \pm 0.2	0.98 \pm 0.1	0.73 \pm 0.1	1.10 \pm 0.7
Total n-6	18.0 \pm 0.4 ^a	25.1 \pm 1.3 ^b	29.6 \pm 2.0 ^{b,c}	33.8 \pm 2.5 ^{c,d}	38.5 \pm 2.0 ^d
Saturated	31.2 \pm 1.6 ^{a,c}	29.3 \pm 0.6 ^a	31.7 \pm 3.0 ^{a,c}	33.2 \pm 1.0 ^c	41.9 \pm 1.7 ^b
DBI					
With <i>t,t</i> -18:2	135.9	147.8	142.1	136.1	126.0
Without <i>t,t</i> -18:2	76.9	99.8	107.9	117.9	126.0

¹ $\bar{x} \pm$ SE. Values in the same row with different superscript letters are significantly different. $P < 0.05$.

cells increased from 5.35% to 15.5%. Cells cultured in MEM plus 75 $\mu\text{mol } c,c\text{-}18:2n-6/\text{L}$ and 25 $\mu\text{mol } t,t\text{-}18:2/\text{L}$ incorporated 15.8% *c,c*-18:2n-6 and 9.1% *t,t*-18:2, respectively. When the endothelial cells were cultured in MEM plus 50 $\mu\text{mol } c,c\text{-}18:2n-6/\text{L}$ and 50 $\mu\text{mol } t,t\text{-}18:2/\text{L}$, the isolated cells contained 13.4% *c,c*-18:2n-6 and 17.1% *t,t*-18:2. Consequently, as the concentration of *t,t*-18:2 in the MEM increased, more *t,t*-18:2 was incorporated into the endothelial cells.

⁴⁵Ca²⁺ influx increased significantly in endothelial cells cultured at a high ratio of *t,t*-18:2 to *c,c*-18:2n-6 (Table 3). Endothelial cells cultured for 3 d in 0.95 mmol Mg/L with various percentages of *t,t*-18:2 and *c,c*-18:2n-6 were tested for calcium influx at 2 different time intervals. With no *t,t*-18:2 or *c,c*-18:2n-6 added to the MEM, an influx of ⁴⁵Ca²⁺ was noted at 3.67 cpm/ μg protein. When the cells were cultured with 100 $\mu\text{mol } t,t\text{-}18:2/\text{L}$, ⁴⁵Ca²⁺ influx increased significantly compared with that from the cells treated with 100 $\mu\text{mol } c,c\text{-}18:2n-6/\text{L}$. When the cells were incubated with decreasing concentrations of *t,t*-18:2, from 97.5 to 80 $\mu\text{mol}/\text{L}$, a significant increase in ⁴⁵Ca²⁺ influx was also noted. However, when the concentration of *t,t*-18:2 decreased to $\leq 75 \mu\text{mol}/\text{L}$, no significant difference in ⁴⁵Ca²⁺ influx was noted.

The increased influx of ⁴⁵Ca²⁺ into endothelial cells cultured in MEM with a high amount of magnesium (0.95 mmol Mg/L) and 12 $\mu\text{mol } t,t\text{-}18:2$, *t*-18:1n-9, or 18:0/L was not significant when compared with the cells cultured without fatty acids (Table 4). However, ⁴⁵Ca²⁺ influx increased significantly when endothelial cells were cultured in medium with a low concentration of magnesium (0.57 mmol/L) plus either 12 $\mu\text{mol } t,t\text{-}18:2$ or *t*-18:1n-9/L, but did not increase significantly with 18:0. ⁴⁵Ca²⁺ influx also did not increase significantly in endothe-

lial cells cultured in medium with 0.57 mmol Mg/L combined with either 18:1n-9 or 18:0. However, a significant increase was noted when endothelial cells were cultured in MEM with *t*-18:1n-9 (Table 5). The efflux of ⁴⁵Ca²⁺ from endothelial cells was not influenced by the presence of *t,t*-18:2 (Table 6).

DISCUSSION

Our tissue culture data indicate that *trans* fatty acids combined with a low amount of magnesium in MEM modify

TABLE 3

⁴⁵Ca²⁺ influx into endothelial cells cultured with various concentrations of *trans,trans* linoelaidic acid (*t,t*-18:2) and *cis,cis* linoleic acid (*c,c*-18:2n-6)¹

Fatty acid ($\mu\text{mol}/\text{L}$)	⁴⁵ Ca ²⁺ influx cpm/ μg protein
0 <i>t,t</i> -18:2 + 100 <i>c,c</i> -18:2n-6	4.76 \pm 0.2 ^a
100 <i>t,t</i> -18:2 + 0 <i>c,c</i> -18:2n-6	6.78 \pm 0.1 ^b
75 <i>t,t</i> -18:2 + 25 <i>c,c</i> -18:2n-6	5.08 \pm 0.1 ^a
50 <i>t,t</i> -18:2 + 50 <i>c,c</i> -18:2n-6	4.74 \pm 0.2 ^a
25 <i>t,t</i> -18:2 + 75 <i>c,c</i> -18:2n-6	4.86 \pm 0.2 ^a
0 <i>t,t</i> -18:2 + 0 <i>c,c</i> -18:2n-6	3.67 \pm 0.2 ^a
97.5 <i>t,t</i> -18:2 + 2.5 <i>c,c</i> -18:2n-6	5.98 \pm 0.3 ^b
85 <i>t,t</i> -18:2 + 15 <i>c,c</i> -18:2n-6	5.29 \pm 0.4 ^b
80 <i>t,t</i> -18:2 + 20 <i>c,c</i> -18:2n-6	4.59 \pm 0.3 ^b

¹ $\bar{x} \pm$ SE of 6 separate experiments. After human arterial endothelial cells were cultured with *t,t*-18:2 and *c,c*-18:2n-6 for 3 d, the cells were pulsed with ⁴⁵Ca²⁺ for 1 h. The monolayers then were washed, digested, and analyzed for radioactivity and protein content. Values in the same column with different superscript letters are significantly different, $P < 0.05$.

TABLE 4

$^{45}\text{Ca}^{2+}$ influx into endothelial cells cultured with *trans* linoleic acid (*t,t*-18:2), *trans* elaidic acid (*t*-18:1n-9), or stearic acid (18:0) with high and low amounts of magnesium¹

Fatty acid	$^{45}\text{Ca}^{2+}$ influx	
	0.95 mmol Mg/L	0.57 mmol Mg/L
	cpm/ μg protein	
0 $\mu\text{mol/L}$	1.82 \pm 0.1	2.07 \pm 0.2
12 $\mu\text{mol t,t-18:2/L}$	2.01 \pm 0.1	2.57 \pm 0.3 ²
12 $\mu\text{mol t-18:1n-9/L}$	1.73 \pm 0.1	2.41 \pm 0.2 ²
12 $\mu\text{mol 18:0/L}$	1.74 \pm 0.3	1.78 \pm 0.2

¹ $\bar{x} \pm \text{SE}$ of 6 separate experiments. After human arterial endothelial cells were cultured with *t,t*-18:2, *t*-18:1n-9, or 18:0 for 3 d, the cells were pulsed with $^{45}\text{Ca}^{2+}$ for 1 h. The monolayers then were washed, digested, and analyzed for radioactivity and protein content.

²Significantly different from 0.95 mmol Mg/L, $P < 0.05$.

endothelial cell membranes, permitting increased $^{45}\text{Ca}^{2+}$ influx into endothelial cells. As indicated in the Introduction, cellular functions and responses are affected when the unsaturated fatty acid content of the cell membrane is modified (26). Magnesium plays a vital role in this process (38). It acts as a cofactor (41) in the activity of the Δ^5 - and Δ^6 -desaturase enzymes, which play a role in decreasing the content of unsaturated fatty acids in the cell membrane. For example, when there was a magnesium deficiency, the fatty acid composition of liver microsomes indicated a slower rate of conversion of 18:2n-6 to 20:4n-6, which is consistent with the decrease in Δ^6 -desaturase activity observed in the liver microsomes of magnesium-deficient rats (42). The decrease in Δ^6 -desaturase activity was attributed to the lower concentration of enzyme molecules as a result of the decreased rate of protein synthesis associated with the magnesium deficiency (43). We found that *trans* 18:1 fatty acids in partially hydrogenated soybean oil have a more inhibitory effect than do saturated acids on essential fatty acid metabolism, even when there is an abundance of essential fatty acids in hydrogenated fat (42). The relevance of the magnesium content of the tissue culture medium to the atherogenic effect of *trans* fatty acids may rest on the pronounced effects of magnesium on the physical state of membrane bilayer lipids. Rayssiguier et al (44) believe that defective membrane function could be the primary reason for the underlying cellular disturbance that occurs in magnesium deficiency. Our data indicate that *t,t*-18:2 and *t*-18:1n-9 contribute to the cellular disturbance of the membrane.

A previous study indicated that cells cultured in MEM with 18:0 contained significantly more 18:1n-9 than did cells cultured with 18:2n-6, or 39.3% and 10.0%, respectively. The increased concentration of 18:1n-9 in cells cultured in 18:0 was necessary to maintain the physical state of the membrane (45). The physical state of the membrane can be conveniently determined by calculating the double bond index (DBI). The DBI is based on the sum of the percentages of unsaturated fatty acids multiplied by the number of double bonds in each unsaturated fatty acid (46). Hypothetically, if one considers *t,t*-18:2 to be an unsaturated fatty acid and if both *trans* and *cis* fatty acids are incorporated into a cell membrane, the DBI of the lipid extracted from the endothelial cells cultured with *t,t*-18:2 would be higher than that from the endothelial cells cultured with *c,c*-18:2n-6, or 135.92 and 126.01, respectively. However,

Mahfouz et al (47) showed that *trans* fatty acids have physical properties similar to those of 18:0 and are inserted into acyl lipids in a manner more similar to that of a saturated acid than to an unsaturated acid. The acyltransferase (ie, 1-acylglycerophosphocholine *O*-acyltransferase) in rat liver microsomes discriminates between the normal *cis* isomers and the unnatural *trans* isomers with respect to their esterification in phospholipids and triacylglycerol, and a chain containing *trans* double bonds is transferred as if it were a saturated carbon chain (48). If the DBI is calculated on the basis of *trans* fatty acids acting similarly to 18:0 in the membrane, the DBI of the data in Table 2 would vary from 76.9 to 126.0. It is evident that the influence of *trans* fatty acids on the physical state of the membranes of endothelial cells cultured in medium with a low amount of magnesium requires further study.

Although the endothelial cells freshly isolated from the umbilical cord contained only 1.2% 18:2n-6, they contained 12.8% 20:4n-6 and more n-6 and n-9 unsaturated fatty acids than did the endothelial cells cultured for 3 d. The DBIs were 142.8 and 54.7, respectively, indicating a significant change in membrane fluidity between freshly isolated and cultured cells. Scott et al (49) found only 2.4% 18:2n-6 in the phospholipid fraction of the lipids extracted from the coronary arteries of infants aged <24 h. This is comparable with the percentage we found (1.2% *c,c*-18:2n-6) in the endothelial cells freshly isolated from the arteries of umbilical cords. Scott et al also found that 18:2n-6 increased from 2.4% to 4.8% and that 20:4n-6 increased from 11.0% to 19.0% in infants and adolescents, respectively.

In 1980, Seelig (50), in a book devoted to the role of magnesium deficiency in cardiovascular pathogenesis, listed >1000 references to ischemic heart disease in infants, primarily due to arteriosclerosis in the coronary arteries. A MEDLINE (National Library of Medicine, Bethesda, MD) search revealed that 195 additional articles dealing with arteriosclerosis in infants have been published since 1980. Seelig et al (51-53) have called attention to the low magnesium content of the US diet, and many other studies have suggested that magnesium intakes are inadequate in the US population (54-56).

Stary et al (35) found fatty streaks (type II lesion) in the aorta of 99% of infants and adolescents tested. According to Stary et al, type II lesions could develop into atherosclerotic type III lesions. Such a change may be governed by plasma factors, eg, excessive amounts of hematin (57) and homocysteine (58). A recent study

TABLE 5

$^{45}\text{Ca}^{2+}$ influx into endothelial cells cultured with *trans* elaidic acid (*t*-18:1n-9), oleic acid (18:1n-9), stearic acid (18:0), or *cis,cis* linoleic acid (*c,c*-18:2n-6) with high and low amounts of magnesium¹

Fatty acid	$^{45}\text{Ca}^{2+}$ influx	
	0.95 mmol Mg/L	0.57 mmol Mg/L
	cpm/ μg protein	
0 $\mu\text{mol/L}$	16.92 \pm 1.9	13.31 \pm 2.5
12 $\mu\text{mol t-18:1n-9/L}$	20.57 \pm 6.1	25.40 \pm 3.0 ²
12 $\mu\text{mol 18:0/L}$	15.67 \pm 1.6	14.01 \pm 2.9
12 $\mu\text{mol 18:1n-9/L}$	15.94 \pm 3.3	14.83 \pm 2.3
12 $\mu\text{mol c,c-18:2n-6/L}$	17.82 \pm 2.1	19.81 \pm 2.0

¹ $\bar{x} \pm \text{SE}$ of 8 separate experiments. After human arterial endothelial cells were cultured with *t*-18:1n-9, 18:0, 18:1n-9, or *c,c*-18:2n-6 for 3 d, the cells were pulsed with $^{45}\text{Ca}^{2+}$ for 1 h. The monolayers then were washed, digested, and analyzed for radioactivity and protein content.

²Significantly different from 0.95 mmol Mg/L, $P < 0.05$.

TABLE 6

⁴⁵Ca²⁺ efflux from endothelial cells cultured with various concentrations of *trans,trans* linoleic acid (*t,t*-18:2) and *cis,cis* linoleic acid (*c,c*-18:2n-6)¹

Fatty acid (μmol/L)	⁴⁵ Ca ²⁺ efflux		
	5 min	10 min	60 min
	<i>cpm/μg protein</i>		
0 <i>t,t</i> -18:2 + 100 <i>c,c</i> -18:2n-6	39.6 ± 9.0	41.1 ± 7.0	18.8 ± 2.1
100 <i>t,t</i> -18:2 + 0 <i>c,c</i> -18:2n-6	34.4 ± 2.1	35.5 ± 1.0	23.3 ± 2.5
75 <i>t,t</i> -18:2 + 25 <i>c,c</i> -18:2n-6	29.9 ± 3.4	31.5 ± 2.4	17.8 ± 1.8
50 <i>t,t</i> -18:2 + 50 <i>c,c</i> -18:2n-6	29.3 ± 4.1	28.8 ± 2.3	17.1 ± 1.2
25 <i>t,t</i> -18:2 + 75 <i>c,c</i> -18:2n-6	25.0 ± 2.6	31.6 ± 3.1	17.3 ± 1.7

¹ $\bar{x} \pm SE$ of 6 separate experiments. After human arterial endothelial cells were cultured with *t,t*-18:2 and *c,c*-18:2n-6 for 3 d, the cells were pulsed with ⁴⁵Ca²⁺ for 1 h. The monolayers then were washed, digested, and analyzed for radioactivity and protein content. There were no significant differences.

indicated that the concentration of homocysteine in plasma is associated with an increased risk of vascular disease (59). A significantly higher concentration ($P < 0.01$) of homocysteine was found in the plasma of young blacks and young Hispanics than in young non-Hispanic whites. In another recent study, Strong et al (60) found type II lesions in the abdominal aorta of 100% of adolescents tested; white adolescents had 1.4% calcification of the aorta. By young adulthood, the calcification had increased to 16.7% in blacks and to 12.6% in whites. Therefore, a high plasma homocysteine concentration, which results from an insufficient dietary intake of vitamin B complex vitamins, may also be a factor that increases the calcification of endothelial cells.

The atherosclerosis in infants noted by Seelig (50) may develop in coronary arteries containing only 2% 18:2n-6 in infants fed human milk high in *trans* fatty acids (25) and low in magnesium. Human milk has a high biological value but has a lower magnesium content than does cow milk: 40 and 130 μg/L, respectively (61). A MEDLINE search found 2365 studies on *trans* fatty acids since 1966. Some of these studies (62) were performed with the liquid-formula diet used by Ahrens et al (63, 64). This liquid formula, which provided 2500 kcal (10460 kJ)/d, lacked sufficient magnesium and contained only 96 mg/d, or <25% of the recommended dietary allowance (65). The magnesium content of the diets in previous studies of hydrogenated fats may be responsible for the observed differences.

Our study defines conditions under which *trans* fatty acids may be a risk factor in the development of atherosclerosis: 1) if there is a low 18:2n-6 concentration in the phospholipid fraction of the endothelial cell membrane, as we found in the arteries of umbilical cords and as Scott et al (49) found in the coronary arteries of infants aged <24 h; 2) if *trans* fatty acids are present in the diet; and 3) if there is insufficient magnesium in plasma. Infants receiving ≈850 kcal (3556 kJ)/d from human milk may only consume 40 mg Mg/d. This inadequate amount of magnesium plus the high content of *trans* fatty acids in human milk may enhance the development of type II lesions into type III lesions (35, 66). When there was an adequate amount of magnesium and *c,c*-18:2n-6 in our MEM, a higher than normal calcium influx into endothelial cells did not occur unless an excessive amount of *trans* fatty acids was added.

Studies in rats showed that *trans* fatty acids from hydrogenated fat did not transfer from the mothers to their young through the placenta (67) but rather from the mother's milk. We found only traces of *trans* fatty acids in human umbilical cords. When hydrogenated fat was removed from the diet of rats, the concentration of *trans* fatty acids decreased rapidly from the fat

tissue of both the mother and her nursing young (68). *trans* Fatty acids in human milk originate from *trans* fatty acids in hydrogenated fats. Lactating women would be well advised to increase their dietary magnesium intake and avoid ingesting hydrogenated fat containing a high concentration of *trans* fatty acids. Our study showed that a diet deficient in magnesium and containing a high amount of *t*-18:1n-9 and *t,t*-18:2 increases the calcification of endothelial cells. Such calcification is a common hallmark of atherosclerosis. 

We thank Mildred Seelig for providing information on arteriosclerosis in infants and Diana Schmidt for the MEDLINE search.

REFERENCES

1. ASCN/AIN Task Force on *Trans* Fatty Acids. Position paper on *trans* fatty acids. *Am J Clin Nutr* 1996;63:663-70.
2. Hu FB, Stampfer MJ, Manson JE, et al. Dietary fat intake and the risk of coronary heart disease in women. *N Engl J Med* 1997; 337:1491-9.
3. Troisi R, Willett WC, Weiss ST. *Trans*-fatty acid intake in relation to serum lipid concentrations in adult men. *Am J Clin Nutr* 1992; 56:1019-24.
4. Willett WC, Stampfer MJ, Manson JE, et al. Intake of *trans* fatty acids and risk of coronary heart disease among women. *Lancet* 1993;341:581-5.
5. Tzonou A, Kalandidi A, Trichopoulou A, et al. Diet and coronary heart disease: a case-control study in Athens, Greece. *Epidemiology* 1993;4:511-6.
6. Aro A, Kaardinaal AFM, Salminen I, et al. Adipose tissue isomeric *trans* fatty acids and risk of myocardial infarction in nine countries: the EURAMIC Study. *Lancet* 1995;345:273-8.
7. Mensink RP, Katan MB. Effect of dietary fatty acids on serum lipids and lipoproteins: a meta-analysis of 27 trials. *Arterioscler Thromb* 1992;12:911-9.
8. Mensink RP, Katan MB. Effect of dietary *trans* fatty acids on high-density and low-density lipoprotein cholesterol levels in healthy subjects. *N Engl J Med* 1990;323:439-45.
9. Zock PL, Katan MB. Hydrogenation alternatives: effect of *trans* fatty acids and stearic acid versus linoleic acid on serum lipid and lipoproteins in humans. *J Lipid Res* 1992;33:399-410.
10. Clevidence BA, Judd JT, Schaefer EJ, et al. Plasma lipoprotein (a) levels in men and women consuming diets enriched in saturated, *cis*-, or *trans*-monounsaturated fatty acids. *Arterioscler Thromb Vasc Biol* 1997;17:1657-61.
11. Judd JT, Clevidence BA, Muesing RA, Wittes J, Sunkin ME, Podczasy JJ. Dietary *trans* fatty acids: effects on plasma lipids and lipoproteins of healthy men and women. *Am J Clin Nutr* 1994;59:861-8.
12. Lichtenstein AH, Ausman LM, Carrasco W, Jenner JL, Ordovas JM, Schaefer EJ. Hydrogenation impairs the hypolipidemic effect of com

- oil in humans. hydrogenation. *trans* fatty acids. and plasma lipids. *Arterioscler Thromb* 1993;13:154-61.
13. Nestel PJ, Noakes M, Belling GB, et al. Plasma lipoprotein lipid and Lp(a) changes with substitution of elaidic acid for oleic acid in the diet. *J Lipid Res* 1992;33:1029-36.
 14. Khosla P, Hayes KC. Dietary *trans*-monounsaturated fatty acids negatively impact plasma lipids in humans: critical review of the evidence. *J Am Coll Nutr* 1996;15:325-39.
 15. Kris-Etherton PM. *Trans* fatty acids and coronary heart disease risk. Report of the Expert Panel on *Trans* Fatty Acids and Coronary Heart Disease. *Am J Clin Nutr* 1995;62(suppl):655S-708S.
 16. Katan MB. Commentary on the supplement *trans* fatty acids and coronary heart disease risk. *Am J Clin Nutr* 1995;62:518-9.
 17. Leveille GA. Commentary on *Trans* fatty acids and coronary heart disease risk. *Am J Clin Nutr* 1995;62:520-1.
 18. Nestel PJ. Comment on *Trans* fatty acids and coronary heart disease risk. *Am J Clin Nutr* 1995;62:522-3.
 19. Willett WC, Ascherio A. Response to the International Life Sciences Institute report on *trans* fatty acids. *Am J Clin Nutr* 1995;62:524-6.
 20. Kummerow FA. Viewpoint on the report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults. *J Am Coll Nutr* 1993;12:2-13.
 21. Enig M, Atal S, Keeney M, Sampugna J. Isomeric *trans* fatty acids in the US diet. *J Am Coll Nutr* 1990;9:471-86.
 22. Enig MG, Pallansch LA, Sampugna J, Keeney M. Fatty acid composition of the fat of selected food items with emphasis on *trans* components. *J Am Oil Chem Soc* 1983;60:1788-95.
 23. Johnston PV, Johnson OC, Kummerow FA. Occurrence of *trans* fatty acids in human tissue. *Science* 1957;126:698-9.
 24. Dotson KD, Jerrell JP, Picciano MF, Perkins EG. High-performance liquid chromatography of human milk triacylglycerols and gas chromatography of component fatty acids. *Lipids* 1992;27:933-9.
 25. Innis SM, King DJ, Werker J, Pegg J. Relation of n-6, n-3 and *trans* fatty acids to growth and visual acuity in exclusively breast fed infants. *FASEB J* 1998;12:A970 (abstr).
 26. Spector AA, Yorek MM. Membrane lipid composition and cellular function. *J Lipid Res* 1985;26:1015-35.
 27. Ferguson KA, Glaser M, Bayer WH, Vagelos PR. Alteration of fatty acid composition of LM cells by lipid supplementation and temperature. *Biochemistry* 1975;14:146-51.
 28. Glaser M, Ferguson KA, Vagelos PR. Manipulation of the phospholipid composition of tissue culture cells. *Proc Natl Acad Sci U S A* 1974;71:4072-6.
 29. Subbaiah PV, Subramanian VS, Liu M. *Trans* unsaturated fatty acids inhibit lecithin:cholesterol acyltransferase and alter its positional specificity. *J Lipid Res* 1998;39:1438-47.
 30. Engelhard VH, Esko JD, Storm DR, Glaser M. Modification of adenylate cyclase activity in LM cells by manipulation of the membrane phospholipid composition in vivo. *Proc Natl Acad Sci U S A* 1976;73:4482-6.
 31. Tongai A, Rayssiguier Y, Motta A, Gueux E, Maurois P, Heaton F. Mechanism of increased erythrocyte membrane fluidity during magnesium deficiency in weanling rats. *Am J Physiol* 1989;257:270-6.
 32. Mahfouz MM, Smith TL, Kummerow FA. Changes of linoleic acid metabolism and cellular phospholipid fatty acid composition in LLC-PK cells cultured at low magnesium concentrations. *Biochim Biophys Acta* 1989;1006:70-4.
 33. Mahfouz MM, Smith TL, Kummerow FA. Changes in phospholipid composition and calcium flux in LLC-PK cells cultured at low magnesium concentrations. *Biochim Biophys Acta* 1989;1006:75-83.
 34. Sary HC, Chandler AB, Dinsmore RE, et al. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis: a report from the Committee on Vascular Lesions of the Council on Arteriosclerosis. *American Heart Association. Arterioscler Thromb* 1995;15:1512-31.
 35. Sary HC, Chandler AB, Glagov S, et al. A definition of initial, fatty streak, and intermediate lesions of atherosclerosis: a report from the Committee on Vascular Lesions of the Council on Arteriosclerosis. *American Heart Association. Special report. Arterioscler Thromb* 1994;14:840-56.
 36. Janowitz WR, Agatston AS, Kaplan G, Viamonte M Jr. Differences in prevalence and extent of coronary artery calcium detected by ultrafast computed tomography in asymptomatic men and women. *Am J Cardiol* 1993;72:247-54.
 37. Wong ND, Kouwabunpat D, Vo AN, et al. Coronary calcium and atherosclerosis by ultrafast computed tomography in asymptomatic men and women: relation to age and risk factors. *Am Heart J* 1994;127:422-30.
 38. Zhou Q, Kummerow FA. The effects of magnesium deficiency on DNA and lipid synthesis in cultured umbilical arterial endothelial cells. *Magnes Res* 1995;8:145-50.
 39. Zhou Q, Kummerow FA. Modifying effect of 26-hydroxycholesterol on low-magnesium-induced atherosclerotic changes in the cultured human arterial smooth muscle cell. *Magnes Res* 1993;6:121-6.
 40. Ott L. An introduction to statistical methods and data analysis. 3rd ed. Boston: PWS-Kent Publishing Co, 1988:459.
 41. Brenner RR. The oxidative metabolism of unsaturated fatty acids. *Mol Cell Biochem* 1974;3:41-52.
 42. Mahfouz MM, Smith TL, Kummerow FA. Effect of dietary fats on desaturase activities and the biosynthesis of fatty acids in rat-liver microsomes. *Lipids* 1984;19:214-22.
 43. Mahfouz MM, Kummerow FA. Effect of magnesium deficiency on Δ^6 desaturase activity and fatty acid composition of rat liver microsomes. *Lipids* 1989;24:727-32.
 44. Rayssiguier Y, Gueux E, Motta C. Evidence for a membrane modification in magnesium nutritional deficiency in the rat: fluorescence polarization study. *Biomembr Nutr* 1989;195:441-52.
 45. Kummerow FA, Benga G, Holmes RP. Biomembranes and cell function. Vol 414. New York: National Academy of Sciences, 1983:44-58.
 46. Lipiello PM, Holloway CT, Garfield SA, Holloway PW. The effect of estradiol on stearyl-GA desaturase activity and microsomal membrane properties in rooster liver. *J Biol Chem* 1979;254:2004-9.
 47. Mahfouz MM, Valicenti AJ, Holman RT. Desaturation of isomeric *trans*-octadecenoic acid by rat liver microsomes. *Biochim Biophys Acta* 1980;618:1-12.
 48. Bickerstaffe R, Annison EF. Lipid metabolism in the perfused chicken liver. The uptake and metabolism of oleic acid, elaidic acid, *cis*-vaccenic acid, *trans*-vaccenic acid and stearic acid. *Biochem J* 1970;118:433-42.
 49. Scott RF, Florentin RA, Daoud AS, Morrison ES, Jones RM, Hutt MS. Coronary arteries of children and young adults. A comparison of lipids and anatomic features in New Yorkers and East Africans. *Exp Mol Pathol* 1966;5:12-42.
 50. Seelig MS. Magnesium deficiency in the pathogenesis of disease. New York: Plenum Press, 1980.
 51. Seelig MS, Berger AR. Range of normal serum magnesium values. *N Engl J Med* 1974;290:974-5.
 52. Seelig MS. Requirement of magnesium by the normal adult. *Am J Clin Nutr* 1964;14:342-90.
 53. Seelig MS, Heggveit HA. Magnesium interrelationships in ischemic heart disease: a review. *Am J Clin Nutr* 1974;27:59-79.
 54. Morgan KJ, Stampley GL, Zabik ME, Fisher DR. Magnesium and calcium dietary intakes of the US population. *J Am Coll Nutr* 1985;4:195-206.
 55. Karppanen H. Epidemiological studies on the relationship between magnesium intake and cardiovascular diseases. *Artery* 1981;9:190-9.
 56. Marier JR. Magnesium content of the food supply in the modern day world. *Magnesium* 1986;5:1-8.
 57. Hulea SA, Wasowicz E, Kummerow FA. Inhibition of metal-catalyzed oxidation of low-density lipoprotein by free and albumin-bound bilirubin. *Biochim Biophys Acta* 1995;1259:29-38.
 58. Olinescu R, Kummerow FA, Handler B, Fleischer L. The hemolytic activity of homocysteine is increased by the activated polymorphonuclear leukocytes. *Biochem Biophys Res Commun* 1996;226:912-6.

59. Jacques PF, Rosenberg IH, Rogers G, et al. Serum total homocysteine concentrations in adolescent and adult Americans: results from the third National Health and Nutrition Examination Survey. *Am J Clin Nutr* 1999;69:482-9.
60. Strong JP, Malcom GT, McMahan CA. Prevalence and extent of atherosclerosis in adolescents and young adults. *JAMA* 1999; 281:727-35.
61. Consumer and Food Economics Institute. Composition of foods, fats and oils. Agriculture handbook no. 8-4. Washington, DC: US Department of Agriculture, 1979.
62. Emken EA. Utilization and effects of isomeric fatty acids in humans. In: Emken EA, Dutton HJ, eds. Geometrical and positional fatty acid isomers. Champaign, IL: American Oil Chemists Society, 1979:99-123.
63. Ahrens E, Hirsh J, Insull W, Tsaltas T, Blomstrand R, Peterson M. The influence of dietary fats on serum-lipid levels in man. *Lancet* 1957;2:943-53.
64. Ahrens E. The use of liquid formula diets in metabolic studies: 15 years experience. *Adv Metab Disord* 1970;4:297-315.
65. National Research Council. Recommended dietary allowance. 10th ed. Washington, DC: National Academy Press, 1989.
66. Fangman RJ, Hellwig CA. Histology of coronary arteries in newborn infants. *Am J Pathol* 1947;23:901.
67. Johnston PV, Johnson OC, Kummerow FA. Non-transfer of *trans* fatty acids from mother to young. *Proc Soc Exp Biol Med* 1957;96:760-2.
68. Johnston PV, Johnson OC, Kummerow FA. Deposition in tissues and fecal excretion of *trans* fatty acids in the rat. *J Nutr* 1958; 65:13-23.

APPENDIX

Hugh Sinclair, a British biochemist, first called attention in 1938 to the possible influence of the trans fatty acids on the development of heart disease. In a paper published by the Journal of Nutrition in 1952 (1) we found that pregnant rats fed a diet which contained 10% hydrogenated fat (Crisco) gave birth to pups which died within 24 hours or died before birth, while pregnant rats fed 10% corn oil gave birth to live pups that were weaned at three weeks of age. This study established that the polyunsaturated fatty acids in corn oil were essential to life and that a hydrogenated fat did not contain these "essential" fatty acids in sufficient amounts. We also found that pregnant rats fed a diet which contained 5% hydrogenated fat plus 5% corn oil gave birth to pups that contained only traces of trans fatty acids in their body tissue. The trans fatty acids were furnished from milk in their mothers. It has recently been shown that the breast milk in Canadian women and women in the USA contained 5-17% trans fatty acids while the breast milk of women in China contained only traces of trans fatty acids. In 1957 we published a paper in Science (2) on the percentage of trans fatty acids in the heart and arteries of humans who died of natural causes and found 14 and 10% trans-acids respectively which was higher than we found in adipose (fat) tissue.

Du Pont et al. stated in their review: "*Trans* FA have physical properties like SFA making them rigid rather than fluid in membranes. *Trans* FA are absorbed as well as oleic acid, stored in adipose tissue proportional to the dietary source similar to other long chain FA and are transported and oxidized for energy in ways similar to other long chain FA (3)."

Soybean and cottonseed oil have been hydrogenated since 1920 and have served as a gradual replacement of lard in the baking of cakes, cookies and pies. It was not until the shortage of fats between 1940-1945 that margarine began to replace butter. Until the 1940s chemists in charge of quality control found it difficult to control the composition of hydrogenated fats as hydrogenation is carried out in 20,000-30,000-lb batches in stainless steel tanks. Very little was known before 1940 about how to control hydrogenation conditions so that one batch had the same SFA composition as another (4). The rate of stirring, temperature of reaction, hydrogen pressure, and number of times the catalyst was reused all determined the SFA content of the product. The instrumentation available, such as the gas chromatograph (GC), identified not only the amount of stearic, oleic and linoleic acid in the final product but also the amount of both geometrical and positional structures of the double bonds in the oleic, linoleic, and linolenic acid in the oils during hydrogenation. This knowledge was especially important to margarine production.

In 1968 Dr. Campbell Moses, medical director of the American Heart Association (AHA), appointed a five-member subcommittee on fats of the AHA nutrition committee to revise the 1961 version of "Diet and Heart Disease." As a member of this subcommittee I urged Dr. Moses to ask the Institute of Shortening and Edible Oils Inc. to have its member organizations decrease the amount of *trans* FA and increase the amount of essential fatty acids (EFA) in their shortenings and margarines. At the time it was known that an increase in EFA composition of a dietary fat would lower plasma cholesterol levels and there was strong evidence that *trans* FA increased plasma cholesterol levels. The first version stated:

“Partial hydrogenation of polyunsaturated fats results in the formation of *trans* forms which are less effective than *cis,cis* forms in lowering cholesterol concentrations. It should be noted that many currently available shortenings and margarines are partially hydrogenated and many contain little polyunsaturated fat of the natural *cis,cis* form.” The Institute of Shortening and Edible Oils Inc. objected to this version. The second and distributed version, omitting reference to *trans* and *cis* FA stated:

“Margarines that are high in polyunsaturates usually can be identified by the listings of a “liquid oil” first among the ingredients. Margarines and shortenings that are heavily hydrogenated or contain coconut oil, which is quite saturated, are ineffective in lowering the serum cholesterol.”

Our tissue culture data (5) indicate that *trans* fat combined with inadequate magnesium in MEM modifies the endothelial cell membrane permitting increased $^{45}\text{Ca}^{++}$ influx into endothelial cells. Cellular functions and responses are affected when the membrane fatty acid unsaturation is modified. Magnesium plays a vital role in this process. It acts as a cofactor in the activity of the Δ^5 , Δ^6 desaturation enzymes that are involved in fatty acid unsaturation of membrane. For example, in magnesium deficiency the fatty acid composition of liver microsomes indicated a slower rate of conversion of 18:2n-6 to 20:4n-6 which was consistent with the decrease of Δ^6 desaturase activity in liver microsomes of magnesium deficient rats. The decrease of Δ^6 desaturase activity was attributed to the lower concentration of enzyme molecules as a result of the decreased rate of protein synthesis in magnesium deficiency. We found that the *trans*-18:1 acids in partially hydrogenated soybean oil have a more inhibitory effect than saturated acids on essential fatty acid (EFA) metabolism. The relevance of the magnesium content of the

tissue culture medium to *trans* fatty acids may rest on the pronounced effects of magnesium on the physical state of membrane bilayer lipids. Defective membrane function could be the primary reason for the underlying cellular disturbance that occurs in magnesium deficiency. Our data indicate that the linoelaidic and elaidic acid contribute to the cellular disturbance of the membrane.

A previous study indicated that cells cultured in MEM with stearic acid contained significantly more 18:1n-9 than cells cultured on 18:2n-6 or 39.3 and 10.0% respectively. The increased concentration of oleic acid in cells cultured in stearic acid was necessary to maintain the physical state of the membrane. The physical state of the membrane can be conveniently determined by calculating the double bond index (DBI). The DBI is based on adding the percentages of unsaturated fatty acids multiplied by the number of double bonds in each unsaturated fatty acid. Hypothetically if one considers *tt* linoelaidic acid as an unsaturated fatty acid and both *trans* and *cis* fatty acids are incorporated into a cell membrane, the DBI of the lipid extracted from the ECs cultured with *tt* linoelaidic would be higher than from the ECs cultured with *cc* linoleic acid or 135.92 and 126.01 respectively. However, Mahfouz et al. (6) have shown that *trans* acids have physical properties similar to stearic acid and are inserted into acyl lipids more like a saturated acid than as an unsaturated acid. The acyl transferase in rat liver microsomes discriminates between the normal *cis* isomers and the unnatural *trans* forms with respect to their esterification in phospholipids and triglycerides, and a chain containing *trans* double bonds is transferred as if it were a saturated carbon chain. It is evident that the influence of *trans* fatty acids on the physical state of the membranes of ECs cultured in a low magnesium medium requires further study.

Although the ECs freshly isolated from the umbilical cord contained only 1.2% 18:2n-6, they contained 12.8% 20:4-n6 and more n-6 and n-9 unsaturated fatty acids than did the ECs cultured for 3 d. The double bond index was 142.8 and 54.7 respectively indicating a significant change in membrane fluidity between freshly isolated and the cultured cells. Scott et al. (7) found only 2.4% linoleic acid in the phospholipid fraction of the lipids extracted from the coronary arteries of infants less than 24 h old. This is comparable to the 1.2% *cc* linoleic acid we found in the ECs freshly isolated from the arteries of umbilical cords. Scott et al. also found that linoleic increased from 2.4% to 4.8 % and arachidonic acid increased from 11.0 to 19.0 % in infants and in adolescents respectively.

In 1980 Seelig, in her book devoted to the role of magnesium deficiency in cardiovascular pathogenesis, listed over a thousand references to infantile ischemic heart disease, primarily due to arteriosclerosis in the coronary arteries (8). A MEDLINE search revealed that 195 additional articles dealing with arteriosclerosis in infants have been published since 1980. Seelig has called attention to the low magnesium content of the U.S. diet, and a number of other studies have suggested that the magnesium intake is inadequate in the U.S. population. Sary et al. (9) found fatty streaks (Type II lesion) in the aorta of 99% of infants and adolescents tested. According to Sary et al., Type II lesions could develop into the atherosclerotic Type III lesion. This may be governed by plasma factors such as an excessive presence of hematin and homocysteine. A recent study indicated that the concentration of homocysteine in the plasma was associated with an increased risk of vascular disease. A significantly higher concentration ($P < 0.01$) of homocysteine was found in the plasma of young African Americans and young Hispanics

medium when both an adequate level of magnesium and *cc* linoleic acid were present, increased calcium influx into ECs did not occur unless an excessive amount of *trans* fatty acid was added.

REFERENCES

1. Kummerow FA, Pan HP, and Hickman H. The effect of dietary fat reproductive performance and the mixed fatty acid composition of fat-deficient rats. *J Nutr* 46; 489-498 (1952).
2. Johnston PV, Johnson OC, and Kummerow FA. Non-transfer of *trans* fatty acids from mother to young. *Proc Soc Exp Biol Med* 96; 760-762 (1957).
3. Du Pont J, White PJ, Feldman EB. Saturated and hydrogenated fats in food in relation to health. *J Am Coll Nutr* 10; 577-592 (1991).
4. Kummerow FA. Viewpoint on the Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults. *J Am Coll Nutr* 12; 2-13 (1993).
5. Kummerow FA. Effect of *trans* fatty acids on calcium influx into human arterial endothelial cells. *Am J Clin Nutr* 70; 832-838 (1999).
6. Mahfouz MM, Valicenti AJ, Holman RT. Desaturation of isomeric *trans*-octadecenoic by rat liver microsomes. *Biochim Biophys Acta* 618; 1-12 (1980).
7. Scott RF, Florentin RA, Daoud AS, Morrison ES, Jones RM, Hutt MS. Coronary arteries of children and young adults. A comparison of lipids and anatomic features in New Yorkers and East Africans. *Exp Mol Pathol* 5; 12-42 (1966).

8. Seelig MS. Magnesium deficiency in the pathogenesis of disease. New York: Plenum Press, 1980.
9. Stary HC, Chandler AB, Glagov S, et al. A definition of initial, fatty streak, and intermediate lesions of atherosclerosis: a report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Special report. *Arterioscler Thromb* 14; 840-856 (1994).
10. Strong JP, Malcom GT, McMahan CA. Prevalence and extent of atherosclerosis in adolescents and young adults. *JAMA* 281; 727-735 (1999).

Breast Milk Fatty Acid Composition: A Comparative Study Between Hong Kong and Chongqing Chinese

Z.Y. Chen^{a,*}, K.Y. Kwan^a, K.K. Tong^a, W.M.N. Ratnayake^b, H.Q. Li^c, and S.S.F. Leung^d

^aDepartment of Biochemistry, and ^dDepartment of Paediatrics, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong; ^bNutrition Research Division, Health Protection Branch, Health Canada, Tunney's Pasture, Ottawa, Canada K1A0L2; and ^cUniversity of Chongqing Medical Sciences, Chongqing, Si Chuan, China

ABSTRACT: The fatty acids of milk samples obtained from 51 Hong Kong Chinese and 33 Chongqing Chinese (Si Chuan Province, China) were analyzed by gas-liquid chromatography. Compared with those of published data for Canadian and other Western countries, the Chinese milk from both Hong Kong and Chongqing contained higher levels of longer-chain polyunsaturated fatty acids, particularly docosahexaenoic acid (22:6n-3) and arachidonic acid (20:4n-6). In contrast, the content of *trans* fatty acids in the Chinese milk was lower compared with those for Canadian and other Western countries. Longitudinally, the concentrations of 22:6n-3 and 20:4n-6 gradually decreased when lactation progressed from colostrum (week 1) to mature (week 6). Over the same interval, linoleic acid (18:2n-6) remained unchanged in Chongqing Chinese but significantly increased in Hong Kong Chinese. Unlike 18:2n-6, linolenic acid (18:3n-3) increased in Chongqing Chinese but remained unchanged in Hong Kong Chinese throughout the study. The total milk fat also increased with the duration of lactation. In addition, the milk of Chongqing Chinese had higher total milk fat than that of Hong Kong Chinese and Canadians. The content of erucic acid (22:1n-9) increased with the progression of lactation in Chongqing Chinese, indicating that there was a switch in dietary consumption from fats of animal origin to rapeseed oil when lactation reached week 6. The present study showed that Hong Kong and Chongqing Chinese had a different fatty acid profile in many ways, which largely reflected a different dietary habit and life-style in these two places.

Lipids 32, 1061-1067 (1997).

Human milk is considered the optimal form of nutrition for infants. The importance of n-6 and n-3 longer-chain polyunsaturated fatty acids (LCP) in human milk for normal brain development, especially during early life, has been emphasized in many studies (1-3). Both human milk and infant formula contain precursor essential fatty acids, linoleic acid (18:2n-6), and linolenic acid (18:3n-3). LCP such as arachidonic acid (20:4n-6) and docosahexaenoic acid (22:6n-3, DHA) are, however, naturally present in milk but they are

generally absent in current infant formulas. These LCP are important for the structural development of the nervous system and are accumulated in large amounts in the developing brain and retina (4,5). There has been increasing interest in adding these LCP to infant formulas (6-10) because formula-fed preterm infants have lower erythrocyte levels of DHA than breast milk-fed preterm infants (1,2), and because the lower tissue levels of this fatty acid are associated with reduced retinal function (11) and reduced visual acuity of preterm infants (12).

The LCP composition in the tissues of growing infants is largely determined by the LCP content of the milk (1), which in turn partially reflects the fatty acid composition in the maternal diet (13-16). Although the fatty acid composition of milk has been reported in many countries (14-23), there is very limited information on fatty acid composition of milk in Chinese (24). The objective of the present study was therefore to investigate if fatty acid composition of milk in Hong Kong Chinese was significantly different from that in Chongqing Chinese, because the diets in the former are partially westernized and in the latter are still traditional.

MATERIALS AND METHODS

Sample and dietary data collection. Fifty-one and 33 lactating women from Hong Kong and Chongqing (Si Chuan Province, China), respectively, were recruited for this study. All mothers were on their usual diets; no dietary modifications were proposed. Donors were requested to express their milk manually after a feed on the assigned dates. The milk was collected at the first 3 d (colostrum milk) followed by 2, 4, and 6 wk after delivery. Milk (10-15 mL) was sampled each time in a bottle washed previously with deionized water. The milk samples were stored immediately at -20°C.

Dietary information was obtained by self-record on blank sheets over 3 d prior to each milk collection. At the beginning of the study, each subject was requested to record the amount of food and drink she consumed. In brief, the mothers were shown the common measuring kitchen utensils, for examples, standard bowl and tablespoon. They were also requested to record the brand name and size of commercially available products they consumed. The total energy and nutrient intake

*To whom correspondence should be addressed.

E-mail: zhenyuchen@cuhk.edu.hk.

Abbreviations: LCP, longer-chain polyunsaturated fatty acids; DHA, docosahexaenoic acid.

were computed using a food composition data base, Nutritionist IV (First Databank, San Bruno, CA). The protocol was approved by the Committee of Human Ethics, the Chinese University of Hong Kong, and signed consent was obtained from each milk donor.

Milk lipid extraction. Milk samples were thawed at room temperature (25°C). Fat from a 2-g sample was extracted using 15 vol of CHCl₃/MeOH (2:1, vol/vol) containing 1.5 mg/mL triheptadecanoin as an internal standard to quantify total milk fat (13). Another 2-g sample was extracted with the same solvent system but without addition of triheptadecanoin to determine the content of endogenous heptadecanoic acid in milk.

Fatty acid analysis. The milk lipids were converted to fatty acid methyl esters using a mixture of 14% BF₃/MeOH reagent (Sigma Chemical Co., St. Louis, MO) and toluene (1:1, vol/vol) at 90°C for 45 min under nitrogen. Fatty acid methyl esters were analyzed by gas-liquid chromatography using an SP-2560 flexible fused-silica capillary column (100 m × 0.25 mm i.d., 20 μm film thickness; Supelco, Inc., Bellefonte, PA) in a Hewlett-Packard 5890 series II gas chromatograph equipped with a flame-ionization detector (Palo Alto, CA). Column temperature was programmed from 150 to 180°C at a rate of 0.5°C/min, and then to 210°C at a rate of 3°C/min. Injector and detector temperatures were 250 and 300°C, respectively. Hydrogen was used as the carrier gas at a head pressure of 20 psi. The gas chromatographic trace of the fatty acid methyl esters' profile of human milk is shown in Figure 1.

Statistics. Data were expressed as mean ± standard deviation. Analysis of variance followed by Student's *t*-test (two-tailed) where applicable was used for statistical evaluation of significant differences between groups.

RESULTS

The total energy intake between Chongqing and Hong Kong lactating women was significantly different (Table 1). The percentage energy intake from fat and protein was significantly higher in Chongqing than in Hong Kong women while the percentage energy derived from carbohydrate was higher in the latter. Traditionally, Chongqing Chinese stay and eat at home after delivery for at least a month to replenish their nutrient stores. Their dietary fat was usually of animal origin including chicken and pork together with 4–10 eggs/d (Table 1). They consumed no dairy products and fruits. In contrast, Hong Kong Chinese lactating women consumed milk and other dairy products, more fruits, and lesser amounts of vegetables than the Chongqing women (Table 1).

The average fatty acid composition of milk pooled over all stages of lactation in Chongqing and Hong Kong Chinese lactating women is shown in Table 2. For comparison, the data published by us for Canadian women are also shown in Table 2 (13). LCP including 22:6n-3 and 20:4n-6 in the milk of both Hong Kong and Chongqing Chinese were higher than those in the milk from Canadian women and milk from the majority of women from other Western countries. In contrast, the milk from both Hong Kong and Chongqing Chinese had

TABLE 1
Dietary Intake of Lactating Women in Hong Kong and Chongqing, Sichuan, China^a

	Hong Kong (n = 51)	Chongqing (n = 33)
Energy (kcal/d)	1809 ± 392	2686 ± 1030 ^b
Carbohydrate (g/d)	247 ± 60	199 ± 125 ^b
%kcal	54 ± 7	31 ± 15 ^b
Protein (g/d)	77 ± 18	196 ± 99 ^b
%kcal	17 ± 2	29 ± 7 ^b
Fat (g/d)	58 ± 19	117 ± 58 ^b
%kcal	28 ± 7	39 ± 9 ^b
Food items (g/d) ^c		
Cooked rice	404	590
Eggs	50	340
Chicken	44	220
Pork	29	54
Fish	26	11
Vegetables	75	278
Fruits (apple and orange)	240	0
Milk	228	0
Bread	53	0

^aValues are mean ± SD. The dietary data (for 3 d) were collected every time before the milk sample was collected.

^bMeans in the same row with different superscripts differ significantly at *P* < 0.05.

^cNo standard deviation could be given because the calculation was made by summing total consumption of each particular food item by all mothers and then dividing by the number of mothers.

lower contents of *trans* fatty acids than that from women of Canada and other Western countries. However, the total *trans* fatty acids in the milk of Hong Kong Chinese were significantly higher than in that of Chongqing Chinese.

Several differences in the milk fatty acid profiles were observed between Hong Kong Chinese and Chongqing Chinese women (Table 2). The milk in Hong Kong Chinese women contained more n-6 fatty acids, including mainly 18:2n-6. Compared with that of the Chongqing Chinese women, the milk of the Hong Kong Chinese women had less *cis*-monounsaturated fatty acids including 18:1n-9, 16:1n-7, 20:1n-9, and 22:1n-9. Among saturates, 16:0, 18:0, 12:0, and 10:0 were lower while 14:0 was higher in the milk of Hong Kong Chinese women than these in Chongqing Chinese women (Table 2). However, there appeared to be no difference in total n-3 fatty acids between these two places.

As lactation progressed in Chongqing and Hong Kong Chinese women, the milk n-6 LCP decreased although their precursor fatty acid, 18:2n-6, remained unchanged in Chongqing Chinese women or significantly increased in Hong Kong Chinese women (Table 3). These n-6 LCP include 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6, and 22:5n-6 (Table 3). Similarly, the milk n-3 LCP including 22:6n-3 and 22:5n-3 also decreased gradually with the duration of lactation although the precursor fatty acid, 18:3n-3, increased in the milk of Chongqing Chinese women. Among saturated fatty acids, 16:0 and 14:0 progressively decreased in the milk of both Chongqing and Hong Kong Chinese women. In contrast, 18:0 and 12:0 increased in the milk of Hong Kong Chinese women, but they decreased in

TABLE 2
Average Fatty Acid Composition (wt% total fatty acids) of Milk Samples Pooled over All Stages of Lactation in Hong Kong and Chongqing Chinese Women Compared with That of Canadian Women^a

Fatty acids	Chongqing (n = 33)	Hong Kong (n = 51)	Canadian ^b (n = 198)
Saturated			
10:0	0.81 (0.40) ^b	0.53 (0.38) ^c	1.39 (0.59) ^a
12:0	4.45 (2.14) ^b	4.23 (2.15) ^b	5.68 (2.01) ^a
14:0	4.22 (2.16) ^c	5.50 (2.01) ^b	6.10 (1.73) ^a
15:0	0.16 (0.05) ^c	0.31 (0.10) ^b	0.37 (0.12) ^a
16:0	22.79 (2.01) ^a	21.29 (2.27) ^b	18.30 (2.25) ^b
17:0	0.25 (0.07)	0.32 (0.08)	0.32 (0.08)
18:0	7.74 (1.39) ^a	5.86 (0.98) ^c	6.15 (0.97) ^b
20:0	0.20 (0.05) ^a	0.20 (0.15) ^a	0.15 (0.09) ^b
Cis-monounsaturated			
14:1n-5	.01 (0.01) ^b	0.01 (0.02) ^b	0.28 (0.08) ^a
16:1n-7	2.46 (0.63) ^a	2.23 (0.59) ^b	2.27 (0.56) ^b
16:1n-9	0.59 (0.08) ^a	0.56 (0.09) ^a	0.41 (0.13) ^b
17:1n-7	0.19 (0.03)	0.22 (0.07)	0.21 (0.06)
18:1n-9	37.15 (3.28) ^a	34.40 (3.90) ^b	30.65 (2.66) ^c
Total other 18:1c	2.25 (0.30) ^b	2.02 (0.78) ^b	4.22 (0.38) ^a
20:1n-9	0.97 (0.41) ^a	0.79 (0.29) ^a	0.39 (0.13) ^c
20:1n-7	0.01 (0.01) ^b	0.03 (0.04) ^b	0.14 (0.09) ^a
22:1n-9	0.58 (0.55) ^a	0.16 (0.14) ^b	0.02 (0.03) ^c
n-6 Polyunsaturated			
18:2n-6	10.44 (2.45) ^b	15.80 (3.01) ^a	10.47 (2.62) ^b
18:3n-6	0.11 (0.08) ^a	0.15 (0.09) ^a	0.08 (0.06) ^b
20:2n-6	0.44 (0.14) ^b	0.68 (0.32) ^a	0.17 (0.37) ^c
20:3n-6	0.37 (0.12) ^a	0.41 (0.16) ^a	0.26 (0.09) ^b
20:4n-6	0.76 (0.24) ^a	0.61 (0.18) ^a	0.35 (0.11) ^b
22:4n-6	0.17 (0.09) ^c	0.23 (0.20) ^a	0.04 (0.05) ^c
22:5n-6	0.10 (0.08) ^a	0.09 (0.11) ^a	0.01 (0.02) ^b
n-3 Polyunsaturated			
18:3n-3	1.17 (0.53)	1.24 (0.54)	1.16 (0.37)
20:5n-3	0.04 (0.05) ^b	0.08 (0.09) ^a	0.05 (0.05) ^b
22:5n-3	0.22 (0.08) ^a	0.23 (0.14) ^a	0.08 (0.05) ^b
22:6n-3	0.54 (0.20) ^a	0.56 (0.23) ^a	0.14 (0.10) ^b
Trans			
14:1t	<0.01	<0.01	0.09 (0.05)
16:1t	<0.01	<0.01	0.18 (0.08)
Total 18:1t	0.20 (0.04) ^c	0.70 (0.51) ^b	5.87 (2.52) ^a
Total 18:2t	0.02 (0.03) ^c	0.18 (0.14) ^b	0.94 (0.42) ^a
Total saturated	40.49 (5.14) ^a	38.07 (4.56) ^b	38.50 (2.94) ^b
Total C ₁₀ -C ₁₃	5.26 (2.43) ^b	4.76 (2.45) ^c	7.11 (2.53) ^a
Total C ₁₄ -C ₁₆	26.80 (3.84) ^a	26.79 (3.14) ^a	24.77 (3.26) ^b
Total n-6	12.38 (2.45) ^b	17.97 (2.94) ^a	11.39 (3.18) ^b
Total n-3	2.12 (0.55) ^a	2.17 (0.74) ^a	1.49 (0.44) ^b
Total n-6 LCP	1.83 (0.51) ^a	2.02 (0.74) ^a	0.83 (0.28) ^b
Total n-3 LCP	0.96 (0.27) ^c	0.92 (0.45) ^a	0.33 (0.24) ^b
Total trans	0.22 (0.06) ^c	0.88 (0.61) ^b	7.19 (3.03) ^a
Total milk fat (g/L)	38.1 (17.1) ^a	32.4 (21.9) ^b	31.6 (9.4) ^b

^aData are mean (SD). Means in the same row with different superscripts (a-c) differ significantly at $P < 0.01$. Abbreviation: LCP, longer-chain polyunsaturated fatty acids.

^bAdapted from Reference 13; milk samples were obtained during 3-4 wk of lactation.

that of Chongqing Chinese women (Table 3). With *cis*-monounsaturated fatty acids, 18:1n-9 remained unchanged in the milk of Hong Kong Chinese women, but it increased in that of Chongqing Chinese women (Table 3). In the milk of Chongqing Chinese women, 20:1n-9 and 22:1n-9 increased but they decreased in that of Hong Kong Chinese women.

The total milk fat was determined by the amount of triheptadecanoin added. As can be seen in Table 2, the total milk fat was very similar for Hong Kong Chinese and Canadian

women, but it was significantly higher in the milk of Chongqing Chinese women. The total milk fat increased significantly when lactation progressed from colostrum (week 1) to week 6 in both Chongqing and Hong Kong Chinese women.

DISCUSSION

Chongqing and Hong Kong Chinese have different dietary habits and life-styles. Populations in Chongqing have a more

TABLE 3
Milk Fatty Acid Composition (wt% total fatty acids) of Hong Kong and Chongqing Chinese Women
at Different Stages of Lactation*

Fatty acids		Colostrum	Week 2	Week 4	Week 6
Saturated					
10:0	Chongqing	0.69 (0.39) ^b	0.94 (0.45) ^a	0.88 (0.36) ^{a,b}	0.76 (0.33) ^b
	Hong Kong	0.33 (0.29) ^{b,b}	0.66 (0.31) ^{a,b}	0.61 (0.39) ^{a,b,b}	0.61 (0.45) ^{a,b}
12:0	Chongqing	4.85 (2.47) ^{a,b}	5.21 (2.55) ^a	4.08 (1.34) ^{a,b}	3.46 (0.97) ^b
	Hong Kong	3.01 (1.93) ^{b,b}	5.32 (1.76) ^a	4.73 (1.78) ^a	4.30 (2.28) ^a
14:0	Chongqing	5.22 (2.60) ^a	4.60 (2.33) ^{a,b}	3.53 (1.17) ^b	3.19 (1.05) ^b
	Hong Kong	5.35 (2.02) ^{a,b}	6.04 (1.94) ^{a,b}	5.48 (2.03) ^{a,b,b}	4.94 (1.83) ^{b,b}
15:0	Chongqing	0.18 (0.04) ^a	0.16 (0.04) ^b	0.16 (0.03) ^b	0.14 (0.06) ^b
	Hong Kong	0.32 (0.09) ^b	0.31 (0.11) ^b	0.30 (0.10) ^b	0.30 (0.10) ^b
16:0	Chongqing	23.25 (1.68) ^a	23.30 (1.67) ^a	23.08 (1.87) ^a	21.45 (2.23) ^b
	Hong Kong	22.64 (2.47) ^a	20.66 (2.02) ^{b,b}	20.68 (1.58) ^{b,b}	20.33 (1.57) ^{b,b}
17:0	Chongqing	0.28 (0.08)	0.25 (0.06)	0.26 (0.06)	0.24 (0.07)
	Hong Kong	0.33 (0.09)	0.32 (0.08)	0.31 (0.07)	0.31 (0.06)
18:0	Chongqing	7.02 (1.19) ^b	8.12 (1.46) ^a	7.95 (1.41) ^a	8.03 (1.14) ^a
	Hong Kong	5.98 (1.07) ^b	5.73 (0.97) ^b	5.77 (0.74) ^b	5.96 (1.02) ^b
20:0	Chongqing	0.19 (0.05)	0.20 (0.05)	0.19 (0.03)	0.22 (0.05)
	Hong Kong	0.18 (0.09)	0.19 (0.07)	0.20 (0.09)	0.21 (0.07)
Cis-monounsaturated					
16:1n-7	Chongqing	2.43 (0.53)	2.42 (0.62)	2.75 (0.67)	2.28 (0.61)
	Hong Kong	1.85 (0.38) ^{b,b}	2.44 (0.55) ^a	2.51 (0.52) ^a	2.34 (0.64) ^a
16:1n-9	Chongqing	0.63 (0.07) ^a	0.61 (0.06) ^{a,b}	0.59 (0.06) ^b	0.52 (0.08) ^c
	Hong Kong	0.59 (0.06) ^{a,b}	0.54 (0.09) ^{b,b}	0.55 (0.10) ^b	0.53 (0.08) ^b
17:1n-7	Chongqing	0.19 (0.03)	0.18 (0.02)	0.19 (0.02)	0.21 (0.04)
	Hong Kong	0.19 (0.07)	0.23 (0.05)	0.26 (0.07)	0.23 (0.06)
18:1n-9	Chongqing	36.27 (3.79) ^b	36.51 (3.42) ^b	37.79 (2.65) ^{a,b}	38.36 (2.52) ^a
	Hong Kong	34.60 (4.76)	34.02 (3.63) ^{a,b}	34.17 (3.39) ^b	34.92 (2.63) ^b
Other 18:1c	Chongqing	2.32 (0.28) ^a	2.15 (0.24) ^b	2.24 (0.32) ^{a,b}	2.27 (0.31) ^{a,b}
	Hong Kong	2.36 (0.92)	2.07 (0.75)	2.00 (0.73)	2.35 (0.28)
20:1n-9	Chongqing	0.89 (0.25) ^b	0.77 (0.23) ^b	0.86 (0.32) ^b	1.39 (0.44) ^a
	Hong Kong	0.99 (0.27) ^a	0.73 (0.25) ^b	0.66 (0.19) ^{b,b}	0.68 (0.21) ^{b,b}
22:1n-9	Chongqing	0.37 (0.26) ^b	0.28 (0.31) ^b	0.41 (0.24) ^b	1.29 (0.52) ^a
	Hong Kong	0.25 (0.18) ^{a,b}	0.13 (0.08) ^{b,b}	0.12 (0.10) ^{b,b}	0.10 (0.08) ^{b,b}
n-6 Polyunsaturated					
18:2n-6	Chongqing	10.30 (2.18)	9.76 (1.78)	10.43 (2.21)	11.34 (3.17)
	Hong Kong	14.85 (2.84) ^{c,b}	15.47 (2.79) ^{b,c,b}	16.75 (2.89) ^{b,b}	17.10 (2.95) ^{a,b}
18:3n-6	Chongqing	0.11 (0.10)	0.09 (0.06)	0.12 (0.06)	0.14 (0.03)
	Hong Kong	0.09 (0.07) ^b	0.18 (0.10) ^{a,b}	0.18 (0.09) ^{a,b}	0.17 (0.07) ^{a,b}
20:2n-6	Chongqing	0.51 (0.09) ^a	0.40 (0.13) ^b	0.39 (0.09) ^b	0.43 (0.09) ^b
	Hong Kong	0.95 (0.23) ^{a,b}	0.60 (0.39) ^{b,b}	0.51 (0.09) ^{b,b}	0.48 (0.01) ^{b,b}
20:3n-6	Chongqing	0.48 (0.09) ^a	0.39 (0.13) ^b	0.34 (0.07) ^c	0.26 (0.08) ^d
	Hong Kong	0.50 (0.17) ^a	0.39 (0.13) ^b	0.36 (0.14) ^b	0.34 (0.11) ^{b,b}
20:4n-6	Chongqing	0.84 (0.24) ^a	0.84 (0.20) ^{a,b}	0.79 (0.23) ^b	0.53 (0.16) ^c
	Hong Kong	0.71 (0.19) ^{a,b}	0.60 (0.17) ^{a,b,b}	0.56 (0.16) ^{b,c,b}	0.52 (0.10) ^c
22:4n-6	Chongqing	0.23 (0.09) ^a	0.19 (0.07) ^{a,b}	0.16 (0.06) ^b	0.10 (0.05) ^c
	Hong Kong	0.39 (0.25) ^{a,b}	0.16 (0.08) ^b	0.13 (0.09) ^b	0.14 (0.08) ^b
22:5n-6	Chongqing	0.12 (0.08) ^a	0.11 (0.08) ^a	0.10 (0.07) ^a	0.04 (0.03) ^b
	Hong Kong	0.11 (0.05)	0.07 (0.06) ^b	0.08 (0.07)	0.08 (0.07)
n-3 Polyunsaturated					
18:3n-3	Chongqing	0.85 (0.28) ^c	0.96 (0.24) ^c	1.21 (0.38) ^b	1.73 (0.61) ^a
	Hong Kong	1.27 (0.42) ^b	1.15 (0.57)	1.23 (0.64)	1.37 (0.50) ^b
20:5n-3	Chongqing	0.01 (0.03)	0.02 (0.03)	0.04 (0.04)	0.03 (0.05)
	Hong Kong	0.07 (0.14) ^a	0.05 (0.01) ^{a,b}	0.05 (0.13) ^{a,b}	0.02 (0.04) ^b
22:5n-3	Chongqing	0.23 (0.09)	0.22 (0.06)	0.23 (0.10)	0.20 (0.06)
	Hong Kong	0.28 (0.17) ^a	0.22 (0.13) ^{a,b}	0.19 (0.10) ^b	0.18 (0.07) ^b
22:6n-3	Chongqing	0.64 (0.16) ^a	0.61 (0.17) ^{a,b}	0.54 (0.18) ^b	0.35 (0.14) ^c
	Hong Kong	0.60 (0.24) ^a	0.58 (0.25) ^a	0.53 (0.23) ^{a,b}	0.48 (0.15) ^{b,b}
Trans					
18:1t	Chongqing	0.18 (0.04) ^b	0.20 (0.04) ^b	0.20 (0.02) ^b	0.23 (0.05) ^a
	Hong Kong	0.66 (0.28) ^b	0.71 (0.28) ^b	0.69 (0.54) ^b	0.75 (0.51) ^b
18:2t	Chongqing	0.01 (0.02)	0.02 (0.02)	0.02 (0.03)	0.02 (0.03)
	Hong Kong	0.15 (0.012) ^b	0.20 (0.16) ^b	0.20 (0.16) ^b	0.21 (0.12) ^b

(continued)

TABLE 3 (continued)

Fatty acids		Colostrum	Week 2	Week 4	Week 6
Total saturated	Chongqing	41.35 (5.62) ^a	42.63 (4.90) ^a	39.95 (3.91) ^{a,b}	37.37 (3.90) ^b
	Hong Kong	37.94 (4.55) ^b	39.08 (4.43) ^b	37.98 (4.81)	36.80 (3.99)
Total C ₁₀ -C ₁₃	Chongqing	5.55 (2.78) ^a	6.15 (2.93) ^a	4.96 (1.63) ^{a,b}	4.22 (1.16) ^b
	Hong Kong	3.34 (2.17) ^{b,b}	5.97 (1.96) ^a	5.34 (2.06) ^a	4.91 (2.65) ^a
Total C ₁₄ -C ₁₆	Chongqing	28.28 (3.01) ^a	27.89 (2.53) ^{a,b}	26.61 (2.43) ^b	24.65 (2.84) ^c
	Hong Kong	28.00 (3.25) ^a	26.71 (3.04) ^{a,b}	26.17 (3.05) ^b	25.28 (1.99) ^b
Total n-6	Chongqing	12.58 (2.29)	11.78 (1.88)	12.33 (2.25)	12.83 (3.09)
	Hong Kong	17.59 (2.79) ^b	17.47 (2.99) ^b	18.58 (2.86) ^b	18.82 (2.84) ^b
Total n-3	Chongqing	1.87 (0.39) ^c	1.96 (0.35) ^{b,c}	2.19 (0.51) ^b	2.54 (0.65) ^a
	Hong Kong	2.33 (0.61) ^b	2.08 (0.85)	2.05 (0.83)	2.12 (0.60) ^b
Total n-6 LCP	Chongqing	2.18 (0.44) ^a	1.93 (0.43) ^b	1.78 (0.42) ^b	1.36 (0.33) ^c
	Hong Kong	2.65 (0.78) ^a	1.82 (0.55) ^b	1.65 (0.37) ^{b,c}	1.55 (0.29) ^c
Total n-3 LCP	Chongqing	1.02 (0.26) ^a	1.00 (0.23) ^a	0.98 (0.29) ^a	0.81 (0.23) ^b
	Hong Kong	1.07 (0.48) ^a	0.93 (0.47) ^{a,b}	0.82 (0.41) ^b	0.75 (0.25) ^b
Total <i>trans</i>	Chongqing	0.19 (0.25) ^b	0.21 (0.04) ^b	0.22 (0.04) ^b	0.26 (0.05) ^a
	Hong Kong	0.81 (0.30) ^b	0.91 (0.79) ^b	0.89 (0.67) ^b	0.97 (0.49) ^b
Total milk fat (g/L)	Chongqing	30.1 (11.8) ^b	39.7 (14.9) ^a	41.3 (19.7) ^a	43.5 (18.6) ^a
	Hong Kong	23.9 (16.1) ^b	38.7 (22.6) ^a	33.8 (19.8) ^a	37.3 (23.6) ^a

^aData are means (SD). Means in the same row with different superscripts (a-d) differ significantly at $P < 0.01$.

^bDifference between Chongqing and Hong Kong is significant at $P < 0.01$.

traditional life-style where people have to rely on foods provided by the nearby farms. The general belief is that for the first month postpartum, women have to replenish their body nutrient stores by consuming more eggs, chicken, and pork. Eating more than eight eggs and 200 g chicken meat per day is not uncommon during the first month after delivery. This unique dietary habit leads to a very high intake of protein and fat in Chongqing women. Fruits are considered to be "cold" food and not commonly consumed during the first month. Milk and dairy products are never food items during pregnancy and lactation. In Hong Kong, however, people have a sedentary life-style and the diet is partly westernized. On average, a lactating Hong Kong Chinese woman drinks a glass of milk and eats a slice of bread for breakfast. During pregnancy and lactation, Hong Kong women consume more fruits than Chongqing Chinese women (Table 1). Furthermore, the amounts of rice, eggs, and meat eaten are not as much as for Chongqing Chinese women because of a lower requirement of energy expenditure in physical activity. This dietary habit clearly contrasts to what was observed for Chongqing women (Table 1). As shown in Table 1, the total energy intake in Hong Kong lactating women was only 1809 kcal/d which is consistent with their desire to get back to their prepregnant body shapes by consuming less total energy.

The present study showed that 22:6n-3 and 20:4n-6 levels in Chinese milk were higher than what has been reported in the majority of Western countries (20,21,25-27). The present values were similar to those in Japan (7), Africa (28), and other Asian countries (29). In some cases, the milk content of 22:6n-3 in Chinese women from fishery areas could reach

2.8% of the total milk fat (24). High seafood intake may contribute to the higher n-3 LCP content in human milk from Hong Kong relative to that from Canada and other Western countries. In contrast, the unique dietary characteristic of consuming more than eight eggs per day may contribute to a higher n-3 LCP content in milk from Chongqing lactating women. It is well known that eggs contain a significant amount of 22:6n-3 ranging from 0.9 to 6.6% of total lipids (30). In addition, the intake of chicken and pork would contribute to a higher 20:4n-6 content in milk from Chongqing Chinese women. The 20:4n-6 and 22:6n-3 contents of foods consumed by the women in this study have not been reported to our knowledge. Furthermore, a higher LCP content in the Chinese milk may be due in part to a very low *trans* fatty acid intake in these two places relative to Western countries. It has been suggested that dietary *trans* fatty acids may inhibit chain elongation and desaturation of 18:2n-6 and 18:3n-3 to form 20:4n-6 and 22:6n-3, respectively, in both maternal liver and mammary glands (31). As seen in Table 1, the two precursor fatty acids, 18:2n-6 and 18:3n-3, were similar in the milk of Chongqing Chinese and Canadian women but 22:6n-3 and 20:4n-6 in the former were much higher than in the latter. In contrast, the total *trans* fatty acid content in the Canadian samples was 32-fold higher than that in the Chongqing Chinese samples.

Our results illustrate a marked difference in dietary 20:4n-6 between Hong Kong and Chongqing Chinese lactating women although the milk content of this fatty acid was very similar (Table 2). Eggs and meats are generally a rich source of 20:4n-6. By assuming the fatty acid content of eggs

and meats consumed by the Chinese women is similar to that reported by Mann *et al.* (32), the Chongqing Chinese women would most likely have been consuming very high levels of 20:4n-6 (700–800 mg/d) compared with the Hong Kong Chinese women (120–140 mg/d). Together with observations made by Mantzioris *et al.* (33) and Makrides *et al.* (34), the present results suggest that breast milk 20:4n-6 may not be sensitive to maternal diet. In fact, it has been known that the breast milk 20:4n-6 level in vegan women (no or very little dietary 20:4n-6) is very similar to that of omnivorous women (35). However, it is important to conduct analyses of local foods to establish a reliable local food composition database before this finding can be confirmed.

The fatty acid composition in the milk of Hong Kong and Chongqing Chinese did not remain constant throughout the 6 wk of lactation. The 22:6n-3 and 20:4n-6 levels decreased gradually as lactation progressed from week 1 to week 6. This observation was in agreement of that of Gibson and Kneebone (18), who showed that 22:6n-3 and 20:4n-6 were significantly decreased from colostrum (22:6n-3, 0.64%; 20:4n-6, 0.71%) to mature milk (22:6n-3, 0.32%; 20:4n-6, 0.31%) in Australian lactating women. It was also evident that these fatty acids in mature milk decreased with the duration of lactation (25). However, the results of the present study were in contrast to those of Clark *et al.* (36), who showed that the total fatty acid composition in the milk of 10 lactating women remained constant throughout 16 wk of lactation. In their study, LCP appeared to decrease with time of lactation although a statistical significance was not found probably because the number of the subjects was relatively small compared to that in the present study. It is likely that the change in milk fatty acid composition is largely due to change in maternal diet as lactation progresses (14,36).

Differences in dietary patterns are also reflected by examining the content of individual fatty acids in the milk from Chongqing and Hong Kong. Firstly, 18:2n-6 in the milk from Hong Kong women was higher than that from Chongqing women. This may be due to the higher consumption of eggs and animal fats in the latter and a higher consumption of corn oil in the former during the first month of lactation. Secondly, eicosapentaenoic acid (20:5n-3) was higher in the milk of Hong Kong Chinese women than that of Chongqing Chinese women. This is possibly because Hong Kong lactating women consume more fish and fish soup presumably for inducing milk letdown. Thirdly, erucic acid (22:1n-9) and 18:3n-3 in the milk of Chongqing Chinese women increased as lactation progressed (Table 3). This is attributed to a change in diet from week 4 to week 6 in Chongqing lactating women. After one month of lactation, the Chongqing lactating women switched from a high animal fat diet to a normal diet, the same as the rest of the family. The 22:1n-9 and 18:3n-3 levels were therefore increased in the milk because high-erucic rapeseed oil is one of the major vegetable oils consumed in the Chongqing population.

In summary, the results from the present study are unique because there are few longitudinal and cross-cultural reports

available of the fatty acids composition of the milk. The milk fatty acid composition, which reflects that in diets, is different in many ways between Chongqing and Hong Kong Chinese lactating women. Compared with that in the milk of women from Canada or other Western countries, the Chinese milk is characterized by having higher LCP and lower *trans* fatty acids contents. The relative higher *trans* fatty acids contents in the milk of Hong Kong Chinese than Chongqing Chinese may reflect the use of partially hydrogenated vegetable oils and dairy products in Hong Kong.

ACKNOWLEDGMENT

We thank the Hong Kong Research Grant Council for support of this research (CUHK 352/95M).

REFERENCES

- Innis, S.M. (1991) Essential Fatty Acids in Growth and Development. *Prog. Lipid Res.* 30, 39–103.
- Carlson, S.E., Rhodes, P.G., and Ferguson, M.G. (1986) Docosahexaenoic Acid Status of Preterm Infants at Birth and Following Feeding with Human Milk and Formula. *Am. J. Clin. Nutr.* 44, 798–804.
- Birch, E.E., Birch, D.G., Hoffman, D.R., and Uauy, R. (1992) Retinal Development in Very-Low-Birth-Weight Infants Fed Diets Differing in Omega-3 Fatty Acids. *Invest Ophthalmol. & Visual Sci.* 33, 2365–2376.
- Daemen, F.J. (1973) Vertebrate Rod Outer Segment Membrane. *Biochim. Biophys. Acta.* 300, 255–288.
- Svennerholm, L. (1968) Distribution and Fatty Acid Composition of Phosphoglycerides in Normal Human Brain. *J. Lipid Res.* 9, 570–579.
- Gibson, R.A. (1992) What Is the Best Fatty Acid Composition for the Fats of Infant Formulas, in *Essential Fatty Acids and Eicosanoids*, (Sinclair, A.J., and Gibson, R.A., eds.), pp. 210–213. American Oil Chemists' Society, Champaign.
- Yonekubo, A., Honda, S., Takahashi, T., and Yamamoto, Y. (1992) Physiological Role of Docosahexaenoic Acid in Mother's Milk and Infant Formula, in *Essential Fatty Acids and Eicosanoids*, (Sinclair, A.J., and Gibson, R.A., eds.), pp. 214–217. American Oil Chemists' Society, Champaign.
- Carlson, S.E., Werkman, S.H., Peeles, J.M., Cooke, R.J., Tolly, E.A., and Wilson, W.M. (1992) Growth and Development of Very Low-Birthweight Infants in Relation to n-3 and n-6 Essential Fatty Acid Status, in *Essential Fatty Acids and Eicosanoids*, (Sinclair, A.J., and Gibson, R.A., eds.), pp. 192–196. American Oil Chemists' Society, Champaign.
- Innis, S.M. (1992) Effect of Different Milk or Formula Diets on Brain, Liver and Blood ω -6 and ω -3 Fatty Acids, in *Essential Fatty Acids and Eicosanoids* (Sinclair, A.J., and Gibson, R.A., eds.), pp. 183–191. American Oil Chemists' Society, Champaign.
- Uauy, R., Birch, D., Birch, E., Hoffman, D., and Tyson, J. (1992) Effect of Dietary Essential ω -3 Fatty Acids on Retinal and Brain Development in Premature Infants, in *Essential Fatty Acids and Eicosanoids* (Sinclair, A.J., and Gibson, R.A., eds.), pp. 197–202. American Oil Chemists' Society, Champaign.
- Uauy, R.D., Birch, D.G., Birch, E.E., Tyson, J.E., and Hoffman, D.R. (1990) Effect of Dietary Omega-3 Fatty Acids on Retinal Function of Very-Low-Birth-Weight Neonates. *Pediatr. Res.* 28, 485–492.
- Birch, D.G., Birch, E.E., Hoffman, D.R., and Uauy, R.D. (1992) Dietary Essential Fatty Acid Supply and Visual Acuity Development. *Invest. Ophthalmol. Visual Sci.* 33, 3242–3253.

13. Chen, Z.Y., Pelletier, G., Hollywood, R., and Ratnayake, W.M.N. (1994) *Trans* Fatty Isomers in Canadian Human Milk, *Lipids* 30, 15-21.
14. Jensen, R.G. (1996) The Lipids in Human Milk, *Prog. Lipid Res.* 35, 53-91.
15. Chardigny, J.M., Wolff, R.L., Mager, E., Sébédio, J.-L., Martine, L., and Juaneda, P. (1995) *Trans* Mono- and Polyunsaturated Fatty Acids in Human Milk, *Eur. J. Clin. Nutr.* 49, 523-531.
16. Wolff, R.L. (1995) Content and Distribution of *trans*-18:1 Acids in Ruminant Milk and Meat Fats. Their Importance in European Diets and Their Effect on Human Milk, *J. Am. Oil Chem. Soc.* 72, 259-272.
17. Waterlow, J.C., Ashworth, A., and Griffiths, M. (1980) Faltering in Infant Growth in Less-Developed Countries, *Lancet* 2, 1176-1178.
18. Gibson, R.A., and Kneebone, G.M. (1981) Fatty Acid Composition of Human Colostrum and Mature Milk, *Am. J. Clin. Nutr.* 34, 252-257.
19. Bitman, J., Wood, L., Hamosh, M., Hamosh, P., and Mehta, N.R. (1983) Comparison of the Lipid Composition of Milk from Mothers of Term and Preterm Infants, *Am. J. Clin. Nutr.* 38, 300-312.
20. Koletzko, B., Mrotzek, M., and Brener, H.J. (1988) Fatty Acid Composition of Mature Human Milk in Germany, *Am. J. Clin. Nutr.* 47, 954-959.
21. Martin, J.C., Bougnoux, P., Fignon, A., Theret, V., Antoine, J.M., Lamise, F., and Couet, C. (1993) Dependence of Human Milk Essential Fatty Acids on Adipose Stores During Lactation, *Am. J. Clin. Nutr.* 58, 653-659.
22. Prentice, A., Prentice, A.M., and Whitehead, R.G. (1981) Milk Fat Concentrations of Rural African Women I. Short-Term Variations Within Individuals, *Br. J. Nutr.* 45, 483-494.
23. Rowland, M.G.M., Paul, A.A., and Whitehead, R.G. (1981) Lactation and Infant Nutrition, *Br. Med. Bull.* 37, 77-82.
24. Chulei, R., Xiaofing, L., Hongsheng, M., Xiulan, M., Guizheng, L., Gianhong, D., Defrancesco, C.A., and Connor, W.E. (1995) Milk Composition in Women from Five Different Regions of China: The Great Diversity of Milk Fatty Acids, *J. Nutr.* 125, 2992-2998.
25. Makrides, M., Simmer, K., Neumann, M., and Gibson, R. (1995) Changes in the Polyunsaturated Fatty Acids of Milk from Mothers of Full-Term Infants Over 30 wk of Lactation, *Am. J. Clin. Nutr.* 61, 1231-1233.
26. Harzer, G., Haug, M., Dieterich, I., and Gentner, P.R. (1983) Changing Patterns of Human Milk Lipids in the Course of the Lactation and During the Day, *Am. J. Clin. Nutr.* 37, 612-621.
27. Finley, D.A., Lonnerdal, B., Dewey, K.G., and Grivetti, L.E. (1985) Milk Composition: Fat Content and Fatty Acid Composition in Vegetarians and Nonvegetarians, *Am. J. Clin. Nutr.* 41, 787-800.
28. Koletzko, B., Thiel, I., and Abiodun, P.O. (1992) The Fatty Acid Composition of Human Milk in Europe and Africa, *J. Pediatr.* 120, 562-570.
29. Kneebone, G.M., and Gibson, R.A. (1985) Fatty Acid Composition of Milk from Three Racial Groups from Penang, Malaysia, *Am. J. Clin. Nutr.* 41, 765-769.
30. Simopoulos, A.P., and Salem, N. (1992) Egg Yolk as a Source of Long-Chain Polyunsaturated Fatty Acids in Infant Feeding, *Am. J. Clin. Nutr.* 55, 411-414.
31. Koletzko, B. (1992) *Trans* Fatty Acids May Impair Biosynthesis of Long-Chain Polyunsaturates and Growth in Man, *Acta Paediatr.* 81, 302-306.
32. Mann, N.J., Johnson, L.G., Warrick, G.E., and Sinclair, A.J. (1995) The Arachidonic Acid Content of the Australian Diet Is Lower Than Previously Estimated, *J. Nutr.* 125, 2528-2535.
33. Mantzioris, E., James, M.J., Gibson, R.A., and Cleland, L.G. (1995) Differences Exist in the Relationships Between Dietary Linoleic Acid and α -Linolenic Acids and Their Respective Long-Chain Metabolites, *Am. J. Clin. Nutr.* 61, 320-324.
34. Makrides, M., Neumann, M.A., and Gibson, R.A. (1996) Effect of Maternal Docosahexaenoic Acid (DHA) Supplementation on Breast Milk Composition, *Eur. J. Clin. Nutr.* 50, 352-357.
35. Sanders, T.A.B., and Reddy, S. (1992) The Influence of a Vegetarian Diet on the Fatty Acid Composition of Human Milk and the Essential Fatty Acid Status of the Infant, *J. Pediatr.* 120, S71-S77.
36. Clark, R.M., Ferries, A.M., Fey, M., Brown, P.B., Hundrieser, E.G., and Jensen, R.G. (1982) Changes in the Lipids of Human Milk from 2 to 16 Weeks Postpartum, *J. Pediatr. Gastroenterol. Nutr.* 1, 311-315.

[Received March 14, 1997, and in final revised form August 8, 1997; revision accepted August 18, 1997]

Trans Fatty Acid Isomers in Canadian Human Milk

Z.-Y. Chen*, G. Pelletier, R. Hollywood and W.M.N. Ratnayake

Nutrition Research Division, Food Directorate, Health Protection Branch, Health Canada, Ottawa, Ontario, K1A 0L2 Canada

ABSTRACT: The fatty acid composition, total *trans* content (i.e., sum of all the fatty acids which may have one or more *trans* double bonds) and geometric and positional isomer distribution of unsaturated fatty acids of 198 human milk samples collected in 1992 from nine provinces of Canada were determined using a combination of capillary gas-liquid chromatography and silver nitrate thin-layer chromatography. The mean total *trans* fatty acid content was $7.19 \pm 3.03\%$ of the total milk fatty acids and ranged from 0.10 to 17.15%. Twenty-five of the 198 samples contained more than 10% total *trans* fatty acids, and thirteen samples contained less than 4%. Total *trans* isomers of linoleic acid were 0.89% of the total milk fatty acids with 18:2 Δ 9c,13*t* being the most prevalent isomer, followed by 18:2 Δ 9c,12*t* and 18:2 Δ 9*t*,12*c*. Using the total *trans* values in human milk determined in the present study, the intake of total *trans* fatty acids from various dietary sources by Canadian lactating women was estimated to be 10.6 ± 3.7 g/person/d, and in some individuals, the intake could be as high as 20.3 g/d. The 18:1 *trans* isomer distribution differed from that of cow's milk fat but was remarkably similar to that in partially hydrogenated soybean and canola oils, suggesting that partially hydrogenated vegetable oils are the major source of these *trans* fatty acids. *Lipids* 30, 15-21 (1995).

Partially hydrogenated fats contain an assortment of geometric and positional isomers of unsaturated fatty acids, of which the *trans* isomers have been reported to raise serum low-density lipoprotein cholesterol (1,2) and lower high-density lipoprotein cholesterol (1). Recent epidemiological and clinical studies have suggested that intake of *trans* fatty acids may be a risk factor in cardiovascular disease (3,4). Some other reports, however, have indicated no such health risk associated with *trans* fatty acids (5,6). *Trans* fatty acids may also interfere with biosynthesis of long-chain polyunsaturated fatty acids during development (7-10). In essential fatty acid deficiency, some of the isomeric fatty acids, particularly the mono-*trans* geometrical isomers of linoleic acid (18:2n-6), are desaturated and elongated to produce unusual longer

chain polyunsaturated fatty acid metabolites in rodents (10-12). The major *trans* isomer of 18:2n-6 in partially hydrogenated vegetable oils is 18:2 Δ 9c,13*t* (13,14). Recently, we have shown that 18:2 Δ 9c,13*t* and three other isomeric fatty acids in partially hydrogenated vegetable oils, viz., 18:2 Δ 12c, 18:2 Δ 9c,12*t* and 18:2 Δ 9*t*,12*c*, are metabolized to unusual C₂₀ polyunsaturated fatty acid isomers even under sufficient intake of 18:2n-6 (1.46% energy; Ref. 15). *Trans* fatty acids have been reported to occur in human milk (16-19). Distribution of *trans* and *cis* isomers has been determined in human tissues (20), but similar data are not yet available for human milk. These *trans* isomers must be of dietary origin, because they cannot be synthesized by mammals. Dietary *trans* fatty acids have also been shown to depress the milk fat content in mice regardless of total fat intake or the dietary level of 18:2n-6 (21,22), but not in swine (23).

To determine the relative contribution of *trans* fatty acid isomers from dietary sources, the present study compared the isomer distribution profile in human milk fat with that in partially hydrogenated vegetable oils and also that in cow's milk. We also sought to ascertain whether dietary *trans* fatty acids inhibit human milk fat production. This was done by correlating the milk fat content with total milk *trans* fatty acid content, which is defined as the sum of all the fatty acids with one or more double *trans* double bonds. Furthermore, *trans* fatty acid consumption of Canadian lactating women was estimated based on the *trans* fatty acid content in their milk.

MATERIALS AND METHODS

Materials. The human milk samples used in this study were from the 1992 collection of Health Protection Branch's ongoing monitoring program of chlorinated hydrocarbon contaminants in the breast milk of Canadian women (unpublished work of Dr. W.H. Newsome, Health Protection Branch, Ottawa, Canada). Samples of "mature milk" (3 to 4 wk after parturition) were collected from donors from nine provinces of Canada (20-25 samples/province) who had resided in Canada for at least 5 yr. Donors were requested to express their milk manually several times during each feeding, alternating between right and left breast. The sample from each donor thus represented the accumulated milk collection of one day. A total of 198 samples was collected in residue-free bottles shipped and stored as reported earlier (24).

*To whom correspondence should be addressed at Department of Biochemistry, The Chinese University of Hong Kong, Shatin, NT, Hong Kong.

Abbreviations: AgNO₃-TLC, silver nitrate thin-layer chromatography; *c*, *cis*; FAME, fatty acid methyl esters; GLC, gas-liquid chromatography; HTM, high-*trans* milk; LTM, low-*trans* milk; MTM, medium-*trans* milk; PHCO, partially hydrogenated canola oil; PHSO, partially hydrogenated soybean oil; *t*, *trans*.

Twelve basestocks. Partially hydrogenated canola (PHCO) and soybean (PHSO) basestocks with different contents of *trans* fatty acids and iodine values were supplied by CanAmera Foods, Inc. (Toronto, Ontario, Canada), which is one of the largest edible oil processors. It also supplies their basestocks to most of the major food chains in Canada. We therefore believe that the basestocks analyzed are representative of what are in the Canadian market. Homogenized cow's milk was purchased locally.

Milk lipid extraction. Milk samples were thawed at room temperature (25°C). Fat from a 5 g sample was extracted using 25 vol of $\text{CHCl}_3/\text{MeOH}$ (2:1, vol/vol) containing 0.02% butylated hydroxytoluene (Sigma Chemicals, St. Louis, MO) as an antioxidant and triheptadecanoic acid (1 mg/mL; Sigma Chemicals) as an internal standard to quantitate total milk fat (25). Another 5 g sample was extracted with the same solvent system but without addition of triheptadecanoic acid to determine the content of endogenous heptadecanoic acid in milk. Cow's milk fat was similarly extracted.

Fatty acid analysis. The extracted lipids were converted to fatty acid methyl esters (FAME) using 14% BF_3/MeOH

reagent (Sigma Chemicals) at 90°C for 50 min under nitrogen. FAME were analyzed by gas-liquid chromatography (GLC) using an SP-2560 flexible fused silica capillary column (100 m \times 0.25 mm i.d., 20 μm film thickness; Supelco, Inc. Bellefonte, PA) in a Hewlett-Packard 5890 series II gas chromatograph equipped with a flame-ionization detector (Palo Alto, CA). Column temperature was programmed from 150 to 180°C at a rate of 0.5°C/min, and then to 210°C at a rate of 3°C/min. Injector and detector temperatures were 250 and 300°C, respectively. Hydrogen was used as the carrier gas at a head pressure of 20 psi.

The gas chromatographic trace of the FAME profile of human milk is shown in Figure 1. Single step, direct GLC cannot determine the total 18:1 *trans* due to overlap of high delta 18:1 *trans* isomers (18:1 Δ 12*t* to 18:1 Δ 16*t*) with 18:1 *cis* isomer peaks (26). We found that an average of 20.8% of total 18:1 *trans* were actually overlapped with 18:1 *cis* fraction. The extent of overlap primarily was dependent on the total *trans* content and ranged from 9–30%. The higher the total 18:1 *trans*, the greater the overlap. Therefore, the total 18:1 *trans* and total 18:1 *cis* contents in the milk samples were de-

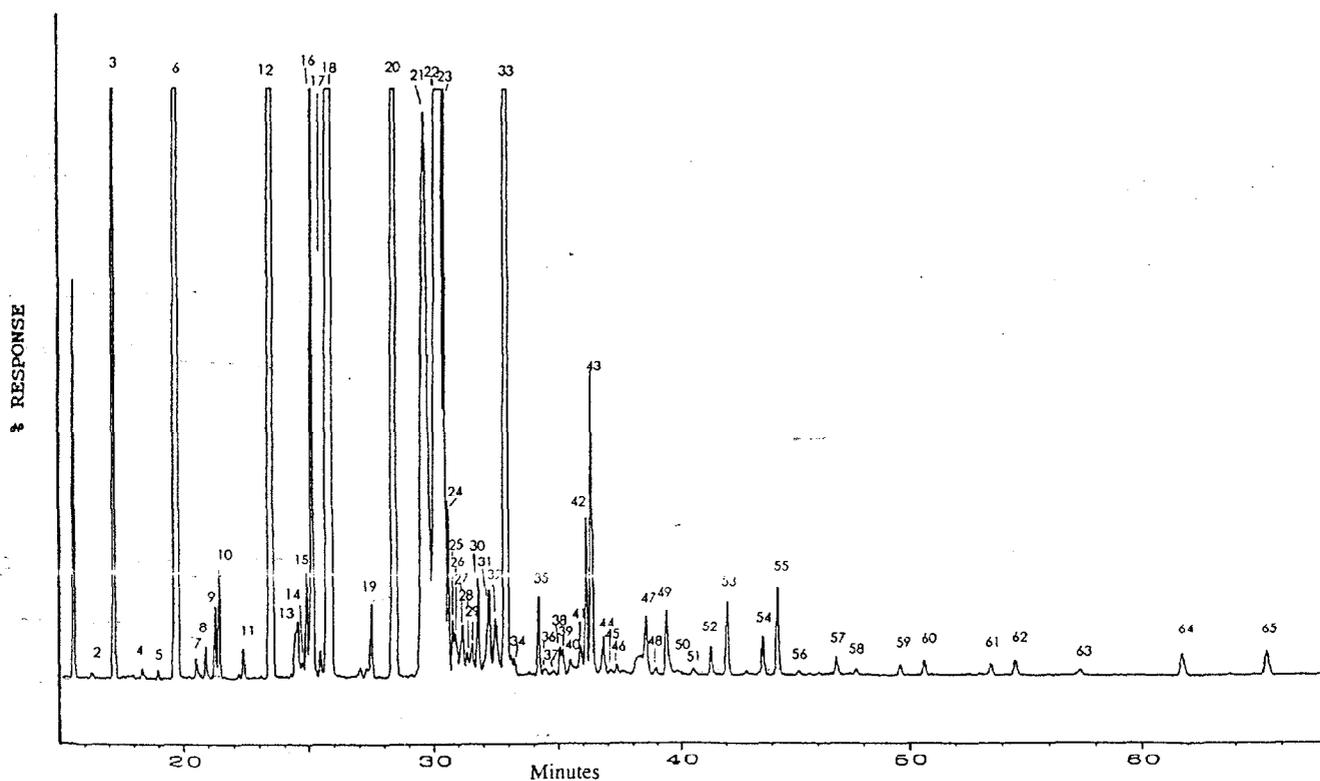


FIG. 1. A typical chromatogram of the C_{10} to C_{22} region of fatty acid methyl esters from a sample of Canadian human milk total lipids on an SP-2560 capillary column (100 m \times 0.25 mm). Peaks were identified as: 1, 10:0; 2, 11:0; 3, 12:0; 4, 13:0; 5, 14:0 *br*; 6, 14:0; 7, 14:1 Δ 9*t*; 8, 14:1 Δ 7*c*; 9, 14:1 Δ 9*c*; 10, 15:0; 11, 16:0 *br* + 15:1 *?*; 12, 16:0; 13, 16:1 Δ 7*t*; 14, 16:1 Δ 9*t*; 15, 16:1 Δ 7*c*; 16, 16:1 Δ 9*c*; 17, unknown; 18, 17:0 (internal standard); 19, 17:1 Δ 10*c*; 20, 18:0; 21, 18:1 Δ 7- Δ 11*t*; 22, 18:1 Δ 7- Δ 10*c* + 18:1 Δ 12- Δ 15*t*; 23, 18:1 Δ 11*c*; 24, 18:1 Δ 12*c*; 25, 18:1 Δ 13*c*; 26, 18:2*t*; 27, 18:1 Δ 14*c* + 18:1 Δ 16*t*; 28, 18:1 Δ 15*c*; 29, 18:1 Δ 16*c* + 18:2 Δ 9*t*, 12*t*; 30, 18:2 Δ 9*c*, 13*t* + 18:2 Δ 8*t*, 12*c*; 31, 18:2 Δ 9*c*, 12*t*; 32, 18:2 Δ 9*t*, 12*c*; 33, 18:2*n*-6; 34, 18:2 Δ 9*c*, 15*c*; 35, 20:0; 36, unknown; 37, unknown; 38, 18:3*n*-6; 39 + 40 + 41, 18:3*t*; 42, 20:1 Δ 11*c*; 43, 18:3*n*-3; 44, 20:1 Δ 13*c*; 45+46, 18:2 conjugates; 47, 20:2 Δ 8*c*, 14*c*; 48, 18:2 conjugate; 49, 20:2*n*-6; 50, 18:2 conjugate; 51, 20:3 Δ 5*c*, 8*c*, 11*c*; 52, 20:3 Δ 5*c*, 11*c*, 14*c*; 53, 20:3*n*-6; 54, 22:1 Δ 13*c*; 55, 20:4*n*-6; 56, 20:4*n*-3; 57, unknown; 58, unknown; 59, 20:5*n*-3; 60, 24:0; 61, 24:1*c*; 62, 22:4*n*-6; 63, 22:5*n*-6; 64, 22:5*n*-3; 65, 22:6*n*-3.

terminated using silver nitrate thin-layer chromatography (AgNO₃-TLC) in conjunction with capillary GLC (26). Preparative AgNO₃-TLC plates were prepared as described previously (26). FAME (~15 mg) were applied to the plates in hexane solution using a TLC streaker (Applied Science Laboratories, State College, PA). The plates were developed in toluene at -20°C for 3 h. The separated bands were visualized under ultraviolet light (254 nm) after spraying with 0.2% dichlorofluorescein in ethanol. The 18:1 *trans* band was scraped off the plate and extracted with hexane/CHCl₃ (1:1, vol/vol). After drying under nitrogen, the 18:1 *trans* fraction was redissolved in hexane, and analyzed by GLC using an SP-2560 capillary column. The proportion of 18:1 *trans* isomers (the high delta value *trans* isomers) that overlapped with the 18:1 *cis* isomer peaks were calculated by comparing the 18:1 region of the GLC chromatogram of the isolated 18:1 *trans* fraction with that of the parent FAME mixture prior to AgNO₃-TLC fractionation. For this purpose, the 18:1 *trans* isomer peaks (18:1Δ7*t* to 18:1Δ11*t*) that were well separated from the *cis* peaks served as the internal standard. The total 18:1 *trans* content was then calculated, by summing up the proportions of the 18:1 *trans* isomers (18:1Δ12*t* to 18:1Δ16*t*) that overlapped with the *cis* isomers and the well separated 18:1 *trans* isomers.

The distribution of 18:1 *trans* and *cis* isomers isolated by AgNO₃-TLC was determined by BF₃/MeOH oxidative ozonolysis (27). Ozone was generated in a Model T-408 Polymetrics Laboratory Ozonator (Polymetrics, Inc., San Jose, CA). Calculation of individual monounsaturated isomers was based on the proportion of the corresponding dimethyl ester products. The dimethyl esters were identified according to Ratnayake and Pelletier (14).

The geometric and positional isomers of 18:2n-6 in human milk were identified and determined as described previously (14).

Statistics. Data were expressed as mean ± standard deviation. Analysis of variance followed by Student's *t*-test (two-tailed) where applicable was used for statistical evaluation of significant difference between groups.

RESULTS

The fatty acid data of Canadian breast milk are given in Tables 1 and 2. The mean fat content of the samples, calculated using triheptadecanoin as the internal standard (25), was 31.6 ± 9.4 g/L. A correlation between the fat content and percentage of total *trans* fatty acids was not observed; however, 18:2n-6 and α-linolenic acid (18:3n-3) were inversely related to the total *trans* fatty acids and 18:1 *trans* isomers (Fig. 2). In addition, total 18:1 *trans* isomers were positively correlated to stearic acid (18:0; *P* < 0.01) but it was negatively correlated with oleic acid (18:1n-9; *P* < 0.05; data not shown). The saturated fatty acids comprised 38.5% total milk fatty acids, approximately half of which was palmitic acid (16:0). The second major group was n-9 monounsaturated fatty acids, of which oleic acid (18:1Δ9) contributed the most (30.7%); 18:2n-6 varied

TABLE 1
Fatty Acid Composition (wt% total fatty acids) of Canadian Human Milk Determined by GC and Ag-TLC Plus GC^a

Fatty acids	Direct GC	Ag-TLC/GC	Range
Saturated fatty acids			
10:0	1.39(0.59)	1.39(0.59)	0.46-4.42
11:0	0.01(0.02)	0.01(0.02)	trace-0.08
12:0	5.68(2.01)	5.68(2.01)	2.32-11.77
13:0	0.03(0.03)	0.03(0.03)	trace-0.14
14:0	6.10(1.73)	6.10(1.73)	2.26-11.68
15:0	0.37(0.12)	0.37(0.12)	0.12-0.67
16:0	18.30(2.25)	18.30(2.25)	12.90-24.06
17:0	0.32(0.08)	0.32(0.08)	0.03-0.44
18:0	6.15(0.97)	6.15(0.97)	3.49-9.85
20:0	0.15(0.09)	0.15(0.09)	trace-0.36
14:0 <i>br</i>	0.14(0.06)	0.14(0.06)	trace-0.26
16:0 <i>br</i>	0.14(0.06)	0.14(0.06)	0.04-0.45
<i>Cis</i>-monounsaturated fatty acids			
14:1Δ9 <i>c</i>	0.28(0.08)	0.28(0.08)	0.06-0.66
16:1Δ9 <i>c</i>	2.27(0.56)	2.27(0.56)	1.11-3.88
16:1Δ7 <i>c</i>	0.41(0.13)	0.41(0.13)	0.20-0.70
17:1Δ10 <i>c</i>	0.21(0.06)	0.21(0.06)	trace-0.44
18:1Δ9 <i>c</i>	32.66(3.22)	30.65(2.66)	23.55-40.64
18:1Δ11 <i>c</i>	1.91(0.17)	1.91(0.17)	1.26-2.33
Total other 18:1 <i>c</i>	1.52(0.12)	2.31(0.23)	1.44-3.97
20:1Δ11 <i>c</i>	0.39(0.13)	0.39(0.13)	0.13-0.65
20:1Δ13 <i>c</i>	0.14(0.09)	0.14(0.09)	trace-0.42
22:1Δ13 <i>c</i>	0.02(0.03)	0.02(0.03)	trace-0.11
n-6 Polyunsaturated fatty acids			
18:2n-6	10.47(2.62)	10.47(2.62)	5.79-21.35
18:3n-6	0.08(0.06)	0.08(0.06)	trace-0.21
20:2n-6	0.17(0.37)	0.17(0.09)	trace-0.35
20:3n-6	0.26(0.09)	0.26(0.09)	trace-0.46
20:4n-6	0.35(0.11)	0.35(0.11)	0.05-0.69
22:4n-6	0.04(0.05)	0.04(0.05)	trace-0.18
22:5n-6	0.01(0.02)	0.02(0.02)	trace-0.16
n-3 Polyunsaturated fatty acids			
18:3n-3	1.16(0.37)	1.16(0.37)	0.58-1.90
20:4n-3	0.06(0.06)	0.06(0.076)	trace-0.26
20:5n-3	0.05(0.05)	0.05(0.05)	trace-0.25
22:5n-3	0.08(0.06)	0.08(0.06)	trace-0.45
22:6n-3	0.14(0.10)	0.14(0.10)	trace-0.53
<i>Trans</i> fatty acids			
14:1Δ7 <i>t</i>	0.09(0.05)	0.09(0.05)	trace-0.47
16:1Δ9 <i>t</i>	0.18(0.08)	0.18(0.08)	trace-0.42
Total 18:1 <i>t</i>	4.65(1.99)	5.87(2.52)	0.10-15.42
18:2Δ9 <i>t</i> ,12 <i>t</i>	0.05(0.08)	0.05(0.08)	trace-0.28
18:2Δ9 <i>c</i> ,13 <i>t</i> /8 <i>t</i> ,12 <i>c</i>	0.36(0.14)	0.36(0.14)	trace-0.76
18:2Δ9 <i>c</i> ,12 <i>t</i>	0.29(0.15)	0.29(0.15)	trace-0.78
18:2Δ9 <i>t</i> ,12 <i>c</i>	0.24(0.12)	0.24(0.12)	trace-0.59
Total 18:3 <i>trans</i>	0.11(0.08)	0.11(0.08)	trace-0.34
Unusual <i>cis</i>-polyunsaturated fatty acids			
18:2Δ9 <i>c</i> ,15 <i>c</i>	0.07(0.05)	0.07(0.05)	trace-0.15
20:2Δ8 <i>c</i> ,14 <i>c</i>	0.19(0.08)	0.19(0.08)	trace-0.49
20:3Δ5 <i>c</i> ,11 <i>c</i> ,14 <i>c</i>	0.02(0.02)	0.02(0.02)	trace-0.12
20:3Δ5 <i>c</i> ,8 <i>c</i> ,11 <i>c</i>	0.05(0.06)	0.05(0.06)	trace-0.18
Others	2.24(0.09)	2.24(0.09)	1.25-2.50

^aData are mean (SD); n = 198. GC, gas chromatography; Ag-TLC, silver thin-layer chromatography; *br* = branched; Other 18:1*c* = 18:1*c* isomers other than 18:1Δ9*c*; trace = trace amount (<0.005% of total fatty acids); Others = this group includes fatty acids <10:0, very minor branched-chain fatty acids, 18:2 conjugated fatty acids and unknowns.

TABLE 2
Total Content (g/L) and Fatty Acid Composition (wt% of total fatty acids) of Canadian Human Milk Determined by Ag-TLC Plus GC^a

Fatty acid class	% of total fatty acids
Total saturated fatty acids	38.50 (2.94)
Total C ₁₀ -C ₁₃ saturated fatty acids	7.11 (2.53)
Total C ₁₄₋₁₆ saturated fatty acids	24.77 (3.26)
Total n-6 fatty acids	11.39 (3.18)
Total n-3 fatty acids	1.49 (0.44)
Total n-6 long-chain fatty acids	0.83 (0.28)
Total n-3 long-chain fatty acids	0.33 (0.24)
Total <i>trans</i> fatty acids	7.19 (3.03)
Total 18:1 <i>trans</i> fatty acids	5.87 (2.52)
Total 18:1 <i>cis</i> other than 18:1n-9	4.22 (0.96)
Total milk fat (g/L)	31.58 (9.37)

^aData are mean (SD); n = 198. Abbreviations as in Table 1.

from 5.8 to 21.4% with an average value of 10.5%; 18:3n-3 accounted for 1.2% total milk fatty acids with a range of 0.6 to 1.9%. The longer-chain products of 18:2n-6 and 18:3n-3 represented 0.8 and 0.3% of total fatty acids, respectively. A correlation between both 18:2n-6 and 18:3n-3 and their corresponding long-chain metabolites was not observed.

Trans fatty acids were found in all the samples and ranged from 0.1 to 17.2% with a mean of $7.2 \pm 3.0\%$. Twenty-five out of the 198 samples contained more than 10% total *trans* fatty acids. Furthermore, more than 15% *trans* fatty acids was found in 2% of the samples.

As expected, 18:1 was the major *trans* isomer group. It ranged from 0.1 to 15.4% of total fatty acids with a mean value of 5.9%. The average 18:1 *trans* isomer distribution is illustrated in Figure 3. In addition, the average 18:1 *trans* iso-

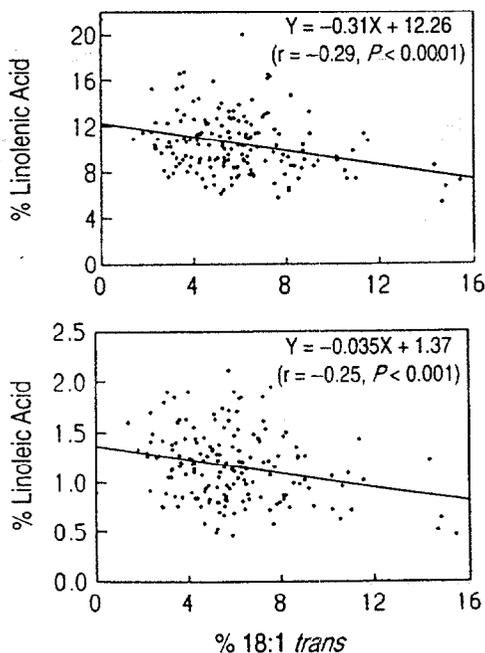


FIG. 2. Correlation of percent 18:2n-6 and percent 18:3n-3 with percent 18:1 *trans* in human milk lipids (n = 198).

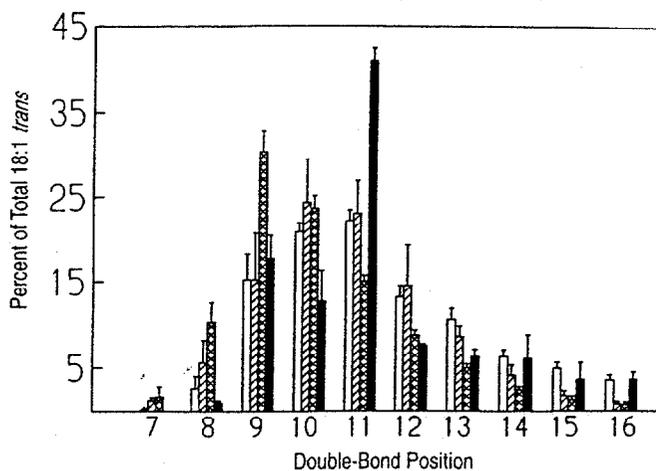


FIG. 3. Distribution of individual 18:1 *trans* isomer in human milk lipids (open bar, n = 198), partially hydrogenated soybean oil (hatched bar, n = 6), partially hydrogenated canola oil (cross-hatched bar, n = 6) and in cow's milk fat (solid bar, n = 6). Data are mean \pm standard deviation. Total 18:1 *trans* isomers (wt% of total fatty acids) were 5.87% in human milk, 31.17% in partially hydrogenated soybean, 32.51% in partially hydrogenated canola oil and 2.10% in cow's milk fat.

mer distributions for PHSO, PHCO and cow's milk fat are also presented in Figure 3 for comparison. In the human milk samples analyzed, the most prevalent isomer was $\Delta 11t$, followed by $\Delta 10t$, $\Delta 9t$, $\Delta 12t$ and $\Delta 13t$ in a decreasing order. The 18:1 *trans* isomer distribution closely resembled that of PHSO, and to a lesser extent to the pattern in PHCO, but differed from that of cow's milk fat. In contrast to human milk fat and partially hydrogenated vegetable oils, cow's milk fat was characterized by having as much as 43% of total 18:1 *trans* isomers represented by 18:1 $\Delta 11t$ and low proportions of other isomers.

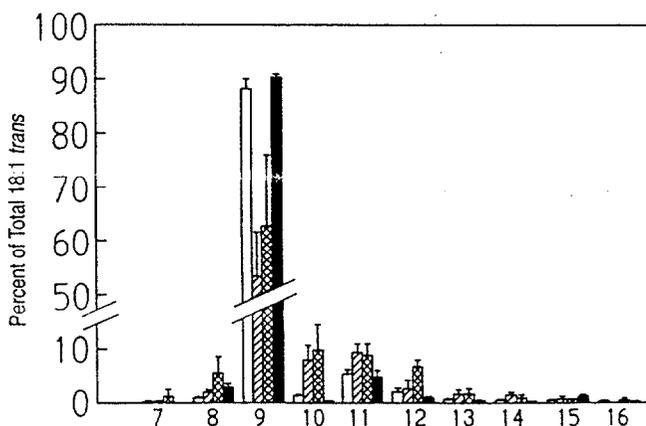


FIG. 4. Distribution of individual 18:1 *cis* isomer in human milk lipids (open bar, n = 198), in partially hydrogenated soybean oil (hatched bar, n = 6), in partially hydrogenated canola oil (cross-hatched bar, n = 6) and in cow's milk fat (solid bar, n = 6). Data are mean \pm standard deviation. Total 18:1 *cis* isomers (wt% of total fatty acids) were 36.88% in human milk, 38.04% in partially hydrogenated soybean, 44.19% in partially hydrogenated canola oil and 22.91% in cow's milk fat.

TABLE 3
Fatty Acid Composition (wt%) of High *Trans* Milk, Low *Trans* Milk and Medium *Trans* Milk Determined by Ag-TLC Plus GC^a

Fatty acid	High <i>trans</i> (n = 25)	Medium <i>trans</i> (n = 155)	Low <i>trans</i> (n = 18)
18:2n-6	9.12 ± 1.96 ^a	10.50 ± 2.51 ^{a,b}	11.70 ± 2.51 ^b
18:3n-3	0.97 ± 0.27 ^a	1.19 ± 0.36 ^{a,b}	1.28 ± 0.32 ^b
18:1t	10.35 ± 2.21 ^a	5.42 ± 1.32 ^b	2.64 ± 1.18 ^c
18:1Δ9c	29.41 ± 3.47	30.75 ± 3.66	30.52 ± 3.59
Total other 18:1c	3.93 ± 0.46	4.24 ± 0.49	4.08 ± 0.48
18:2Δ9c,13t/8t,12c	0.51 ± 0.14 ^a	0.36 ± 0.13 ^a	0.13 ± 0.11 ^b
18:2Δ9c,12t	0.42 ± 0.15 ^a	0.28 ± 0.13 ^a	0.12 ± 0.11 ^b
18:2Δ9t,12c	0.37 ± 0.11 ^a	0.24 ± 0.12 ^a	0.07 ± 0.07 ^b
20:4n-6	0.33 ± 0.06	0.35 ± 0.13	0.36 ± 0.12
22:6n-3	0.13 ± 0.06	0.14 ± 0.12	0.16 ± 0.07
Total <i>trans</i>	12.40 ± 2.41 ^a	6.92 ± 1.50 ^b	3.10 ± 0.97 ^c

^aData are mean ± SD. Means in the same row with different superscripts (a, b, c) differ, $P < 0.01$. Total other 18:1c is the sum of 18:1 *cis* isomers excluding 18:1Δ9c. Abbreviations as in Table 1.

The distribution patterns of 18:1 *cis* isomers in human milk fat, partially hydrogenated vegetable oil and cow's milk fat were not comparable (Fig. 4). In human milk, the total 18:1 *cis* isomer group was largely oleic acid (Δ9c; 88.2%), followed by *cis*-vaccenic acid (Δ11c; 5.5%) with a small amount of other isomers ranging from Δ8 to Δ16. This is in contrast to PHSO and PHCO, where 18:1Δ9c accounted for 54–63% of total 18:1 *cis* isomers followed by Δ12c (7–18%), Δ11c (8–9%) and Δ10c (7–8%). In cow's milk, the Δ9c isomer represented 90% of the total 18:1 *cis* isomers with Δ11c and Δ8c being less than 5 and 3%, respectively (Fig. 4).

Trans isomers of 18:2n-6 amounted to 0.9% of total milk fatty acids (Table 1). This group primarily was composed of four isomers, 18:2Δ9c,13t/8t,12t (0.4%), 18:2Δ9c,12t (0.3%) and 18:2Δ9t,12c (0.2%). In the partially hydrogenated vegetable oils analyzed in the present study, these isomers accounted for 2.0, 1.1 and 1.0% of total fatty acids, respectively. The 18:2Δ9c,13t and 18:2Δ8t,12c are not separable by GLC or silver TLC, and hence are listed together (Tables 1 and 3). Of these two isomers, however, 18:1Δ9c,13t is the major one in PHSO and PHCO; and 18:2Δ8t,12c normally presents at extremely low level (14).

DISCUSSION

Significant regional differences in the average fatty acid composition data of Canadian human milk samples were not observed in this study (data not shown). However, within this population there were wide variations in the content of *trans* fatty acid in breast milk. Thus, we have reclassified the human milk samples into three categories (Table 3) according to the content of *trans* fatty acids; high-*trans* milk (HTM) for samples containing >10% *trans*, medium-*trans* milk (MTM) for samples containing ≥4% but ≤10% *trans*, and low-*trans* milk (LTM) for samples containing <4% *trans*. The high content of *trans* fatty acids in HTM was associated with lower

levels of 18:2n-6 and 18:3n-3 than in MTM and LTM (Fig. 2; Table 3).

The total number of HTM accounted for 12% of total samples analyzed, and MTM accounted for 78%. This may indicate that the diet of a very large (~90%) segment of Canadian lactating women contains moderate to high levels of *trans* fatty acids. The fatty acid composition of milk from lactating women reflects to a certain extent the fatty acid composition of diets from previous days. This is particularly applicable to 18:1 *trans* fatty acids which fluctuate in human milk lipids according to their amount in the diet (18,28). Craig-Schmidt *et al.* (28) have reported a linear correlation between 18:1 *trans* in milk and in the diet among eight lactating women consuming different levels of partially hydrogenated fats. Using their linear equation, $Y = 1.49 + 0.42X$ [where Y and X represent the percentage 18:1 *trans* in total milk fat and dietary fat, respectively (28)], total *trans* intake can be estimated provided total *trans* fatty acids in diet and milk have similar relationship as 18:1 *trans*. Application of this equation indicates that HTM lactating women consumed diets in which 26.0% of fat was *trans* fatty acids. Thus, the total *trans* fatty acid intake for HTM women, ingesting a total energy of 2350 kcal in which 30% of the energy is derived from fat (29), could be estimated to be 7.7% of the total dietary energy or 20.3 g/person/d. Similarly, total *trans* fatty acid intake for MTM women could be estimated to be 3.9% total energy or 10.1 g/person/d, whereas total *trans* intake for LTM lactating women could be 1.1% total energy or 3.0 g/person/d. If the calculation is based on the mean *trans* fatty acid content (7.2%) for all the 198 human milk samples, then the mean total *trans* fatty acid intake for Canadian lactating women could be estimated to be 4.0% total energy or 10.6 g/person/d. This value is 16.5% higher than the value of 9.1 g/person/d estimated for the Canadian general population in 1981 (30). From data reported in four studies involving up to 57 breast milk samples (16,18,31,32), a mean value of 4.5 g/person/d can be deduced for lactating women in the United States. A similar *trans* fatty acid consumption has been estimated for British women (33). Thus, these data may suggest that Canadian women are consuming more *trans* fatty acids than are women in the United States or Britain. However, the estimated *trans* intake for women in the United States is far below the recent estimates of 12.5–15.2 g (34) or 8.1 g/person/d (35,36) for the general United States population.

In the present study a correlation between total *trans* fatty acids and total milk fat content was not found, which may indicate that dietary *trans* fatty acids do not suppress milk fat production in humans. This suggestion is consistent with the findings of Jensen *et al.* (37) for United States women. These findings with humans are also consistent with those in a laboratory study of ours in which partially hydrogenated canola oil did not suppress percent fat in lactating rats (Chen and Ratnayake, unpublished data). Similar observation was found in swine (23). Contrary to these findings, suppression of milk fat percentage by dietary *trans* fatty acids has been reported in mice (21,22). Thus, it appears that the influence of dietary

trans fatty acids on fat production in milk may be species-dependent.

The 18:1 *trans* isomer distribution was remarkably similar to that of partially hydrogenated vegetable oils. Since *trans* fatty acids are not *de novo* synthesized, the *trans* isomer distribution is a reflection of that in the diet. Under conditions of energy deficiency, lactating women produce milk which resembles the fatty acid pattern of their adipose tissue, but when energy is adequate, the dietary fatty acids are the major influence (38). The fatty acid composition of adipose tissue has also been suggested to reflect the long-term fatty acid profile of the diet (39-43). We have found a similar 18:1 *trans* isomer pattern between partially hydrogenated vegetable oil and adipose tissue in Canadian subjects (Chen, Ratnayake, Fortier, Ross, and Cunnane, unpublished data). A similar observation was also made by Ohlrogge *et al.* (20) for the United States population. Although complete dietary records were unavailable in the present study, this close correlation between the distribution of individual 18:1 isomers in partially hydrogenated vegetable oils and in human milk fat or adipose tissue, suggests that the major source of these fatty acids in the Canadian population is partially hydrogenated vegetable oil, whereas contribution from dairy products is relatively minor.

It is difficult to compare the pattern of 18:1 *cis* isomers in human milk with that in the diet because of the *de novo* synthesis of $\Delta 9c$ and $\Delta 11c$ isomers. However, comparison of the relative proportions of $\Delta 8c$, $\Delta 9c$ and $\Delta 12c$ isomers in partially hydrogenated vegetable oils with those in human milk indicates that $\Delta 10c$ isomer is preferentially metabolized or discriminated by mammary gland. The low incorporation or apparent selective metabolism of the $\Delta 10c$ isomer in human milk fat was also observed in human (20,44) and animal tissues (45,46).

Chappell *et al.* (19) studied the influence of margarine on fatty acid composition and found a significant elevation in milk 18:2n-6 levels with a depression in 18:1 *trans* following ingestion of margarine containing lower levels 18:1 *trans* fatty acids. This pattern in milk fatty acid was reversed by feeding margarine high in *trans* fatty acids and low in 18:2n-6 (19). We also found a similar inverse relationship between 18:2n-6 and 18:1 *trans* in human adipose tissue (Chen, Ratnayake, Fortier, Ross, and Cunnane, unpublished data). The linear inverse relationship between 18:2n-6 or 18:3n-3 and 18:1 *trans* isomers observed in this study indicates the elevation of *trans* fatty acids in Canadian human milk is at the expense of 18:2n-6, 18:3n-3 because high *trans* products contain less 18:2n-6 and 18:3n-3.

Many geometric and positional isomers of 18:2n-6 are formed during partial hydrogenation of vegetable oils (13,14) or during the deodorization of oils (47,48). 18:2 $\Delta 9c$,13*t*, 18:2 $\Delta 9c$,12*t* and 18:2 $\Delta 9t$,12*c* are the major 18:2 isomers. Significant levels of these isomers are present in margarine and other dietary fats (13,14). The interest in these 18:2 isomers is related to their possible chain-elongation and desaturation to unusual 20:4 isomers (11,12,15). In essential fatty acid-de-

ficient rats fed partially hydrogenated soybean oil, the formation of 20:4 isomers was extensive and was even more than the arachidonic acid produced (11). We have studied the relationship between chain-elongation and desaturation of the 18:2 isomers and dietary level of 18:2n-6 in rats. Our data have shown that elongation and desaturation of these isomers are inversely related to the level of 18:2n-6 in the diet (49). In another study (15), we found that 18:2 $\Delta 9c$,13*t* and 18:2 $\Delta 9c$,12*t* were metabolized to 20:4 $\Delta 5c$,8*c*,11*c*,15*t* and 20:4 $\Delta 5c$,8*c*,11*c*,14*t*, respectively, even under sufficient intake of 18:2n-6 (1.46% energy). Although these unusual 20:4 isomers were less than 0.4% of the total liver phospholipids, it is conceivable that they might exert some physiological effects. For example, a *trans* isomer of docosahexaenoic acid (DHA) derived from a mono *trans* isomer of 18:3n-3 has been found to incorporate into the retina of rats (50) and consequently altered the electroretinograph (51). We found that several milk samples in the present study had as much as 1.7% of total fat as 18:2 isomers. It is generally believed that dietary *trans/cis* isomers of 18:2n-6 and 18:3n-3 are not elongated and desaturated to C_{20} and C_{22} polyunsaturated fatty acids in humans, under conditions of adequate intake of essential fatty acids (5,6,32,52). Contrary to this, Chardigny *et al.* (53) found *trans* isomers of eicosapentaenoic acid and DHA in human platelets. Although 20:4 *trans* isomers are not present in human milk (present study), future studies should focus on the possible formation of C_{20} and C_{22} *trans* isomers in infants ingesting HTM. It will also be necessary to determine whether or not incorporation of such *trans* isomers into infants tissues may have physiological implications.

In addition to the possible chain-elongation and desaturation of 18:2 isomers, some 18:1 *cis* isomers may also be metabolized to unusual C_{20} isomers. For example, our previous study showed that 18:1 $\Delta 12c$ was desaturated/elongated subsequently to 20:2 $\Delta 8c$,14*c* and 20:2 $\Delta 5c$,8*c*,14*c* in rats fed the partially hydrogenated oils under sufficient intake of 18:2n-6 (15). In the human milks we analyzed, 18:1 $\Delta 12c$ accounted for 2.0% of total 18:1 *cis* fatty acids (Fig. 4). In addition, its two corresponding C_{20} metabolites were found to account for 0.2% of total milk fat (Table 1). The presence of the two metabolites in human milk suggests that the metabolic pathway of 18:1 $\Delta 12c$ may be similar in rats and humans.

In conclusion, it is clear that *trans* fatty acid isomers present in partially hydrogenated vegetable oils are transferred to human milk through maternal diets and, subsequently, to infants. Feeding diets high in *trans* fatty acids to newborn animals led to rapid accumulation of *trans* fatty acids in adipose tissue, liver and heart (54-56). The significance of an early supply of *trans* fatty acids in large amounts to human neonates is still unknown.

ACKNOWLEDGMENTS

Z.Y. Chen was a T.K. Murray Fellow of the National Institute of Nutrition. The authors thank Bureau of Field Operations and Dr. W.H. Newsome for procuring the samples. Thanks are also due to the

mothers who donated milk samples. T. Mag of CanAmera Food, Inc. (Toronto, Ontario, Canada) is thanked for supplying partially hydrogenated canola and soybean oil basestocks.

REFERENCES

1. Mensink, R.P., and Katan, M.B. (1990) *N. Engl. J. Med.* 323, 439-445.
2. Judd, J.T., Clevidence, B.A., Muesing, R.A., Wittes, J., Sunkin, M.E., and Podczasy, J.J. (1994) *Am. J. Clin. Nutr.* 59, 861-868.
3. Willet, W.C., Stampfer, M.J., Manson, J.E., Golditz, G.A., Speizer, F.E., Rosner, B.A., Sampson, L.A., and Hennekens, C.H. (1993) *Lancet* 341, 581-585.
4. Siguel, E.N., and Lerman, R.H. (1993) *Am. J. Cardiol.* 71, 916-920.
5. Emken, E.A. (1983) *J. Am. Oil. Chem. Soc.* 60, 995-1004.
6. Senti, F., *Health Effects of Dietary trans Fatty Acids*, Bethesda, MD, Federation of American Societies for Experimental Biology, August 1985 (Report of HHS Contract No. FDA 223-87-2020).
7. Koletzko, B. (1992) *Acta. Paediatr.* 81, 302-306.
8. Koletzko, B., and Muller, J. (1990) *Biol. Neonate* 57, 172-178.
9. Anderson, R.L., Fullmer, C.S., and Hollenbach, E.J. (1975) *J. Nutr.* 105, 393-400.
10. Beyers, E.C., and Emken, E.A. (1991) *Biochim. Biophys. Acta.* 1082, 275-284.
11. Holman, R.T., Pusch, F., Svingen, B., and Dutton, H.J. (1991) *Proc. Natl. Acad. Sci. (USA)* 88, 4830-4834.
12. Hill, E.G., Johnson, S.B., Lawson, L.D., Mahfouz, M.M., and Holman, R.T. (1982) *Proc. Natl. Acad. Sci. (USA)* 79, 953-957.
13. Ratnayake, W.M.N., Hollywood, R., O'Grady, E., and Pelletier, G. (1993) *J. Am. Coll. Nutr.* 12, 651-660.
14. Ratnayake, W.M.N., and Pelletier, G. (1992) *J. Am. Oil. Chem. Soc.* 69, 95-105.
15. Ratnayake, W.M.N., Chen, Z.Y., Pelletier, G., and Weber, D. (1994) *Lipids* 29, 707-714.
16. Picciano, M.F., and Perkins, E.G. (1977) *Lipids* 12, 407-408.
17. Koletzko, B., Maria, M., and Bremer, H.J. (1988) *Am. J. Clin. Nutr.* 47, 954-959.
18. Atchison, J.M., Kunkley, W.L., Canotly, N.L., and Smith, L.M. (1977) *Am. J. Clin. Nutr.* 30, 2006-2015.
19. Chappell, J.E., Clandinin, M.T., and Kearney-Volpe, K. (1985) *Am. J. Clin. Nutr.* 42, 49-56.
20. Ohlrogge, J.B., Emken, E.A., and Gulley, R.M. (1981) *J. Lipid Res.* 22, 955-960.
21. Teter, B.B., Sampugna, J., and Keeney, M. (1990) *J. Nutr.* 120, 818-824.
22. Teter, B.B., Sampugna, J., and Keeney, M. (1992) *Lipids* 27, 912-916.
23. Pettersen, J., and Opstvedt, J. (1991) *Lipids* 26, 711-717.
24. Mes, J. (1981) *Internat. J. Environ. Anal. Chem.* 9, 283-299.
25. Chen, Z.Y., and Cunnane, S.C. (1992) *Am. J. Physiol.* 27, 912-916.
26. Ratnayake, W.M.N., and Beare-Rogers, J.L. (1990) *J. Chromatogr. Sci.* 28, 633-639.
27. Ackman, R.G., Sebedio, J.L., and Ratnayake, W.N. (1981) *Methods Enzymol.* 72, 253-276.
28. Craig-Schmidt, M.C., Weete, J.D., Faircloth, S.A., Wickwire, M.A., and Livant, E.J. (1984) *Am. J. Clin. Nutr.* 39, 778-786.
29. Health and Welfare Canada (1990) *Nutrition Recommendations: The Report of the Scientific Review Committee*, Canadian Government Publishing Centre, Ottawa, Canada.
30. Brisson, G.J. (1981) in *Lipids in Human Nutrition*, Jack K Burgess, Inc., pp. 41-71, Englewood.
31. Finley, D.A., Lonnerdal, B., Dewey, K.G., and Grivetti, L.E. (1985) *Am. J. Clin. Nutr.* 41, 787-800.
32. Hunderieser, K.E., Clark, R.M., and Brown, P.B. (1983) *J. Ped. Gastro. Nutr.* 2, 635-639.
33. Gregory, J., Foster, K., Tyler, H., and Wiseman, M. (1990) *The Dietary and Nutritional Survey of British Adults*, The Social Survey Division, Office of Population Censuses and Survey, The United Kingdom.
34. Enig, M.G., Atal, S., Keeney, L., and Sampugna, J. (1990) *J. Am. Coll. Nutr.* 9, 471-486.
35. Hunter, J.E., and Applewhite, T.H. (1986) *Am. J. Clin. Nutr.* 44, 707-717.
36. Hunter, J.E., and Applewhite, T.H. (1991) *Am. J. Clin. Nutr.* 54, 363-369.
37. Jensen, R.G., Hagerty, M.M., and McMahon, K.E. (1978) *Am. J. Clin. Nutr.* 31, 990-1016.
38. Insull, W., Hirsh, T.I., James, T., and Ahrens, E.H. (1959) *J. Clin. Invest.* 28, 443-450.
39. Martin, F.C., Bougnoux, P., Fignon, A., Theret, V., Antoine, J.M., Lamisse, F., and Gouet, C. (1993) *Am. J. Clin. Nutr.* 58, 653-659.
40. Dayton, S., Hashimoto, S., Dixon, W., and Pearce, M.L. (1966) *J. Lipid Res.* 7, 103-106.
41. Beynen, A.C., Herms, R.J.J., and Hautvost, J.G.A.J. (1980) *Am. J. Clin. Nutr.* 33, 81-85.
42. Hirsh, J.J., Farquhar, W., Ahrens, E.H., Peterson, M.L., and Stoffel, W. (1956) *Am. J. Clin. Nutr.* 8, 499-511.
43. Huggins, L.C., Hirsch, J., and Emken, E.A. (1991) *Am. J. Clin. Nutr.* 53, 474-482.
44. Emken, E.A., Rohwedder, W.K., Adlof, R.O., Dejarlis, W.J., and Gully, R.M. (1985) *Biochim. Biophys. Acta.* 836, 233-245.
45. Wood, R. (1979) in *Geometrical and Positional Fatty Acid Isomers* (Emken, E.A., and Dutton, H.J., eds.) pp. 213-276, American Oil Chemists' Society, Champaign.
46. Reichwald-Hacker, I., Grosse-Oetringhaus, S., Kiewitt, I., and Mukherjee, K.D. (1979) *Biochim. Biophys. Acta.* 575, 327-334.
47. Ackman, R.G., Hooper, S.N., and Hooper, D.L. (1974) *J. Am. Oil. Chem. Soc.* 51, 42-49.
48. Wolf, R.L. (1992) *J. Am. Oil. Chem. Soc.* 69, 106-110.
49. Chen, Z.Y., Pelletier, G., Hollywood, R., and Ratnayake, W.M.N. (1994) *INFORM* 5, 473.
50. Grandgirard, A., Bourre, J.M., Julliard, F., Homayoun, P., Dumont, O., Piciotti, M., and Sebedio, J.L. (1994) *Lipids* 29, 251-258.
51. Chardigny, J.M., Bonhomme, B., Sebedio, J.L., Juaneda, P., Doly, M., and Grandgirard, A. (1994) *INFORM* 5, 472.
52. Applewhite, T.H., and Hunter, J.E. (1992) *Lipids* 27, 486.
53. Chardigny, J.M., Sebedio, J.L., Juaneda, P., Vatele, J.M., and Grandgirard, A. (1993) *Nutr. Res.* 13, 1105-1111.
54. Pettersen, J., and Opstvedt, J. (1989) *Lipids* 24, 616-624.
55. Moore, C.E., and Dhopeswarkar, G.A. (1980) *Lipids* 15, 1023-1028.
56. Rastogi, B.K., Fryer, B.A., Allee, F., and Fryer, H.C. (1980) *Nutr. Rep. Inter.* 22, 925-933.

[Received August 26, 1994, and in revised form November 15, 1994; Revision accepted November 23, 1994]

Incorporation of *trans*-Fatty Acids into Tissue Lipids¹

DEMETRIOS SGOUTAS AND FRED A. KUMMEROW

THREE MAJOR INDUSTRIES, dairy, meat, and oil seed, are economical sources of calories for our diet. The soybean oil industry alone contributes one-half of the more than 10 billion pounds of "visible" fats and oils consumed per year in the United States (1). Approximately 3 billion pounds are hydrogenated to various congeal points and blended with unhydrogenated oils in proportions to produce margarines or shortenings of a desirable solid fat index (Fig. 1). For example, a margarine should contain a low proportion of high-melting triglycerides to prevent brittleness at refrigeration temperatures and to permit complete melting at body temperature. But it must also contain enough high-melting triglycerides so that it will not be too soft at room temperature. Such a margarine can only be produced from hydrogenated fats that contain *trans*-fatty acids or from completely hydrogenated fats that are rearranged with unhydrogenated fats or oils (2). Because rearrangement adds to the processing steps, it involves added cost.

The use of *trans*-fatty acids in a margarine base stock is considered desirable from a technological viewpoint, because those acids have a higher melting point than does the equivalent *cis*-acid. During the hydrogenation process, the horseshoe-like physical configuration of the natural *cis*-unsaturated fatty acids is converted into the straighter spatial configuration of the *trans*-isomer, thereby extending the molecule and increasing its melting point. For

example, the melting points of the *cis*-isomers of oleic and linoleic acid are 14 C and 12 C, those of *trans*-isomers, 52 C and 29 C, respectively. The *trans*-fatty acids, therefore, add to the proportion of high-melting triglycerides, that is, they provide the same technological function as that of the saturated fatty acids in animal fats. In actual practice, hydrogenated soybean oil with as much as 63% *trans*-fatty acids (IV 68) and 0% essential fatty acids (EFA) is blended with hydrogenated soybean oil of 2.5% *trans*-fatty acids (IV 6.5) and unhydrogenated corn oil, cottonseed, or safflower oil. The iodine values, percentage of *trans*-fatty acids, and percentage of EFA of typical margarines are given in Table 1. American margarines contain more EFA than do Canadian or European margarines. We found, however, that the percentage of *trans*-fatty acids and EFA content were not related to price. A well-advertised margarine that contained 14% linoleic acid cost twice as much as an unadvertised brand that contained 27% linoleic acid (3). A cup-type margarine prepared from a blend of safflower oil and hydrogenated soybean oil had the highest EFA content. All of the margarines in Table 1 had a good solid fat index.

The polyunsaturated fatty acid (PUFA) content of margarine has become important because a diet high in PUFA has been claimed to lower serum lipid levels (4-7). Spritz and Mishkel (8) believe that the lipid-lowering effect of unsaturated fat is related to the differences between the spatial configurations of saturated and unsat-

¹ From The Burnside Research Laboratory, University of Illinois, Urbana, Illinois.

urated fatty acids. Since unsaturated fatty acids occupy a greater area than do saturated fatty acids, Spritz and Mishkel hypothesized that fewer lipid molecules can

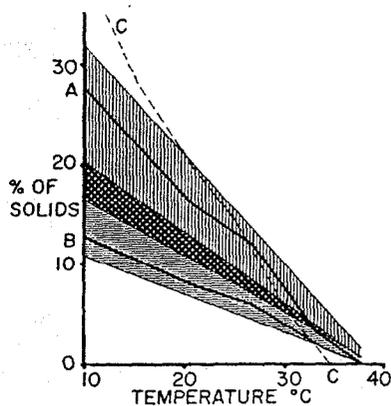


FIG. 1. Solid fat-index range for margarine blends: vertical striped area shows that found in commercial stick margarines, horizontal striped area that in commercial cup margarines, and checked area that for harder cup margarines. C = butterfat.

TABLE I
Characteristics of margarines produced in the United States, Poland and Canada (2)

Margarine type	Iodine number	<i>trans</i> , %	EFA, %
Hydrogenated cottonseed-soybean oil	95.7	40.5	27.7
Hydrogenated corn oil	82.9	46.9	14.5
Hydrogenated soybean, liquid corn oil	94.8	34.4	3.17
Liquid and hydrogenated soy-cotton oil	114.5	20.5	47.6
Safflower, hydrogenated soybean oil	125.1	18.1	66.4
Liquid cottonseed and soybean oil	97.7	37.1	40.9
Canadian (hydrogenated rapeseed oil)	77.2	41.5	3.0
Polish (hydrogenated rapeseed oil)	74.0	41.1	5.0
Polish (palm peanut oil)	96.3	43.1	23.0
Polish (hydrogenated rapeseed oil)	83.0	54.8	10.1

EFA = essential fatty acids.

be accommodated by the apoprotein of the low-density lipoprotein (LDL), and thus the total lipid content of the LDL is lowered. It was reported (9) that the fatty acids in low-density lipoproteins isolated from the serum of chickens fed coconut oil contained approximately the same percentages of long-chain saturated fatty acids as those of chickens fed corn oil. However, the LDL from those fed corn oil contained 10% less oleic, four times more arachidonic, and three times more linoleic acid than did that from chickens on coconut oil. These data seem to indicate that a shift in the type of unsaturated fatty acids rather than a replacement by saturated fatty acids (Table II) may be responsible for differences in the lipid accommodation of the apoprotein.

If some of the PUFA in the LDL had a *trans* configuration, these fatty acids may take on a spatial configuration closer to that of oleic acid than of the original *cis,cis* structures. Rand and Quackenbush (10) have shown that rats fed purified

TABLE II
Fatty acid composition in dietary fats and in serum low density lipoproteins in chicks (9)

Fatty acid component	Fatty acid composition			
	Hydrogenated coconut oil, %	Corn oil, %	Of serum low density lipoproteins: dietary supplements	
			Hydrogenated coconut oil, %	Corn oil, %
Caprylic	6.3			
Capric	5.8			
Lauric	49.7		2.6	
Myristic	18.5		4.7	
Palmitic	8.7	11.5	22.8	23.3
Palmitoleic			9.3	2.3
Stearic	9.3	2.3	15.4	14.8
Oleic	1.7	28.8	31.5	20.5
Linoleic		57.4	9.7	33.4
C ₂₀ Trienoic			2.8	1.0
C ₂₀ Tetranoic			1.2	4.7

ed by the apoprotein of the apoprotein (LDL), and thus the content of the LDL is low-reported (9) that the fatty density lipoproteins isolated of chickens fed coconut oil approximately the same percentage saturated fatty acids as those fed corn oil. However, those fed corn oil contained four times more arachidonic, six more linoleic acid than those on coconut oil. This indicates that a shift in saturated fatty acids rather than by saturated fatty acids is responsible for differences in the composition of the apopro-

tein. PUFA in the LDL had a similar configuration closer to that of the original. Rand and Quackenbush found that rats fed purified

TABLE II
Composition in dietary fats and low density lipoproteins in chicks (9)

Fatty acid composition			
Hydrogenated coconut oil, %	Corn oil, %	Of serum low density lipoproteins: dietary supplements	
		Hydrogenated coconut oil, %	Corn oil, %
6.3			
5.8			
10.7		2.6	
8.5		4.7	
3.7	11.5	22.8	23.3
		9.3	2.3
9.3	2.3	15.4	14.8
1.7	28.8	31.5	20.5
	57.4	9.7	33.4
		2.8	1.0
		1.2	4.7

trans-fatty acids had higher serum cholesterol levels than did those fed the corresponding purified *cis*-fatty acids. It is, therefore, possible that the *trans*-fatty acids in margarine counteract the cholesterol-lowering effect of the *cis*-fatty acids. High PUFA margarines have been claimed to lower serum cholesterol levels (11); however, if such margarines contain large amounts of *trans*-fatty acids, they may have overall influence on the serum lipid level. Erickson et al. (11) fed margarines of various P/S ratios to groups of men for 5-week periods and found no significant differences in serum lipid levels between groups unless egg yolk was also included in the diet as a source of cholesterol. One of the margarines contained 10% *trans*-fatty acids, which may have been too low to influence the serum lipid levels.

Whether high PUFA margarines, which contain substantial amounts of *trans*-fatty acids, lower serum cholesterol levels may be a debatable question (12, 13). Studies on the biological utilization of *trans*-fatty acids have, however, brought out some interesting observations on the absorption, metabolism, and incorporation of those acids into tissue lipid components that are totally divorced from the influence of PUFA on the serum cholesterol level.

ABSORPTION AND DEPOSITION OF *trans*-FATTY ACIDS

Ono and Fredrickson (14) fed ^{14}C isomers of *cis*- and *trans*-oleic and linoleic acid, simultaneously with ^3H -palmitic acid, in 0.5 ml of olive or corn oil, to rats with cannulated thoracic ducts, and collected the chyle over an 8-hr period. They found no differences in the absorption of the *cis*- and *trans*-isomers and palmitic acid. About 85–90% appeared as triglycerides, 4–6% in phospholipids, an equal amount in mono- and diglycerides, and less than 1% in cholesterol esters. These data are in agreement with those of Coats (15), who recovered 97.4% of ^{14}C -linoleic acid in the gly-

cerides, 2.2% in the phospholipids, and 0.3% in the sterol esters, from the lymph of rats fed *trans*, *trans*- ^{14}C -linoleic acid.

The percentage of *trans*-fatty acids that can be deposited in the tissue has been shown to depend on the percentage of *trans*-fatty acids in the dietary fat (16). Rats fed a diet, for 1 month, that included 10% of a margarine base stock of 40% *trans*-fatty acid content had incorporated 16.7% of *trans*-fatty acids in the carcass fat. When the margarine stock was fed at a 5% level with 5% olive oil, only 10% of *trans*-fatty acids was found in the carcass fat. When *trans*-fatty acids were removed from the diet, the *trans*-fatty acids decreased in 2 months to 4.4 and 2.8%, respectively. Autopsy and biopsy tissues from human subjects all contained *trans*-fatty acids (17), the highest percentage in liver and adipose tissue (up to 12 and 14.4%, respectively), which reflects a substantial intake (Table III).

The lipid components in the tissue lipids of animals fed *trans*-fatty acids all contained substantial amounts of the acids. Sinclair (18) and Barbour (19) were the first investigators to show that the lipids from animals fed elaidin contained a higher percentage of *trans*-fatty acids in the cholesterol esters than in the triglycerides—43 and 35%, respectively (20). Weigensberg and McMillan (12) found that the serum from rabbits fed elaidinized linoleic acid for 84 days contained 13.2% *trans*-isomers in the fatty acids of the cholesterol

TABLE III
trans-Fatty acid content of human tissues (17)

Tissue	<i>trans</i> , %	Acids, % (17)
Adipose	2.4	12.2
Liver	4.0	14.4
Heart	4.6	9.3
Aorta	2.3	8.8
Atheroma	2.3	8.8

esters, 11.3% in the triglycerides, and 2.5% in the phospholipids.

METABOLISM OF THE *trans*-FATTY ACIDS

Trans-unsaturated fatty acids have been shown to oxidize as readily to CO₂ as do their *cis*-isomers (14, 15). The *trans*-isomers of linoleic acid were catabolized to CO₂ to a somewhat greater extent, however, than was *cis,cis*-linoleic acid (21-23). Anderson (24) believed this to be due to a lower rate of oxidation of the alkyl chain on the methyl side of the *trans*-double bond; both *cis*- and *trans*-acids provided the same fragments in the Krebs cycle.

Anabolism or elongation of the *trans*-fatty acids seems to be selective. The *cis*-9, *trans*-12, octadecadienoic acid has been reported (25) to elongate to an isomer of arachidonic acid believed to be *cis*-5, *cis*-8, *cis*-11, *trans*-14 eicosatetraenoic acid, and *trans,trans*-linoleic acid seemed to elongate to *cis*-5, *cis*-8, *trans*-11, *trans*-14 eicosatetraenoic acid (26). Selinger and Holman (27) found that *trans,trans*-linoleic acid did not increase the level of polyunsaturated fatty acids normally derived from the *cis,cis*-isomer. The studies of DeTomas et al. (28) indicated that elongated nonessential fatty acids can esterify the β -position in lecithin. A number of studies (27, 29, 30) have demonstrated, however, that *trans,trans*-linoleic acid cannot function as an essential fatty acid, presumably because its elongated derivative does not have the functional characteristics of the elongated *cis,cis*-isomer.

Privett and Blank (31) reported that the *cis,trans*-isomer depressed the conversion of *cis,cis*-linoleic to arachidonic acid (to 33%), as did the *trans,cis*-isomer (to 12%), although not so drastically as the *trans,trans*-isomer (to 1.4%). The presence of 50% *cis,cis*-linoleic acid did not counteract completely the depressing effect of *trans,trans*- (13.5%) or *cis,trans*- (17.5%) linoleic acid on the conversion of linoleic to arachidonic acid. The major share of the ara-

chidonic acid was present in a α -saturated β -tetraene triglyceride. In animals that had been fed *cis,trans*- or *trans,trans*-linoleic acid, the percentage of arachidonic acid incorporated at the β -position was greatly depressed when compared with that of animals fed *cis,cis*-linoleic acid (14.1, 1.6, and 42.4%, respectively). In those fed *cis,trans*-linoleic acid, an almost equal amount (12.5%) trienoic acid was incorporated at the β -position.

POSITIONAL SPECIFICITY IN ESTERIFICATION OF TRIGLYCERIDES AND PHOSPHOLIPIDS

Pancreatic lipase did not appear to differentiate between the *trans*- and *cis*-isomers of oleic acid (32, 33). However, the acyl-glycerol-3-phosphoryl-choline (GPC) transferase in liver microsomes could discriminate (34) between the normal *cis*- and the *trans*-isomers. Lands et al. (35) found, in in vitro studies, that the *trans*-9, *trans*-12-isomer was esterified rapidly at the 1-position. When a *cis* configuration was present (in the *trans*-9, *cis*-12-isomer), the rate of esterification at the 1-position of 2-acyl-GPC was considerably diminished. When the *cis* configuration was in the 9-position, it interfered even more seriously with that acylation. The effect of configuration was not so marked on the rates of reaction at the 2-position although the *trans*-dienoates reacted significantly less rapidly than did the *cis*-isomers. Similar results were noted in the microsomal fractions obtained from EFA-deficient rats that had been fed *cis*- and *trans*-isomers. The observed distribution of octadecadienoate between the secondary and primary positions in triglycerides and lecithin differed according to the isomer fed. The *cis,cis*-isomer was found predominantly at the 2-position, the *trans,trans*- at the 1-position, and the *cis,trans*-isomer gave values intermediate between those two. Lands suggested that these differences in acyltransferase specificities pointed to future studies that would show differences in acyltransferase activity

was present in a α -saturated glyceride. In animals that had *trans*- or *trans,trans*-linoleic entage of arachidonic acid at the β -position was greatly compared with that of *cis*-linoleic acid (14.1, 1.6, respectively). In those fed *cis*, an almost equal amount of *cis* acid was incorporated at

SPECIFICITY IN ESTERIFICATION OF FATTY ACIDS AND PHOSPHOLIPIDS

ase did not appear to differ between the *trans*- and *cis*-isomers (32, 33). However, the phosphoryl-choline (GPC) liver microsomes could distinguish between the normal *cis*-isomers. Lands et al. (35) reported studies, that the *trans*-9, was esterified rapidly at the 1-position. When a *cis* configuration was present, the esterification at the 1-position is considerably diminished. Esterification was in the 9-position even more seriously affected. The effect of configuration was marked on the rates of esterification although the *trans*-11 significantly less rapidly than the *cis*-isomers. Similar results were obtained in the microsomal fractions of FA-deficient rats that had predominantly *trans*-isomers. The order of octadecadienoate bearing and primary positions on lecithin differed accordingly. The *cis, cis*-isomer was esterified predominantly at the 2-position, and the *trans* gave values intermediate between the two. Lands suggested that in acyltransferase specificity studies that would measure acyltransferase activity

for the positional isomers of the unsaturated fatty acids. Sgoutas (36) has shown this to be true for rat liver acyl-CoA-cholesteryl acyltransferase. The extent of cholesterol esterification, the composition of synthesized cholesterol esters, and the rate of cholesterol esterification with several geometrical isomers of the commonly found oleic and linoleic acids indicated that the enzyme is highly specific to a single *cis* double bond at a proximal distance of 9 carbon atoms from the carboxyl group. *trans*-Fatty acids were not rapidly esterified and resembled stearic acid.

SPECIFICITY IN HYDROLYSIS OF CHOLESTEROL ESTERS

Sgoutas found that the cholesterol esterases in the microsomal and soluble fractions of rat liver hydrolyzed the ^{14}C -cholesterol esters of *cis*- and *trans*-octadecenoic and octadecadienoic acids at different rates (37).

The substrate concentration curves of the hydrolysis of cholesteryl-oleate, -linoleate, -elaidate, -linelaidate, -palmitate, and -stearate, by the soluble enzyme fractions, showed that unsaturated cholesterol esters were hydrolyzed faster than saturated ones, in agreement with previous reports (38, 39). They also showed that the unsaturated esters were clearly divided into two groups: those with *cis* double bond and those with *trans*, with the former being hydrolyzed more rapidly than the latter. The substrate concentration curves indicated that the order of their hydrolysis by the soluble protein fraction was: *cis*-unsaturated > *trans*-unsaturated > saturated cholesterol esters. A similar pattern of substrate preference was observed when the crude soluble fraction or the microsomal fraction was used as the enzyme source.

CHANGES IN CHOLESTEROL ESTERIFICATION ACTIVITY

Rat liver microsomes have been shown (10-12) to contain an enzyme system that

esterifies cholesterol in the presence of ATP and CoA. When this enzyme system was obtained from the liver microsomes of rats that had been fed a hydrogenated fat of 48% *trans* and 0% EFA content, a decrease in the ability of these liver microsomes to esterify ^{14}C -cholesterol in vitro was observed (unpublished data). In this study, groups of rats in our laboratory were fed hydrogenated soya-bean oil (10%), corn oil (10%), fat free diet and stock diet for a period of 10 weeks. Liver microsomes were prepared according to Goodman et al. (41) as described by Sgoutas (36).

For the enzyme assay, the reaction mixture contained microsomes, 0.6 mg protein; ATP, 5 μ moles; CoA, 50 μ moles; phosphate buffer (pH 7.4), 300 μ moles; and ^{14}C -cholesterol 30 μ moles in 0.1 ml acetone, in a final volume of 3 ml. Incubations were carried out on a metabolic shaker for 1 hr at 37 C. The reaction was stopped by the addition of chloroform-methanol and the lipids were extracted and analyzed for total and free cholesterol (36). The esterified cholesterol was calculated from the radioactivity in the cholesterol ester, the total radioactivity in the sample, and the specific activity of the introduced ^{14}C -cholesterol.

The enzyme activities in terms of μ moles of free ^{14}C -cholesterol esterified in the livers of rats fed corn oil, the stock diet, hydrogenated fat and the fat free diet are given in Table IV.

TABLE IV
Acyl-CoA-cholesteryl ester acyltransferase activity in rat liver microsomes^a

Diet	Enzyme activity ^b
Stock	25.0
Corn oil	29.0
Fat-free	8.0
Hydrogenated fat	10.0

^a Conditions of incubation are given in the text.

^b Expressed in millimicromoles cholesterol esters synthesized per milligram protein per hour. Values represent averages of three experiments.

The difference in esterification activity could not have been due to EFA deficiency or to the simple addition of fat to the diet, because the addition of 10% corn oil did not change the esterification rate of the liver microsomes from that of rats fed the stock diet. The threefold difference in esterification rate of the liver microsomes of rats fed 10% corn oil as compared with those fed 10% hydrogenated fat pointed to a significant decrease in esterification in the latter. Analytical data indicated that *trans*-fatty acids had been incorporated into all classes of lipids in the liver microsomes from rats fed the hydrogenated fat. A somewhat analogous observation was made by Sugano and Portman (43) in the liver microsomes isolated from EFA-supplemented and EFA-deficient rats. These workers proposed that the differences in the liver esterification activity induced by EFA deficiency were secondary to alteration in the stability of liver organelles (43).

FUNCTIONAL INFLUENCE OF INCORPORATED *trans*-FATTY ACIDS

The functional influence on the serum lipoproteins and cell membranes into which phospholipids and cholesterol esters containing *trans*-fatty acids have been incorporated remains an area for further study. Chapman et al. (44) found that the replacement of a *cis*- by a *trans*-acid in the 2-position of phosphatidylethanolamine or phosphatidylcholine caused monomolecular films to be appreciably more condensed in character *in vitro*. The association of cholesterol with *trans*-phospholipids was also believed to differ from the association of cholesterol with the isomeric *cis*-phospholipids. Mulder and Van Deenen (45) have shown that there is a free interchange, *in vitro*, of fatty acids between the lipids in the plasma and the lipids in the red blood cell. Such an exchange may also occur *in vivo*, as Walker and Kummerow (46) have shown that the plasma

and the red cells isolated from fat-deficient rats contained 15.6 and 24.9% eicosatrienoic acid, respectively.

A highly significant difference between animals fed elaidinized and nonelaidinized olive oil was noted when the red blood cells from such animals were incubated with lecithinase (47). After a lag period of 2 min, erythrocytes containing *trans*-fatty acids hemolyzed at a rate 5 times faster than that of the control cells. It is possible that the more rapid rate of hemolysis observed in the cells from rats fed *trans*-fatty acids or from rats deficient in dietary linoleate was due to an increase (46) in osmotic fragility, and that these cells were unable to sustain so large an increase in volume without hemolyzing as were those taken from animals fed olive or corn oil.

Korn has recently speculated (48) "Is the order of the macromolecular components random within the membrane, or is there a unique arrangement under cellular control?" The studies to date on the specificity in the esterification of triglycerides, phospholipids, and cholesterol indicate that the order of synthesis of these components of the macromolecules is highly selective and that the incorporation of *trans*-fatty acids into the macromolecular components within the membrane influences its biophysical properties. The mere presence of *trans*-fatty acids *in vivo* influenced the elongation of the natural *cis*, *cis*-polyunsaturated fatty acids (25, 27, 31) and their incorporation at specific positions (35, 36), which in turn influenced the permeability of the membrane into which they were incorporated (47). In fact, Van Deenen and co-workers (49, 50) have shown that even a difference in chain length and its degree of unsaturation influenced the interfacial characteristics of phospholipids significantly.

Other fat-soluble factors, such as the fat-soluble vitamins, may also be involved in the arrangement or positional specificity of the lipid components in cell membranes.

cells isolated from fat-deficient rats (15.6 and 24.9% eicosatrienoic acid) respectively.

Significant difference between hydrogenated and nonhydrogenated red blood cells was observed when the red blood cells from rats were incubated with hydrogenated cells. After a lag period of 2 hours, cells containing *trans*-fatty acids were lysed at a rate 5 times faster than control cells. It is possible that the rapid rate of hemolysis observed in cells from rats fed *trans*-fatty acids is due to an increase (46) in osmotic pressure that these cells were unable to regulate, or an increase in volume regulation as were those taken from olive or corn oil.

Recently speculated (48) "It is possible that the macromolecular composition within the membrane, or its arrangement under cellular conditions, studies to date on the specific incorporation of triglycerides, and cholesterol indicate that the synthesis of these components in the membrane is highly selective. The incorporation of *trans*-fatty acids into the membrane influences its biological function. The mere presence of *trans*-fatty acids in vivo influenced the elongation of *cis*, *cis*-polyunsaturated fatty acids (7, 31) and their incorporation into phospholipids (35, 36), which in turn influences the permeability of the membrane to which they were incorporated. Van Deenen and co-workers (49) have shown that even a small amount of *trans*-fatty acids in length and its degree of unsaturation influenced the interfacial characteristics of phospholipids significantly. Other factors, such as the presence of cholesterol, may also be involved in the distribution of *trans*-fatty acids in cell membranes.

Norman et al. (51), for example, have suspected vitamin D of acting or stimulating "the biochemical expression of genetic information," and evidence has been obtained for the binding, through hydrophobic linkage, of β -carotene to membrane proteins (52). Studies on the amino acid and the nucleic acid sequences in proteins have advanced our knowledge of the assembly of the proteins that are incorporated into cell membranes. A similar advancement may result from studies on the in vivo assembly of lipid components that are incorporated into cell membranes (53), by the use of model systems containing the essential fat-soluble factors and *trans*- instead of *cis*-fatty acids.

In summary, the *trans*-fatty acids that are produced during hydrogenation are deposited in the tissue and are metabolized as easily as the naturally occurring *cis*-fatty acids. They differ from the naturally occurring *cis*-polyunsaturated fatty acids, however, in the manner in which they incorporate into triglycerides and phospholipids and in the specificity of their cholesterol esters to cholesterol esterases. They seem to alter the permeability characteristics of cell membranes, which may result in changes in their biological function. The biological utilization of the *trans*-fatty acid, therefore, seems worthy of further study.

REFERENCES

1. Fats and Oils Situation, Economic Research Service, Division of the U.S. Dept. Agr., January, 1968.
2. ZALEWSKI, S., AND F. A. KUMMEROW. Rapeseed oil in a two-component margarine base stock. *J. Am. Oil Chemists' Soc.* 45: 87, 1968.
3. KUMMEROW, F. A. The role of polyunsaturated fatty acids in nutrition. *Food Technol.* 18: 49, 1964.
4. ADRENS, E. H., JR., J. HIRSCH, W. INSCHE, JR., T. T. TSALTAS, R. BLOMSTRAND AND M. L. PETERSON. The influence of dietary fats on serum lipids in man. *Lancet* 1: 943, 1957.
5. KEYS, A., J. T. ANDERSON AND F. GRANDE. Serum cholesterol response to dietary fat. *Lancet* 1: 787, 1957.
6. CONNOR, W. E., D. B. STONE AND R. E. HODGES. The interrelated effects of dietary cholesterol and fat upon human serum lipid levels. *J. Clin. Invest.* 43: 1691, 1961.
7. WOOD, P. D. S., R. SHIODA AND L. W. KINSELL. Dietary regulation of cholesterol metabolism. *Lancet* 2: 604, 1966.
8. SPRITZ, N., AND M. A. MISHKEL. Effects of dietary fats on plasma lipids and lipoproteins: an hypothesis for the lipid lowering effect of unsaturated fatty acids. *J. Clin. Invest.* 48: 78, 1969.
9. NISHIDA, T., AND F. A. KUMMEROW. Effect of dietary fats and vitamin E on oxidative denaturation of serum low density lipoproteins. *Proc. Soc. Exptl. Biol. Med.* 109: 724, 1962.
10. RAND, P. G., AND F. W. QUACKENBUSH. Effects of purified *cis*- and *trans*-fatty acid derivatives on the hypercholesterolemic rat. *J. Nutr.* 87: 489, 1965.
11. ERICKSON, A., R. H. COOTS, F. H. MATTSON AND A. M. KLIGMAN. The effect of partial hydrogenation of dietary fats, of the ratio of polyunsaturated to saturated fatty acids, and of dietary cholesterol upon plasma lipids in man. *J. Clin. Invest.* 43: 2017, 1964.
12. WEIGENBERG, B. I., AND G. C. McMILLAN. Serum and aortic lipids in rabbits fed cholesterol and linoleic acid stereoisomers. *J. Nutr.* 83: 314, 1964.
13. McOSKER, D. E., F. H. MATTSON, H. B. SWERINGEN AND A. M. KLIGMAN. The influence of partially hydrogenated dietary fats on serum cholesterol levels. *J. Am. Med. Assoc.* 180: 380, 1962.
14. ONO, K., AND D. S. FREDRICKSON. The metabolism of ¹⁴C-labeled *cis*- and *trans*-isomers of octadecenoic and octadecadienoic acids. *J. Biol. Chem.* 239: 2482, 1964.
15. COOTS, R. H. A comparison of the metabolism of *cis*, *cis*-linoleic, *trans*, *trans*-linoleic, and a mixture of *cis*, *trans*- and *trans*, *cis*-linoleic acids in the rat. *J. Lipid Res.* 5: 473, 1964.
16. JOHNSTON, P. V., O. C. JOHNSON AND F. A. KUMMEROW. Deposition in tissue and fecal excretion of *trans*-fatty acids in the rat. *J. Nutr.* 65: 13, 1958.
17. JOHNSTON, P. V., O. C. JOHNSON AND C. H. WALTON. Origin of the *trans*-fatty acids in human tissue. *Proc. Soc. Exptl. Biol. Med.* 99: 735, 1958.
18. SINCLAIR, R. G. The metabolism of the phospholipids. VIII. The passage of elaidic acid into tissue phospholipids. Evidence of the intermediary role of liver phospholipid in fat metabolism. *J. Biol. Chem.* 111: 515, 1935.
19. BARBOUR, A. D. The deposition and utilization

- of hydrogenation isooleic acid in the animal body. *J. Biol. Chem.* 101: 63, 1933.
20. SINCLAIR, R. G., AND L. CHAPMAN. The composition of the cholesterol esters and glycerides in the liver of the rat. *J. Biol. Chem.* 167: 773, 1947.
 21. ANDERSON, R. L., AND R. H. COOTS. The catabolism of the geometric isomers of uniformly ^{14}C labeled Δ^9 -octadecenoic acid and uniformly ^{14}C labeled $\Delta^{9,12}$ -octadecadienoic acid by the fasting rat. *Biochim. Biophys. Acta* 144: 525, 1967.
 22. ANDERSON, R. L. Oxidation of the geometric isomers of $\Delta^{9,12}$ -octadecadienoic acid by rat liver mitochondria. *Biochim. Biophys. Acta* 152: 531, 1968.
 23. ANDERSON, R. L. Oxidation of the geometric isomers of $\Delta^{9,12}$ -octadecadienoic acid by rat liver mitochondria. *Biochim. Biophys. Acta* 152: 531, 1968.
 24. ANDERSON, R. L. Oxidation of the geometric isomers of Δ^9 -octadecenoic acid by rat liver mitochondria. *Biochim. Biophys. Acta* 144: 18, 1967.
 25. BLANK, M. L., AND O. S. PRIVETT. Studies on the metabolism of *cis,trans*-isomers of methyl linoleate and linolenate. *J. Lipid Res.* 4: 470, 1963.
 26. KNIPPRATH, W. G., AND J. F. MEAD. The metabolism of *trans,trans* octadecadienoic acid. Incorporation of *trans,trans*-octadecadienoic acid into the C_{20} polyunsaturated acids of the rat. *J. Am. Oil Chemists' Soc.* 41: 437, 1964.
 27. SELINGER, Z., AND R. T. HOLMAN. The effects of *trans,trans* linoleate upon the metabolism of linoleate and linolenate and the positional distribution of linoleate isomers in liver lecithin. *Biochim. Biophys. Acta* 106: 56, 1965.
 28. DETOMAS, M. E., R. R. BRENNER AND R. O. PELUFFO. Position of eicosatrienoic acid in the phosphatidylcholine and phosphatidylethanolamine from rats deficient in essential fatty acids. *Biochim. Biophys. Acta* 70: 472, 1963.
 29. HOLMAN, R. T. Metabolism of isomers of linoleic and linolenic acids. *Proc. Soc. Exptl. Biol. Med.* 76: 100, 1951.
 30. MATTSON, F. H. An investigation of the essential fatty acid activity of some of the geometrical isomers of unsaturated fatty acids. *J. Nutr.* 71: 366, 1960.
 31. PRIVETT, O., AND M. L. BLANK. Studies on the metabolism of linolenic acid in the essential fatty acid deficient rat. *J. Am. Oil Chemists' Soc.* 41: 292, 1964.
 32. CLEMENT, G., J. BELLEVILLE, C. LORLETTE AND J. RAULIN. Application de la méthode enzymatique à l'étude de la structure de triglycérides comportant des acides gras à liaisons éthylenique de foie *trans*. *Bull. Soc. Chim. Biol.* 45: 1433, 1963.
 33. JENSEN, R. G., J. SAMPUGNA AND R. L. PEREIRA. Pancreatic lipase. Lipolysis of synthetic triglycerides containing a *trans*-fatty acid. *Biochim. Biophys. Acta* 84: 481, 1964.
 34. LANDS, W. E. M. Effects of double bonds configuration on lecithin synthesis. *J. Am. Oil Chemists' Soc.* 42: 465, 1965.
 35. LANDS, W. E. M., M. L. BLANK, L. J. NUTTER AND O. S. PRIVETT. A comparison of acyltransferase activities *in vitro* with the distribution of fatty acids in lecithin and triglycerides *in vivo*. *Lipids* 1: 224, 1966.
 36. SGOUTAS, D. S. Effect of geometry and position of ethylenic bond upon acyl-CoA-cholesterol-O-acyltransferase. *Biochemistry* 9: 1826, 1970.
 37. SGOUTAS, D. S. Hydrolysis of synthetic cholesterol esters containing *trans*-fatty acids. *Biochim. Biophys. Acta* 164: 317, 1968.
 38. DEYKIN, D., AND D. S. GOODMAN. The hydrolysis of long chain fatty acid esters of cholesterol with rat liver enzymes. *J. Biol. Chem.* 237: 3649, 1962.
 39. NIEFT, M. L., AND H. J. DEUEL, JR. Studies on cholesterol esterases. I. Enzyme systems in rat tissues. *J. Biol. Chem.* 177: 143, 1949.
 40. MUKHERJEE, S., G. KUNITAKE AND R. B. ALFINSLATER. The esterification of cholesterol with palmitic acid by liver homogenates. *J. Biol. Chem.* 230: 91, 1958.
 41. GOODMAN, D. S., D. DEYKIN, AND T. SHIRATORI. The formation of cholesterol esters with rat liver enzymes. *J. Biol. Chem.* 239: 1335, 1964.
 42. SWELL, L., M. D. LAW AND C. R. TREADWELL. Esterification of cholesterol in rat liver microsomes. *Arch. Biochem. Biophys.* 104: 128, 1964.
 43. SUGANO, M., AND D. W. PORTMAN. Essential fatty acid deficiency and cholesterol ester activity of plasma and liver *in vitro* and *in vivo*. *Arch. Biochem. Biophys.* 109: 302, 1965.
 44. CHAPMAN, D., N. F. OWENS AND D. A. WALKER. Physical studies of phospholipids. II. Monolayer studies of some synthetic 2,3-diacyl-*sn*-phosphatidylethanolamines and phosphatidylcholines containing *trans* double bonds. *Biochim. Biophys. Acta* 120: 148, 1966.
 45. MULLER, E. AND L. L. VAN DEENEN. Metabolism of red-cell lipids. I. Incorporation *in vitro* of fatty acids into phospholipids from mature erythrocytes. *Biochim. Biophys. Acta* 106: 106, 1965.
 46. WALKER, B. L., AND F. A. KUMMEROW. Erythrocyte fatty acid composition and apparent permeability to non electrolytes. *Proc. Soc. Exptl. Biol. Med.* 115: 1099, 1964.
 47. DECKER, W. J., AND W. MERTZ. Effects of dietary elaidic acid on membrane function in rat mitochondria and erythrocytes. *J. Nutr.* 91: 324, 1967.

4. J. SAMPUGNA AND R. L. PEREIRA. Lipase. Lipolysis of synthetic triglyceride containing a *trans*-fatty acid. *Biochim. Acta* 84: 481, 1964.
5. M. Effects of double bonds on lecithin synthesis. *J. Am. Oil Chem. Soc.* 42: 465, 1965.
6. M., M. L. BLANK, L. J. NUTTER RIVETT. A comparison of acyl-*trans*-fatty acids *in vitro* with the distribution of a lecithin and triglycerides *in vivo*. 1966.
7. Effect of geometry and position bond upon acyl-CoA-cholesterol-Oase. *Biochemistry* 9: 1826, 1970.
8. Hydrolysis of synthetic cholesterol containing *trans*-fatty acids. *Biochim. Biophys. Acta* 4: 317, 1968.
9. AND D. S. GOODMAN. The hydrolysis of *trans*-fatty acid esters of cholesterol by enzymes. *J. Biol. Chem.* 237: 1968.
10. AND H. J. DECEL, JR. Studies on esterases. I. Enzyme systems in rat liver. *J. Biol. Chem.* 177: 143, 1949.
11. G. KUNITAKE AND R. B. ALFINGER. Esterification of cholesterol with *trans*-fatty acids by liver homogenates. *J. Biol. Chem.* 233: 1958.
12. S., D. DEYKIN, AND T. SHIRATORI. Hydrolysis of cholesterol esters with rat liver enzymes. *J. Biol. Chem.* 239: 1335, 1964.
13. D. LAW AND C. R. TREADWELL. Hydrolysis of cholesterol in rat liver microsomes. *Biochem. Biophys. Acta* 104: 128, 1964.
14. AND D. W. PORTMAN. Essential fatty acid and cholesterol ester activity of rat liver microsomes *in vitro* and *in vivo*. *Arch. Biochem. Biophys.* 109: 302, 1965.
15. N. F. OWENS AND D. A. WALKER. Monolayers of phospholipids. II. Monolayers of synthetic 2,3-diacyl-DL-phosphatidylcholines and phosphatidylcholines containing double bonds. *Biochim. Biophys. Acta* 148: 1966.
16. D. L. L. VAN DEENEN. Metabolism of phospholipids. I. Incorporation *in vitro* of phospholipids from mature erythrocytes to phospholipids. *Biochim. Biophys. Acta* 106: 106, 1966.
17. AND F. A. KUMMEROW. Erythrocyte composition and apparent permeability to electrolytes. *Proc. Soc. Exptl. Biol. Med.* 1964.
18. L. AND W. MERTZ. Effects of *trans*-fatty acid on membrane function in erythrocytes. *J. Nutr.* 91: 1966.
48. KORN, E. D. Cell Membranes: Structure and Synthesis. *Ann. Rev. Biochem.* 38: 263, 1969.
49. DEMEL, R. A., L. L. M. VAN DEENEN AND B. A. PETHICA. Monolayer interactions of phospholipid and cholesterol. *Biochim. Biophys. Acta* 135: 11, 1967.
50. EIBL, H., R. A. DEMEL AND L. L. M. VAN DEENEN. Monolayers of lysolecithins and analogs. *J. Colloid Interface Sci.* 29: 381, 1969.
51. NORMAN, A. W., M. R. HAUSSLER, T. H. ADAMS, J. F. MYRTLE, P. ROBERTS AND K. A. HIBBERD. Basic studies on the mechanism of action of vitamin D. *Am. J. Clin. Nutr.* 22: 396, 1969.
52. JI, T. H., J. L. HESS AND A. A. BENSON. Studies on chloroplast membrane structure. I. Association of pigments with chloroplast lamellar protein. *Biochim. Biophys. Acta* 150: 676, 1968.
53. DOBLASOVA, M., AND N. S. RADIN. Uptake of cerebroside, cholesterol and lecithin by brain myelin and mitochondria. *Lipids* 3: 439, 1968.

71-4
Egwin, Peter O., and Demetrios S. Sgoutas

1971. The Fatty Acids of Adrenal Lipids from Rats Fed
Partially Hydrogenated Soybean Fat

J. Nutrition, v. 101, no. 3, March
Pages 315-322.

The Fatty Acids of Adrenal Lipids from Rats Fed Partially Hydrogenated Soybean Fat^{1,2}

PETER O. EGWIM AND DEMETRIOS S. SGOUTAS³
The Burnside Research Laboratory, University of Illinois,
Urbana, Illinois 61801

ABSTRACT The effect of partially hydrogenated soybean fat on the lipid composition of rat adrenals has been determined. The experimental fat contained 48% *trans* fatty acids and traces of linoleic acid. When fed at levels of 10 and 20% by weight of the diet for 5 months, an increase in the concentration of cholesterol esters was observed and elaidic acid incorporated in amounts of 22% of total fatty acids in cholesterol esters, 17% in triglycerides, and 7% in phospholipids. In addition, an acid identified as 9,13-docosadienoic acid occurred in the cholesterol ester and the phospholipid fractions. In particular, when the experimental fat was fed at 20% level, the acid amounted to 24 and 16% concentration in adrenal cholesterol esters and phospholipids, respectively. On supplementing the experimental diet with 2% corn oil, the 9,13-docosadienoic acid diminished whereas the concentration of *trans* fatty acids remained practically unchanged. *J. Nutr.* 101: 315-322, 1971.

Recently, we fed rats partially hydrogenated soybean fat containing 48% of its fatty acids as *trans* acids and among other analyses that we performed (1), we determined the adrenal lipids in these rats. Since no information is available on the adrenal lipids from rats fed fats rich in *trans* fatty acids, the object of this communication is to describe the results obtained from our experiments.

MATERIALS AND METHODS

Male weanling rats of the Holtzman strain, 21 days old, were divided into six groups, nine rats in each group. Groups 1 and 2 were fed basic fat-free diets containing 10 and 20% partially hydrogenated soybean fat,⁴ respectively. Groups 3 and 4 were fed the above diets, respectively, supplemented with 2% corn oil; group 5 was fed a diet containing 10% corn oil and group 6 the basic fat-free diet (1). The dietary fats were all commercial products. Major components for corn oil were: (% of total fatty acids) 16:0, 11.5%; 18:0, 3.2%; 18:1, 25.1%; 18:2, 58.5%; and 18:3, 1.2%. For the partially hydrogenated soybean fat the major components were: 16:0, 8.1%; 18:0, 13.2%; *cis* 18:1, 28%; *trans* 18:1, 48.0%; and 18:2, 0.6%. De-

tailed gas-chromatographic analyses of these dietary fats were published in a previous paper (1). Dietary fat was added at the expense of sucrose in all cases (1). Diets and water were provided ad libitum for 5 months and the rats were weighed and inspected weekly.

At the end of this period the animals were killed under ether anesthesia, and as rapidly as possible thereafter the adrenal glands were removed. The adhering perirenal fat was carefully removed; the glands were weighed and then pooled so that for each dietary treatment there were three pooled groups containing the adrenal glands obtained from three rats. The lipids were extracted with chloroform-methanol (2:1 v/v) after homogenizing the glands in 0.9% saline.

The yield of lipid was determined for each group. The lipid extract was placed onto a silicic acid column (1 by 8 cm) and

Received for publication August 10, 1970.

¹ Supported by a grant (HE 10779-04) from the National Institute of Health and by the National Dairy Council.

² Taken from a thesis submitted by P. O. Egwim in partial fulfillment of the Ph.D. degree in Food Science, University of Illinois at Urbana-Champaign.

³ Present address: Department of Pathology, Woodruff Medical Center, Emory University, Atlanta, Georgia 30322.

⁴ Kindly supplied by Humko Company, Champaign, Ill.

the column was eluted with the following solvent mixtures at a flow rate of 1.0 to 1.5 ml/minute: 18% benzene in petroleum ether (50 ml), 60% benzene in petroleum ether (50 ml), chloroform (100 ml) and methanol (100 ml). Four fractions were recovered containing, respectively, cholesterol esters, triglycerides, free cholesterol, and phospholipids. The purity of each fraction was checked by thin-layer chromatography (2). Cholesterol in cholesterol ester and as free cholesterol was determined by the method of Sperry and Webb (3), phospholipid phosphorus by the method of Bartlett (4), and triglyceride content was calculated by difference. The fatty acids present in all fractions (except cholesterol) were converted to the corresponding methyl esters by the transesterification procedure of Stoffel et al. (5). Methyl esters were purified on small silicic acid columns and their analysis was performed by gas-liquid chromatography as previously described (1). Qualitative analysis was accomplished primarily through comparison with standard mixtures of known methyl esters.⁵ Components for which standards were not available were identified through the use of semilog plots of retention time versus carbon number. The equivalent chain length (ECL) of these compounds was also compared with those published by Hofstetter et al. (6) for further verification. Quantification was by means of triangulation and the detector was calibrated using a standard⁶ containing saturated even chain length esters from C₄ to C₂₄. Experimental values agreed with calculated values with an error of less than 4% for all components.

Preparative gas-liquid chromatography was performed as previously described (1).

Trans fatty acids were measured by gas chromatography on a capillary column (1) and whenever the amount of material available was large enough, by infrared absorption spectroscopy (7).

Double bonds were located by either oxidative degradation (8) or by ozonization followed by reduction of the ozonides (9). The resulting acids, aldehydes and aldehyde esters were subjected to gas-liquid chromatography. Reference acids and aldehydes were used as an aid in the identification. For aldehyde esters, methyl

esters of known structure (18:1 ω 7, 18:1 ω 12, 20:5 ω 3) were cleaved and a plot of carbon number against log retention time was constructed.

RESULTS

The adrenal lipid composition of rats fed the different diets is shown in table 1. The lipid content per gram of wet adrenal gland was almost constant and independent of the dietary regimen. However, feeding diets deficient in essential fatty acids (EFA) resulted in a marked increase in the weight of the cholesterol ester fraction, at the expense of the other lipid fractions.

Table 2 shows the fatty acid compositions of adrenal cholesterol esters of all groups. The fatty acid compositions of cholesterol esters from rats fed the fat-free and the 10% corn oil diet are included for the sake of comparison. In accord with previous studies (10, 11), polyunsaturated fatty acids⁷ predominated in the adrenal cholesterol ester fraction, 20:4 ω 6 and 22:4 ω 6 acids in rats fed corn oil and 20:3 ω 9 and 22:3 ω 9 acids in rats fed the fat-free diet.

Adrenal cholesterol esters from rats fed the partially hydrogenated soybean fat at 10 and 20% levels contained elaidic acid in a concentration of 19.0 and 22.5% of total fatty acids, respectively. In addition, a peak tentatively identified as 22:2 accounted for approximately 11.0 and 24% of the total fatty acids in rats fed the 10 and 20% hydrogenated fat, respectively. Other polyunsaturated acids present, although at lower concentrations, were: 22:3 ω 9 and 22:4 ω 6. Supplementation of the test diets with 2% corn oil decreased the amount of docosadienoic acid, but it did not change the concentration of either 22:3 ω 9 or 22:4 ω 6. Also, palmitic and stearic acids were substantially increased, whereas elaidic acid decreased slightly.

⁵ Reference methyl esters were obtained from the Hormel Institute, Austin, Minn. and Applied Science Laboratories, State College, Pa.

⁶ Applied Science Laboratories, State College, Pennsylvania.

⁷ Fatty acids are designated by number of carbon atoms; number of double bonds. In acids with methylene-interrupted sequence of double bonds, the digit after ω states the number of carbon atoms from the methyl end of the acyl chain to the nearest double bond. In all other cases, the position of double bond is given by a number preceding the number of carbon atoms, and denotes the number of carbon atoms from the methyl end to the double bond.

structure (18:1 ω 7, cleaved and a plot against log retention

TS

composition of rats is shown in table 1. A comparison of wet adrenal stant and independent. However, feed-essential fatty acids marked increase in sterol ester fraction, other lipid fractions. Fatty acid composition of sterol esters of all diets and compositions of rats fed the fat-free diet are included. In accord with (1), polyunsaturated acids in the adrenal diet, 20:4 ω 6 and fed corn oil and acids in rats fed the

sters from rats fed soybean fat at elaidic acid 19.0 and 22.5% of ively. In addition, a ified as 22:2 ac- tely 11.0 and 24% in rats fed the 10 d fat, respectively. acids present, al- centration, were: upplementation of corn oil decreased dienoic acid, but it centration of either also, palmitic and tentially increased, creased slightly.

ere obtained from the n. and Applied Science es, State College, Penn-

1 by number of carbon is. In acids with meth- double bonds, the digit carbon atoms from the 1 to the nearest double position of double bond g the number of carbon r of carbon atoms from bond.

TABLE 1
Adrenal lipid composition¹

	Corn oil, 10%	Fat-free	Partially hydrogenated fat			
			10%	20%	10% + 2% Corn oil	20% + 2% Corn oil
Total lipids ²	9.6(8.0-11.2)	10.8(9.5-12.2)	12.2(9.4-14.0)	11.6(9.8-14.1)	10.8(9.0-12.3)	11.8(9.9-13.5)
Cholesterol esters	10.4(8.1-11.5)	19.1(17.3-24.1)	17.5(15.8-20.0)	22.2(20.6-24.3)	12.3(11.5-14.2)	14.1(12.0-15.6)
Triglycerides	3.1(2.2-3.8)	1.8(0.9-2.5)	2.4(1.8-3.0)	1.7(0.6-2.1)	2.8(2.0-4.2)	2.2(1.6-2.9)
Cholesterol	3.9(2.8-4.1)	1.8(1.2-2.3)	2.0(0.9-3.2)	1.0(0.5-1.8)	3.5(3.1-4.5)	1.9(1.5-2.8)
Phospholipids	6.9(5.7-7.8)	3.5(3.1-4.0)	3.8(2.8-4.0)	2.6(1.9-3.3)	4.8(3.8-5.2)	3.0(2.1-4.6)

¹ Average value (range of values) of individual determinations of three samples. Each sample represents three pooled pairs of adrenals.
² Percentage wet weight of tissue.

Furthermore, and in order to characterize the acid, the total dienoic ester fraction from the adrenal cholesterol ester was isolated by argentation column chromatography (12). A small sample of this fraction was hydrogenated and a mixture of 18:0, 20:0, and 22:0 acids resulted. A second sample was subjected to preparative gas-liquid chromatography and the fraction corresponding to the retention time of 22:2 was isolated. Subsequent gas-liquid chromatographic analysis indicated, however, that 22:2 was still contaminated with 18:2 and 20:2 methyl esters. An aliquot was oxidatively degraded and the major products of this oxidation were nonanoic, tetranedioic, and nonanedioic acids, suggesting the structure 9,13 and 4,13 for the 22:2 acid. Ozonization and reduction (7) of the ozonides of the dienoic fraction, on the other hand, gave nonanal and C₉ aldehyde-ester as the predominant products suggesting that 9,13-22:2 is the correct structure. Another aliquot was subjected to infrared spectrophotometric analysis, and failure to detect any absorption at 10.3 m μ indicated the absence of *trans* double bonds.

Table 3 summarizes the fatty acid composition of the triglyceride and phospholipid fractions of adrenals from rats fed the hydrogenated fat. In both groups, elaidic acid accounted for 17 and 8% of total fatty acids in triglycerides and phospholipids, respectively. Docosadienoic acid increased from a concentration of 5 to 17% in phospholipids from rats fed the 10 and 20% hydrogenated fat, respectively, and it was absent in triglycerides. The concentration of 20:4 ω 6 did not change appreciably whereas that of 20:3 ω 9 decreased from 13 to 6%, in rats fed the 10 and the 20% hydrogenated fat, respectively. In adrenal phospholipids from rats fed the fat-free diet, 20:3 ω 9 accounted for 20%, 20:4 ω 6 for 18%, and 22:3 ω 9 was absent. In rats fed the 10% corn oil diet, 20:4 ω 6 accounted for 26% of total fatty acids and only traces of 22:4 ω 6 were detected. These data were in accord with those previously published (10, 11) and they are not shown here in tabular form.

We have recently undertaken a second experiment with another group of rats fed the same diets in order to study the

TABLE 2
The composition of fatty acids of the adrenal cholesterol esters¹

Fatty acid	ECL ²	Partially hydrogenated fat					
		Fat-free	10% Corn oil	10%	20%	10% + 2% Corn	20% + 2% Corn
14:0	14.0	3.0	1.0	2.4	—	0.6	2.1
16:0	16.0	10.7(8.5-12.6)	13.6(10.2-16.1)	11.1(9.8-13.0)	10.4(8.8-11.8)	15.2(14.0-17.1)	17.8(15.5-17.0)
16:1 ω 7	16.6	4.2	2.9	2.9	2.6	7.1	3.1
18:0	18.0	3.9(2.8-4.0)	5.5(4.1-6.3)	3.7(2.8-4.4)	5.3(4.6-6.2)	11.7(10.5-12.6)	15.8(14.1-17.2)
<i>cis</i> 18:1 ³	18.6	19.9(17.1-22.0)	16.2(14.8-18.1)	12.3(11.0-14.1)	6.2(5.5-7.8)	3.1(2.2-4.4)	6.5(5.2-7.8)
<i>trans</i> 18:1	18.6	—	—	19.0(16.5-22.0)	22.5(20.2-24.6)	18.0(16.4-20.0)	20.3(18.1-22.2)
18:2 ⁴	18.8	—	—	2.8	2.9	0.5	1.2
18:2 ω 6+18:2 ω 9	19.2	4.5	10.7	1.6	1.8	5.4	5.5
18:3 ω 6	19.6	—	—	—	—	4.0	2.9
20:0	20.0	—	—	—	1.4	—	—
18:3 ω 3	20.2	0.2	1.0	—	—	—	—
20:1 ω 9	20.4	5.7	3.0	3.1	6.0	1.2	—
20:2 ⁵	20.8	—	—	3.4	2.6	2.5	3.8
20:2 ω 6	21.0	2.9	0.2	—	—	3.4	3.1
20:3 ω 9	21.3	12.9(10.9-14.2)	—	7.4(6.8-8.2)	3.0(2.2-3.8)	4.7(3.2-5.6)	6.2(5.5-7.2)
20:3 ω 6	21.6	0.6	1.5	—	—	—	—
20:4 ω 6	22.2	2.5(1.7-3.1)	17.1(15.5-19.2)	2.0(1.2-2.8)	1.6(1.0-2.1)	5.2(4.8-5.8)	3.6(2.8-4.2)
22:1 ω 9	22.4	3.4	—	—	0.9	—	—
22:2	22.7	0.8(0.4-1.2)	—	11.2(10.8-11.6)	24.3(21.8-26.5)	2.6(1.8-4.0)	2.8(2.0-3.5)
20:5 ω 3	22.9	0.2	1.6	—	—	—	—
22:3 ω 9	23.3	16.0(14.5-18.3)	—	5.0(4.1-6.2)	4.5(3.1-5.4)	4.5(3.6-5.2)	5.2(4.5-6.7)
22:4 ω 6	23.8	3.9(2.9-4.8)	18.4(16.1-20.2)	2.0(1.3-2.8)	1.3(0.7-1.8)	7.6(5.0-8.8)	0.5(0.3-0.8)
22:5 ω 3	25.3	0.4	3.2	—	1.0	1.6	—
22:6 ω 3	25.9	2.0	6.0	3.5	2.3	2.0	—

¹ In g/100 g of total fatty acids. Values represent mean of individual determinations of each of three samples, each sample consisting of three pooled pairs of adrenals. Range of values (in parentheses) is presented only where relevant to the discussion.

² Equivalent chain length on EGS column, see Methods.

³ Approximately 80% 18:1 ω 9 and 20% 18:1 ω 7.

⁴ Tentatively identified as 5.9-18:2.

⁵ Identified as a mixture of 20:2 acids with no methylene-interrupted double bond sequence.

TABLE 3
The fatty acid composition of adrenal lipids of triglycerides and phospholipids from rats fed the partially hydrogenated soybean fat diets¹

Fatty acids	ECL ²	10%		20%	
		Triglycerides	Phospholipids	Triglycerides	Phospholipids
14:0	14.0	2.1	—	2.4	0.5
16:0	16.0	14.8(12.9-16.1)	22.0(19.2-24.3)	23.0(20.5-25.3)	16.5(14.1-17.9)
16:1 ω 7	16.6	4.4	1.7	7.0	0.8
17:0	—	—	—	—	1.3
18:0	18.0	19.8(16.5-22.0)	27.5(24.5-30.2)	8.5(6.4-10.2)	26.6(23.0-28.5)
<i>cis</i> 18:1 ³	18.6	32.0(28.0-35.6)	3.8(1.9-4.3)	38.0(34.5-41.2)	7.5(6.0-9.8)
<i>trans</i> 18:1	18.6	17.4(15.2-19.0)	7.8(6.0-9.2)	17.0(15.2-19.1)	8.3(6.2-10.0)
18:2 ω 6+18:2 ω 9	19.2	2.4	0.7	1.5	1.7
20:2 ⁴	20.8	1.5(0.6-2.4)	0.2	1.8(0.8-2.4)	0.6(0.3-1.0)
20:3 ω 9	21.3	4.0(2.8-5.9)	12.8(10.4-14.8)	0.5(0.2-0.8)	6.1(4.8-8.1)
20:4 ω 6	22.2	1.0(0.6-1.4)	11.6(8.2-14.0)	0.2	12.8(10.1-14.2)
22:2	22.7	—	5.4(4.2-6.7)	—	17.3(15.1-19.2)
22:4 ω 6	23.8	—	1.3(0.8-2.2)	—	—
22:5 ω 6	24.4	—	5.2	—	—

¹ In g/100 g of total fatty acids. Values represent mean of individual determinations of each of three samples, each sample consisting of three pooled pairs of adrenals. Range of values (in parentheses) are presented only where relevant to the discussion.

² Equivalent chain length on EGS column, see Methods.

³ Approximately 80% 18:1 ω 9 and 20% 18:1 ω 7.

⁴ Identified as a mixture of 20:2 with no methylene-interrupted double bond sequence.

incorporation of radioactive fatty acids into adrenal lipids. Analyses performed on the adrenal lipids agreed well with the present data confirming our observations as presented in this paper.

DISCUSSION

Earlier studies have also shown that the composition of adrenal gland lipids can be drastically modified by dietary means. Thus, feeding rapeseed oil, or erucic acid to rats, resulted in a threefold increase in adrenal cholesterol ester concentration (13) and a similar increase was observed when rats were fed either a fat-free diet (10) or a diet containing 10% hydrogenated coconut oil (11) but not corn oil.

It has also been known that rat adrenals require the presence of polyunsaturated fatty acids of longer than C₂₀ chain length, particularly in their cholesterol ester fraction. In normal rats, the acid is 22:4 ω 6 (10) and in essential fatty acid-deficient rats, it is 22:3 ω 9 (9, 10). Feeding rapeseed oil resulted in the accumulation of cholesteryl erucate (13) and it appeared as if this acid with moderate amounts of 22:4 ω 6, also present, satisfied the aforementioned requirement.

Our data indicated that feeding rats partially hydrogenated soybean fat resulted in a twofold increase in the concentration of adrenal cholesterol esters with a

concomitant accumulation of cholesteryl elaidate and cholesteryl docosadienoate. Docosadienoic acid, its structure shown to be 9,13-22:2, occurred in phospholipids but not triglycerides and in that respect resembled 22:4 ω 6 and 22:3 ω 9 acids. Concerning the formation of this acid, the absence of *trans* absorption and the 9-terminal structure would suggest that it derived from oleic acid. However, this can be conclusively demonstrated only with the use of radioactive tracers. It is of particular interest that the acid has its double bond in a different arrangement than the familiar methylene-interrupted mode. Fatty acids with a similar double bond pattern have been shown to occur in rats fed either elaidic or hydrogenated fat (14, 1) and in one case their biosynthetic route has been established (15). Nothing is known, however, about the factors governing the formation of fatty acids with this arrangement of double bonds. One possibility is that dietary fats high in *trans* fatty acid induce alterations in the enzymes responsible for the elongation-desaturation of long-chain fatty acids.

In that context, and since lipoproteins are probably involved in the desaturation-elongation of long-chain fatty acids, it is reasonable to assume that the structure of this lipid constituent is an important factor governing their enzymic activities. Incorporation

Values represent mean of individual determinations of each of three samples, each sample consisting of three pooled pairs of adrenals. Range of values (in parentheses) is presented only where relevant to the discussion.

² Equivalent chain length on EGS column, see Methods.

³ Approximately 80% 18:1 ω 9 and 20% 18:1 ω 7.

⁴ Tentatively identified as 5,9-18:2.

⁵ Identified as a mixture of 20:2 acids with no methylene-interrupted double bond sequence.

22:3 ω 9	23.3	10.0(11.0-10.0)	18.4(16.1-20.2)	2.0(1.3-2.8)	1.3(0.7-1.8)	7.6(5.0-8.8)	0.5(0.3-0.8)
22:4 ω 6	23.8	3.9(2.9-4.8)	3.2	—	1.0	1.6	—
22:5 ω 3	25.3	0.4	6.0	—	2.3	2.0	—
22:6 ω 3	25.9	2.0	—	3.5	—	—	—

ration of *trans* fatty acids undoubtedly results in changes in the fatty acid composition of the lipoprotein lipid constituents and these changes may well modify yields and routes of the reaction. It has already been established that desaturation of fatty acids, occurring in mammalian organism, is markedly depressed in the liver of fasted, diabetic and aged animals (15-17).

Furthermore, when diets were supplemented with 2% corn oil, in which case the rats attained an adequate essential fatty acid status, docosadienoic acid did not occur at noticeable concentrations. Under these conditions, the concentration of 20:4 ω 6 and 22:4 ω 6 acids in adrenal cholesterol esters was low, suggesting that linoleic acid from the corn oil, by itself and not by its metabolites, inhibited the formation of 22:2 acid. At present, the manner by which this control is exercised is not known. An increase in palmitic and stearic acid concentration observed in the same experiment may be related to the increase in linoleic and 18:3 ω 6 acid concentration. It is well known that when a particular fatty acid is increased by dietary means the rest of the fatty acids do not maintain the same percentage distribution relative to each other.

Our data show that *trans* fatty acids were incorporated into adrenal cholesterol ester, triglycerides, and phospholipids at 22, 17, and 7% levels, respectively. The preferential incorporation of *trans* fatty acids in cholesterol esters points to similar preferences in their incorporation in other tissues, like liver as opposed to plasma lipids (18). If adrenal lipids are synthesized *in situ*, then the same factors mentioned in an earlier discussion (19) could control the fatty acid composition of adrenal lipids. If we assume, on the other hand, that cholesterol elaidate is rather derived from plasma cholesterol esters, then differences in ease of disposal of the ester could account for its accumulation. This aspect is currently under investigation in this laboratory.

ACKNOWLEDGMENT

The authors wish to thank Dr. F. A. Kummerow for his interest in this work.

LITERATURE CITED

- Egwim, P., and D. S. Sgoutas 1970 Occurrence of eicosadienoic acids in liver lipids of rats fed partially hydrogenated soybean fat. *J. Nutr.* 101: 307-314.
- Mangold, H. K. 1961 Thin layer chromatography of lipids. *J. Amer. Oil Chem. Soc.* 38: 708.
- Sperry, W., and M. Webb 1950 Revision of Schoenheimer-Sperry method for cholesterol determination. *J. Biol. Chem.* 187: 97.
- Bartlett, G. R. 1959 Phosphorus assay in column chromatography. *J. Biol. Chem.* 234: 466.
- Stoffel, W., F. Chu and E. H. Ahrens, Jr. 1959 Analysis of long chain fatty acids by gas liquid chromatography: micromethod for preparation of methyl esters. *Anal. Chem.* 31: 307.
- Hofstetter, H. H., N. Sen and R. T. Holman 1965 Characterization of unsaturated fatty acids by gas-liquid chromatography. *J. Amer. Oil Chem. Soc.* 42: 537.
- Swern, D., H. B. Knight, O. D. Shreve and M. R. Heather 1950 Comparison of infrared spectrophotometric and lead salt alcohol methods for determination of *trans* octadecenoic acid and esters. *J. Amer. Oil Chem. Soc.* 27: 17.
- Moore, J. H., and D. L. Williams 1966 Studies on the cholesterol esters of the adrenal glands and other tissues of the rabbit. *Biochim. Biophys. Acta* 125: 352.
- Beroza, M., and B. A. Bierl 1967 Rapid determination of olefin position in organic compounds in microgram range by ozonolysis and gas chromatography-alkylidene analysis. *Anal. Chem.* 39: 1131.
- Gidez, L. I. 1964 Occurrence of a docosatrienoic acid in the cholesterol esters of adrenals of rats on essential fatty acid deficient diets. *Biochem. Biophys. Res. Commun.* 14: 413.
- Walker, B. L. 1970 The fatty acids of adrenal lipids from essential fatty acid-deficient rats. *J. Nutr.* 100: 355.
- De Vries, B. 1963 Quantitative separation of higher fatty acid methyl esters by absorption chromatography on silica impregnated with silver nitrate. *J. Amer. Oil Chem. Soc.* 40: 184.
- Carroll, K. K. 1962 Studies on the mechanism by which erucic acid affects cholesterol metabolism. *Can. J. Biochem. Physiol.* 40: 1115.
- Lemarchal, P., and N. Munsch 1965 Étude sur le métabolisme de l'acide élaidique chez des rats carencés on acides gras indispensables. *Comptes Rendus* 260: 714.
- Lemarchal, P. 1966 Deshydrogenation de l'acide élaidique *in vitro* par des homogénats de foie de rat. *Comptes Rendus* 262: 816.
- De Gomes Dumm, I. N. T., M. J. T. De Alaniz and R. R. Brenner 1970 Effect of diet on linoleic acid desaturation and on some enzymes of carbohydrate metabolism. *J. Lipid Res.* 11: 96.
- Inkpen, C. A., R. A. Harris and F. W. Quackenbush 1969 Differential responses to fasting and subsequent feeding by micro-

- lly hydrogenated soybean
07-314.
- 1961 Thin layer chroma-
J. Amer. Oil Chem. Soc.
- Webb 1950 Revision of
ry method for cholesterol
biol. Chem. 187: 97.
- 1959 Phosphorus assay in
graphy. J. Biol. Chem.
- u and E. H. Ahrens, Jr.
long chain fatty acids by
ography: micromethod for
ethyl esters. Anal. Chem.
- N. Sen and R. T. Holman
ation of unsaturated fatty
chromatography. J. Amer.
: 537.
- Knight, O. D. Shreve and
1950 Comparison of infra-
etric and lead salt alcohol
rmination of *trans* octa-
esters. J. Amer. Oil Chem.
- d D. L. Williams 1966
lesterol esters of the adre-
her tissues of the rabbit.
Acta 125: 352.
- B. A. Bierl 1967 Rapid
olefin position in organic
rogram range by ozonoly-
atography-alkylidene anal-
39: 1131.
- 1 Occurrence of a doco-
the cholesterol esters of
n essential fatty acid defi-
m. Biophys. Res. Commun.
- 970 The fatty acids of
om essential fatty acid-
lutr. 100: 355.
- 3 Quantitative separation
cid methyl esters by ab-
graphy on silica impreg-
nitrate. J. Amer. Oil Chem.
- 62 Studies on the mech-
ucic acid affects cholesterol
J. Biochem. Physiol. 40:
- and N. Munsch 1965
olisme de l'acide élaidique
nce's on acides gras indis-
s Rendus 260: 714.
- 1966 Deshydrogenation de
n *vitro* par des homoge-
at. Comptes Rendus 262:
- I. N. T., M. J. T. De Alaniz
1970 Effect of diet on
uration and on some en-
lrate metabolism. J. Lipid
- A. Harris and F. W.
1959 Differential responses
sequent feeding by micro-
somal systems of rat liver: 6- and 9-desatura-
tion of fatty acids. J. Lipid Res. 10: 277.
18. Privett, O. S., and M. L. Blank 1964 Stud-
ies on the metabolism of linoleic acid in
the essential fatty acid deficient rat. J.
Amer. Oil Chem. Soc. 41: 292.
19. Goller, H. J., D. S. Sgoutas, I. A. Ismail and
F. D. Gunstone 1970 Dependence of sterol
ester hydrolase activity on the position of
ethylenic bond in cholesteryl *cis*-octadeceno-
ates. Biochemistry 9: 3072.

Viewpoint on the Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults

Biochemical studies at the molecular level have not to date confirmed a causal relationship between dietary saturated fatty acids (SFA) and coronary heart disease (CHD). I shall point out that the metabolism of SFA involves a complex process and is dependent on essential cofactors. My discussion will call particular attention to the essential role of magnesium (Mg) in lipid metabolism and to the ambiguity of dietary recommendations.

The Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults (NCEP) [1] provides guidelines for the evaluation and treatment of high blood cholesterol in adults 20 years of age and over: "The report specifies the LDL-cholesterol at which dietary therapy should be started and the goals of therapy, and provides detailed guidance on the nature of the recommended dietary changes."

The report also states that the general aim of dietary therapy is "to reduce elevated cholesterol levels while maintaining a nutritionally adequate eating pattern" [1]. In a recent review entitled *Dietary Influences on Serum Lipids and Lipoproteins*, Grundy and Denke [2] conclude that three identified nutritional factors raise serum LDL levels: 1) saturated fatty acid (SFA), 2) cholesterol itself, and 3) excess caloric intake leading to obesity. The influence of the various fatty acids (FA) in dietary lipids on total and LDL plasma cholesterol levels was thoroughly reviewed by Grundy and Denke. More recently Du Pont et al [3] estimated the total availability of SFA in the United States at 13%, with 7.7% from meat, poultry, and fish, and 5.3% from other sources. They estimate that fats and oils in visible and invisible forms and in pure and processed forms contribute 21% of the total SFA.

The total US per capita per year availability of edible fats and oils in 1987 was 62.4 lbs, which decreased 1.5 lbs/capita/year by 1989, possibly due to more commercial reuse of cooking oil. In Europe, a law requires the application of a simple "cooking life" test. Such a test is not

required in the United States and the cooking oil may have been used for a longer period of time during the present recession. Salad and cooking oil production has increased from 7.7 to 25.2 lbs, shortenings from 17.4 to 21.3 lbs, and margarine from 8.9 to 10.5 lbs/capita/year, whereas butter, lard and tallow production have all decreased since 1959 [4,5]. Fat consumption has shifted towards more salad and cooking oils and more margarine and shortening, which increased total fat consumption by 16.4 lbs/capita/year (Fig. 1) since 1959 in spite of dietary recommendations to lower fat intake that were first published by the American Heart Association in 1961 [6].

The ambiguity of dietary recommendations has, in my opinion, contributed to the increase in total fat consumption per capita during the last 30 years. The slogan "low in saturates and low in cholesterol" has actually increased SFA consumption. Salad oil consumption has not increased significantly but the consumption of cooking oils that are used in the frying of chicken, fish, other seafoods, corn chips, potato chips, doughnuts, and french fries has increased significantly. To label all cooking oils as "oils" is not correct; many are vegetable oils stabilized by hydrogenation to increase their heat stability as indicated by Du Pont et al [3]. Furthermore, if total visible and invisible fat consumption per capita is considered, vegetable fats supply approximately the same amount of SFA to the diet as do animal fats.

At the request of the editor of *Paroi Arterielle/Arterial Wall* I wrote an editorial entitled "Dietary Recommendations to Reduce Cholesterol Consumption May Have Undesirable Consequences," which was translated into French and German [7]. I stated the following in this 1981 editorial:

I disagree with the well-meaning advice of prestigious organizations that recommend a reduction [8] of dietary cholesterol consumption from 600 to 300 mg/day. I disagree because such a reduction would result in a less nutritious diet for most people; it would not prevent human

Address reprint requests to F.A. Kummerow, PhD, 205 Burnside Research Laboratory, University of Illinois, 1208 W. Pennsylvania Ave., Urbana, IL 61801.

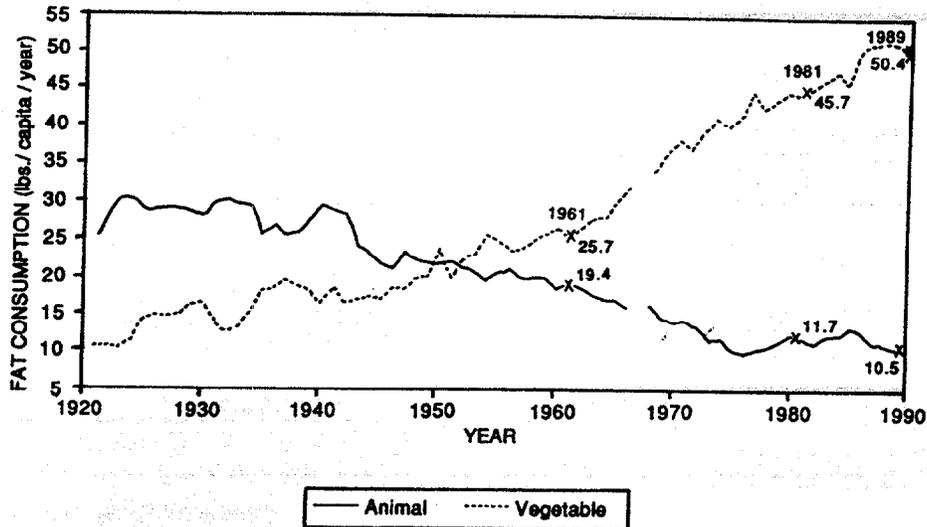


Fig. 1. Comparison of fat sources and consumption.

56.4
 10.5
 60.9
 1.5
 62.4

atherosclerosis, and it would be disastrous for agriculture in the US, Canada and Northern Europe. Reducing the cholesterol intake from 600 to 300 mg/day would reduce animal protein intake from the present 70 to 35 g/day/capita in the US since the cholesterol is supplied by animal protein sources such as meat, milk and eggs. Although the animal protein intake in Northern Europe is not as high as in the US or Canada, decreasing the animal protein intake to one-half of its present level would be as undesirable for Europe as it is for the US and Canada.

The calories supplied by eggs, meat and milk *would probably be replaced by low-protein high-fat food items* with the possibility that more rather than less calories would be consumed. Sufficient high-protein vegetable food items at less cost and the equivalent in nutritional value to eggs, meat and milk are not available. The low-calorie foods such as vegetables and fruits, which are recommended as a replacement for animal protein foods, are expensive and are not "satisfying" as high-calorie foods such as soft drinks, potato chips, french fries and candy bars. The result may well be a diet higher in fat and more deficient in protein, vitamins and minerals, thus facilitating more rather than less atherosclerosis [9].

In addition to the three factors that Grundy and Denke believe raise serum LDL, I would like to add five more: 1) a low level of dietary magnesium (Mg) which may influence desaturase activity and apo A-I, A-IV, β , C and E synthesis, 2) the order parameter of the lipids in the plasma lipoproteins, 3) the level of "trans" FA in the diet, 4) the influence of amino acid composition on plasma cholesterol levels, and 5) the importance of trace minerals.

LOW LEVEL DIETARY MAGNESIUM MAY INFLUENCE DESATURASE ACTIVITY AND APO A-1, A-2, β , AND E SYNTHESIS

Nutritionists have considered Mg an essential element for optimum health [10-13], and biochemists have found that Mg activates the enzymes in both the glycolytic and tricarboxylic acid cycles, both of which are essential to life [14]. Rayssiguier et al [15-22] showed Mg to be an active participant in lipid metabolism. Mg²⁺-deficiency produced hypercholesterolemia, hypertriglyceridemia and dyslipoproteinemia characterized by an increase of very-low-density lipoprotein (VLDL) and LDL and a decrease of high-density lipoprotein (HDL) [16]. In Mg²⁺-deficient rats an increase of plasma-free cholesterol and a decrease of esterified cholesterol as a result of reduced lecithin-cholesterol acyltransferase activity (LCAT) were observed [16]. Changes in plasma FA were also reported in Mg²⁺-deficient rats. These changes were characterized by a decrease of stearic and arachidonic (20:4 ω 6) acids and an increase of oleic and linoleic (18:2 ω 6) acids [17]. They were related to the hypertriglyceridemia and the increase of VLDL and LDL and decrease of HDL since a notable FA alteration can occur as the density of the lipoprotein increases [17].

Many cellular functions and responses are affected when membrane cholesterol or FA unsaturation is modified [23]. These include carrier-mediated transport, membrane-bound enzymes and receptor properties. In Mg deficiency, the FA composition of the liver microsomes indicated a slower rate of conversion of 18:2 ω 6 to 20:4 ω 6 in vivo [24], which is consistent with the decrease of Δ 6

desaturase activity in liver microsomes of Mg-deficient rats as measured in vitro. The decrease of $\Delta 6$ desaturase activity was attributed to the lower concentration of enzyme molecules as a result of the decreased rate of protein synthesis in Mg deficiency. On a high-cholesterol diet, Mg-deficient rats developed hypercholesterolemia and lower HDL levels than did control rats on the same diet [25].

Fluorescence polarization has been used to compare fluidity of membrane preparations from Mg-deficient and control rats [26]. Erythrocyte membranes, lymphocytes and hepatocyte plasma membranes from Mg-deficient rats were more fluid than were those of control rats, and Mg deficiency induced a significant decrease in anisotropy of both intact hepatic mitochondria and inner mitochondrial membranes. Several functional alterations in the membrane occurred in parallel to the physical changes measured by the increase in fluidity [27]. The loss of Mg from membranes may contribute to the increased fluidity owing to the direct binding of the cation to phospholipid (PL) head groups, but metabolic alterations of the lipid composition are also involved in the modification of membrane fluidity that occurs during Mg deficiency.

We have found that the molar concentration of Mg in the medium influenced both the PL head group composition [28] and the FA composition of mammalian cells in tissue culture [29]. The FA composition of the cellular PL showed a significant decrease in 20:4 ω 6 and a significant increase in 18:2 ω 6 in Mg-deficient cells compared to Mg-sufficient cells, which is consistent with the decrease of $\Delta 6$ desaturase activity in liver microsomes of Mg-deficient rats as measured in vitro. We also found Mg deficiency produces hypercholesterolemia and hypertriglyceridemia characterized by an increase of VLDL and LDL, and a decrease of HDL in rats [24]. Plasma total cholesterol and triglyceride (TG) concentrations were significantly elevated in Mg-deficient rats, and the increase in TG was much higher than the increase in total cholesterol. In human studies, plasma Mg and cholesterol [30] and free fatty acid levels [31] were negatively correlated.

Recent experiments by Gueux et al [32] indicate that apo-mRNA (messenger ribonucleic acid) in the liver of Mg-deficient rats is significantly increased as compared to control rats. These results showing a stimulatory effect of Mg deficiency on apo-gene expression are of interest since Gueux et al [32] found secretion of VLDL to be totally dependent upon apo- β . Therefore, one cause of hyperlipidemia may be overproduction of apo- β entering the circulation. Mg deficiency induced a decrease in the percent composition of apo-E and a relative increase in the apo-C for VLDL and HDL. The proportion of apo A-I was higher than normal, apo A-IV was lower than normal, and apo-E was virtually absent in Mg deficiency [33]. (For a more detailed discussion of the role of Mg in the structure and function of plasma membrane, see [34]).

Advances in understanding lipoprotein metabolism and causes for the pathogenesis of hyperlipoproteinemia have been due in part to studies of patients with unusual defects in lipoprotein metabolism [35]. Other studies on lipoprotein metabolism have been based on plasma cholesterol responses to dietary fats [36-45]. Early results in which the plasma cholesterol-raising effect of SFA was first noted may have been due to a deficiency in dietary Mg. Aherns et al [46] used liquid diets composed of sugar, milk protein and the test fat. The basic formula consisted of milk protein (15%), fat (40%), and carbohydrate (45%) of total calories. Milk is a relatively poor source [47] of Mg (13 mg/100 ml). The test formula of 2500 cal/day contained 96 mg Mg [48], which is one-third to one-fourth the 1980 National Research Council recommendation, depending on a subject's lean weight. Subjects used in these studies were hypercholesterolemic and were fed the test diet for up to 8-10 weeks. Tongai et al [26] have found that weanling rats fed a Mg-deficient diet for only 8 days began to show signs of Mg deficiency with changes in lipid metabolism. It is possible that in all of the experimental studies in which liquid diets deficient in Mg were used, activity of $\Delta 5$ and $\Delta 6$ desaturating enzymes was reduced and not available for the conversion of the SFA in coconut oil or butter fat to unsaturated FA. Corn oil is already desaturated and had little influence on plasma cholesterol [46].

The NCEP stated that "margarine represents partially hydrogenated vegetable oil and is preferable to butter." This statement is probably based on the experimental studies of Ahrens et al [49] diets and the studies of Keys et al [50-52] and others [53,54] with men on institutional diets of unknown Mg content. It is possible that these institutional diets were also deficient in Mg. Seelig [55-58] has called attention to the low Mg content of the American diet and a number of studies have suggested that Mg intake is inadequate in the US population [12,59,60]. Furthermore, the plasma Mg level is not a reliable index of tissue Mg deficiency [47].

We have recently tested the influence of dietary Mg level on the plasma lipid levels in swine fed butter vs margarine and found that the level of Mg in the diet was more significant to the total plasma cholesterol, LDL and HDL level than was the dietary FA content of an animal or a vegetable fat [61]. On a dietary Mg level considered adequate for swine by the National Research Council [62], there was a significant difference in total plasma cholesterol level between those fed margarine and butter: 115 and 126 mg %, respectively; the LDL plasma cholesterol level was higher in those fed butter [63]. However, when an additional 248 mg Mg/kg of diet was fed, there was no significant difference in total plasma cholesterol: 115 and 112 mg % and no significant difference in LDL or HDL level. Flynn et al [64] found no significant differences in plasma cholesterol levels in humans on adequate

diets which contained either margarine or butter.

Epidemiological studies have shown that the consumption of mineral rich "hard" drinking water protects against cardiovascular disease (CVD), including strokes and hypertension [37,65-69]. Schroeder [70] and Durlach et al [71] have presented evidence that water Mg (a component of water hardness) actually correlates better than total water hardness with protection against CVD. Mg intake has been progressively decreasing in industrialized countries over the past century [72,73], and this decrease has been related to the increase in CVD and hypertension [73]. In countries such as France, Italy, Greece, India, China and Japan with less CVD than the United States, ocean salt—which contains Mg, calcium (Ca) and potassium, in addition to sodium—is used as table salt. The use of ocean salt was discontinued in Japan in 1970, but is now being reintroduced there as a table salt [34].

Whether clinical trials such as "The Lipid Research Clinics Coronary Primary Trial" (LRC-CPPT) may have been compromised by the Mg and Ca content of the water supply at the 12-trial center requires a more controlled study. This trial was conducted at medical schools in 11 geographical areas of the United States and one in Canada (Table 1). The original report [74] stated on page 355, "The cholestyramine-treated group at seven clinics had at least 18% fewer primary end points than placebo-treated men. At four clinics there was essentially no treatment difference; only one clinic showed an excess of events in the drug group."

The Stanford Medical School (Table 1) clinic showed an excess of events in the drug group, with more deaths

from coronary heart disease (CHD) in the cholestyramine treatment group and with more definite myocardial infarctions (Def MI) and definite coronary heart disease and myocardial infarctions (Def CHD/MI). Oklahoma had one and Washington University Medical Schools had three more definite deaths in the cholestyramine group. The Universities of Cincinnati, Iowa and Toronto showed no difference in death. At the six remaining clinics there were more deaths in the placebo group. The largest difference between placebo and cholestyramine groups was at the University of Minnesota.

Significance calculated by the O'Brien Fleming method [75], which was used in the LRC/CPPT, indicated a statistical significance at the $\alpha=0.05$ for this study. The statistically significant differences between the placebo and cholestyramine groups may have been due to the pharmaceutical action of cholestyramine rather than differences in total serum cholesterol level or due to possible differences in the Ca and Mg content of the water supply. I telephoned the chemist in charge of the water analysis at each of the cities where the medical schools were located to check the Mg and Ca in the water supply that might have contributed to differences in the CHD/MI (Table 2). The Mg and Ca in the water supply at the 12 centers did differ, but due to variables in the sources, even within a city, it is not possible to draw any firm conclusions on the influence of Mg on the total serum cholesterol in individuals during the 7 years of the study.

The men recruited for this study were all middle aged with primary hypercholesterolemia (type II hyperlipoproteinemia) free of CHD but at high risk because of elevated

Table 1. Differences in Percent Change in Total Serum Cholesterol (TSC) from Visit 2 to Visit 5 by Clinic and Treatment (September 1977)

Clinic	Treatment group											
	Placebo						Cholestyramine					
	No. at risk	Diff. TSC	Def CHD death	Def MI	DEF CHD/MI	All mort.	No. at risk	Diff. TSC	Def CHD death	Def MI	Def CHD/MI	All mort.
Baylor	150	-3.8	6	10	16	8	153	-2.8	3	13	16	4
Univ. Cincinnati	160	-3.7	2	15	17	5	163	-3.3	2	16	18	5
G. Washington	118	-3.1	1	9	10	5	118	-1.5	0	8	8	1
Univ. Iowa	184	-4.2	2	16	17	7	188	-5.5	2	11	12	6
Johns Hopkins	125	-2.4	3	10	12	7	130	-1.9	1	12	13	4
Univ. California	147	-4.2	5	11	13	5	145	-4.2	2	8	10	6
Univ. Minnesota	201	-3.4	8	19	26	12	202	-4.1	3	12	14	5
Oklahoma Medical	152	-5.1	2	13	15	5	148	-2.3	3	13	16	8
Univ. Washington	171	-4.5	2	10	11	6	169	-4.2	1	8	9	4
Stanford	180	-2.0	0	11	11	1	176	-1.8	3	14	17	6
Univ. Toronto	149	-5.5	5	19	22	8	151	-3.4	5	11	14	10
Washington Univ.	182	-4.8	2	15	17	2	164	-4.5	5	4	8	9
Total	1899	-3.9	38	158	187	71	1907	-3.4	30	130	155	68

Data courtesy of Dr. C.N. Davis, Department of Biostatistics, North Carolina Medical School, Chapel Hill.

Definite coronary heart disease death (Def CHD death). Definite nonfatal myocardial infarction (Def MI). Definite CHD deaths and/or definite nonfatal myocardial infarctions (Def CHD/MI). All mortality (All mort).

Table 2. Average Water Composition at Clinic Locations

Clinic	Location	Ca (mg/100 ml)	Mg (mg/100 ml)	Water co. phone
Baylor	Houston, TX	3.6	1.72	713-880-2444
Univ. Cincinnati	Cincinnati, OH	3.95	0.6	513-352-4651
George Washington	Washington, DC	3.8	0.7	202-282-2741
Univ. Iowa	Iowa City, IA	2.15	0.36	319-356-5160
Johns Hopkins	Baltimore, MD	1.99	0.55	301-396-5352
Univ. California	La Jolla, CA	5.4	2.1	619-463-0327
Univ. Minnesota	Minneapolis, MN	0.63	2.25	612-788-3907
Oklahoma Medical	Oklahoma City, OK	7.9	2.6	405-297-2535
Univ. Washington	Seattle, WA	1.2	0.82	206-684-7404
Stanford	Stanford, CA	1.2	0.27	415-329-2241
Univ. Toronto	Toronto, Canada	4.00	0.84	416-392-8224
Washington Univ.	St. Louis, MO	3.04	0.29	314-868-5640

Analysis of the water supply at the respective cities was supplied through the courtesy of the chemist in charge of water analysis at the cities involved in the Lipid Research Clinics Coronary Primary Prevention Trails (JAMA 251:351-374, 1984). All 12 cities used river water. Cities near lakes, dams or deep wells of higher Ca and Mg content blend this water with river water.

LDL-cholesterol levels. The total serum cholesterol level among Chinese varies between 150-160 mg %, yet cerebral vascular disease (also due to atherosclerosis) is the leading cause of death in Beijing followed by cancer and CHD [76-79]. Data obtained from coronary bypass operations question whether low total serum cholesterol level can prevent CHD in the United States [80]. Men suffering from hypercholesterolemia are at risk. However, as cholestyramine seemed to decrease the risk of CHD/MI at six of the 12 clinics, there may be factors in the plasma other than the total serum cholesterol level which influence CHD development. In my opinion, the cause for CHD due to atherosclerosis is far more complex than the total serum cholesterol level or SFA levels or the amount of cholesterol in the diet.

THE ORDER PARAMETER OF THE PLASMA LIPOPROTEIN

The NCEP has not taken into consideration the dynamics involved in lipid metabolism at the molecular level. As previously indicated, changes in acyl unsaturation or cholesterol content can result in an altered organizational state of the lipid in the lipoprotein or a cell membrane which can be detected by measurement of the order parameter (S_{DPH}) [81]. In the plasma, the lipoproteins exist as spherical micelles-like particles rather than as a rigid PL and cholesterol/protein interlaced bilayer structures of the type found in cell membranes. The more saturated acyl chains in lipoproteins may increase the rigidity in the lipoprotein organization, while unsaturated acyl chains in the lipid portion of lipoproteins may increase the freedom of motion [63]. In cholesterol-lecithin bilayers, cholesterol moderates both effects, making unsaturated chains less random

and saturated chains less rigid [82]. We noted no significant difference in the S_{DPH} parameter in the plasma LDL or HDL from swine fed margarine vs butter [63]. Order parameters measured by diphenyl hexatriene (DPH) provides an indication of the degree of organization of the lipids being measured (S_{DPH}) [81]. Cholesterol may moderate the relationship between cholesterol and the saturated and unsaturated acyl chains in the lipid portion of the LDL and HDL lipoprotein [63,81,82].

The NCEP believes that for every 1 mg % lowering plasma cholesterol level, replacing an SFA by polyunsaturated fat (PUFA) would lower the risk of heart disease by 2%. However, there may be another explanation for this phenomenon, i.e., the role of Mg in SFA metabolism and the role of microviscosity (S_{DPH}). The plasma cholesterol level may be lowered by PUFA because less cholesterol is needed for the plasma to keep the microviscosity (S_{DPH}) of the LDL lipoprotein in the plasma in the lumen recognizable to the receptor sites on cell membranes. Although the explanation for enzyme action as a lock and key arrangement is no longer in vogue [83], it may serve as an explanation for the lower plasma cholesterol level in the presence of increased PUFA intake. The liver must secrete LDL particles that are recognized by the receptor sites on cell membranes (the lock). The order parameter of the lipid portion of the LDL lipoprotein (the key) may be as important as the apoprotein portion for the recognition of LDL for the key to work in the lock properly (the receptor site).

THE LEVEL OF TRANS FATTY ACIDS IN THE DIET

Dupont et al [3] stated in their review: "Trans FA have physical properties like SFA making them rigid rather than

fluid in membranes. *Trans* FA are absorbed as well as oleic acid, stored in adipose tissue proportional to the dietary source similar to other long chain FA and are transported and oxidized for energy in ways similar to other long chain FA."

Enig et al [84] in a review of current data on *trans* FA in the US diet reported the total availability of vegetable fat as 62.9 g and animal fat as 62.1 g, or a total of 124 g fat which furnishes 12.83 g *trans* FA/day. The wastage of vegetable fats (mainly in frying) was estimated at 15%, leaving approximately 114.7 g or 1032 calories for consumption. Whether that many calories from fat are actually consumed is debatable [85]; however, if the consumption of 32 g (128 calories)/capita/day of sugar [86] is added, 1160 (or more than one-third) of the calories in the US diet are supplied by fat and sugar. (For further discussion on the role of calories in the American diet see [9]).

Soybean and cottonseed oil have been hydrogenated since 1920 and have served as a gradual replacement of lard in the baking of cakes, cookies and pies. It was not until the shortage of fats between 1940-1945 that margarine began to replace butter. Until the 1940s chemists in charge of quality control found it difficult to control the composition of hydrogenated fats as hydrogenation is carried out in 20,000-30,000-lb batches in stainless steel tanks. Very little was known before 1940 about how to control hydrogenation conditions so that one batch had the same SFA composition as another [87]. The rate of stirring, temperature of reaction, hydrogen pressure, and number of times the catalyst was reused all determined the SFA content of the product. The instrumentation available, such as the gas chromatograph (GC), identified not only the amount of stearic, oleic and linoleic acid in the final product but also the amount of shifting of both geometrical and positional structures of the double bonds in the oleic, linoleic and linolenic acid in the oils during hydrogenation. This knowledge was especially important to margarine production.

In 1968 Dr. Campbell Moses, medical director of the American Heart Association (AHA), appointed a five-member subcommittee on fats of the AHA nutrition committee to revise the 1961 version of "Diet and Heart Disease." As a member of this subcommittee I urged Dr. Moses to ask the Institute of Shortening and Edible Oils Inc to have its member organizations decrease the amount of *trans* FA and increase the amount of essential fatty acids (EFA) in their shortenings and margarines. At the time it was known that an increase in EFA composition of a dietary fat would lower plasma cholesterol levels and there was strong evidence that *trans* FA increased plasma cholesterol levels. The first version stated:

"Partial hydrogenation of polyunsaturated fats results in the formation of *trans* forms which are less effective than *cis,cis* forms in lowering cholesterol concentrations. It should be noted that many currently available shorten-

ings and margarines are partially hydrogenated and many contain little polyunsaturated fat of the natural *cis,cis* form." The Institute of Shortening and Edible Oils Inc objected to this version. The second and distributed [88] version, omitting reference to *trans* and *cis* FA stated:

"Margarines that are high in polyunsaturates usually can be identified by the listings of a "liquid oil" first among the ingredients. Margarines and shortenings that are heavily hydrogenated or contain coconut oil, which is quite saturated, are ineffective in lowering the serum cholesterol."

Industry agreed to lower the *trans* FA and increase the level of EFA in shortenings and margarine (Table 3). Dr. R. I. Levy, director of the National Heart, Lung, and Blood Institute at the time, believed 1968 a watershed, as the incidence of CHD has steadily decreased in the US since that year [89]. Why it decreased remains unknown [90]; however, a publication in 1981 by Kinsella et al [91] may provide a partial explanation for the decreased incidence in CHD. Kinsella et al [91] explained, "Because of the important role of linoleic acid as the principal precursor of cyclic endoperoxides, prostaglandins and leukotrienes, the potential deleterious effects of *trans* isomers of this acid are discussed. High levels of dietary *trans,trans* linoleate can impair $\Delta 6$ desaturase activity and decrease prostaglandin production in rats on experimental diets." Kinsella et al further stated in this paper: "It is extremely important to carefully reexamine the role of *trans* fatty acids on desaturase inhibition in vivo, because even small changes in fatty acid concentrations may result in large changes in the levels of PGs synthesized. Since PGs have been shown to play a critical role in modulating many physiological events, the importance of reexamining the influence of dietary *trans* FA (as well as other dietary substances) on EFA metabolism and subsequent PG biosynthesis is extremely important." We showed in 1984 [92] that the *trans* 18:1 acid in partially hydrogenated soybean oil has a more inhibitory effect than SFA on EFA metabolism, even in the presence of adequate amounts of EFA.

As indicated by a lipid analysis the linoleic acid content of the red cells (erythrocytes) of Americans has increased from 9% in 1964 [93] to 13.7% in 1979 [9] and to 20.5% in 1980 [94]. One would assume that the possible combination of FA in the phospholipid head group of mammalian cell membranes brought on by excessive dietary fats in the American diet allows for an optimum microviscosity (S_{DPH}) in the plasma membrane. Holman et al [95] have recently stated:

"Although a recent study did not discuss positional isomers present in hydrogenated fat, *trans* isomers of 18:1 were found to be at least as unfavorable as that of the cholesterol-raising saturated fatty acids, because they not only raise LDL cholesterol levels but also lower HDL cholesterol levels [96]. It is now clear that uncommon isomers of PUFA occur in the lipids of animals fed partially

Table 3. Composition of Shortenings and Margarines Before 1968 (Old) and After 1968 (New) Compared to Butterfat and Tallow

Fatty acid composition (%)	Shortening ¹		Margarine ¹		Butter ²	Tallow ²
	Old	New	Old	New		
Saturated	27	25	22	21	61	46
Monounsaturated	61	47	62	50	31	47
Polyunsaturated	12	28	16	29	4	4
<i>Cis-cis</i> linoleic	8	24	11	25	>3	<3
<i>Trans</i> acids	30	20	40	27	2-4	5-10

¹ Courtesy of Procter & Gamble Co, 1968.

² Sommerfeld M: *Trans* unsaturated fatty acids in natural products and processed foods. *Prog Lipid Res* 22:221-233, 1983. Cholesterol: 240 mg/100 g in butter, 90 mg/100 g in tallow. [*Trans* fatty acids act like saturated fatty acids in vivo. Lands et al: Distribution of fatty acids in lecithins and triglycerides in vivo and in vitro. *Lipids* 8:111-118, 1973.]

hydrogenated fat and that they inhibit the metabolism of PUFA at many steps in the normal metabolic cascade. It would, therefore, seem wise to avoid foods that contain unusual or unnatural isomeric PUFA or their isomeric monoenoic fatty acid (FA) precursors. The latter, both *cis* and *trans* positional isomers of 18:1, occur abundantly in partially hydrogenated vegetable oils now commonly consumed by Western populations. The large scale hydrogenation of vegetable oils reduces ω 3 and ω 6 essential fatty acids (EFA) and replaces them by saturated and isomeric 18:1 acids that interfere with the ω 3 and ω 6 metabolism, including significant partial deficiencies of EFA. It would seem wise to preserve the essential nutrients and to avoid producing inhibitors of their metabolism by hydrogenation. Evidence is growing for the essentiality of ω 3 PUFA and the occurrence of deficiencies of ω 3 acids in humans under stress conditions [97]. It would, therefore, be wise economy to use oils containing linolenic acid directly as foods and to avoid their hydrogenation." The hydrogenated fat used by Holman et al [95] was fed at an adequate level of Mg. As we have shown that an inadequate level of Mg inhibits the conversion of 18:2 ω 6 to 20:4 ω 6 [24], it seems essential to study in greater detail the influence of inadequate Mg on effects of high SFA diets in Western populations.

THE INFLUENCE OF AMINO ACID COMPOSITION ON PLASMA CHOLESTEROL LEVELS

In the American diet, eggs, meat and dairy products furnish amino acids that are necessary for the synthesis of body tissue and serum lipoproteins which "carry" the excessive load of dietary fat [9]. Approximately 50% of the amino acids in the various apoprotein portion of lipoproteins are "essential amino acids," that is, they must be furnished by the daily diet. An unbalanced dietary amino acid level can increase serum cholesterol levels. For ex-

ample [98], crystalline amino acid mixtures which were deficient or made deficient by the elimination of a specific essential amino acid invariably elevated serum cholesterol levels and depressed growth in chicks. Upon correction of the deficiency, improved growth and lower serum cholesterol levels were observed. Since casein is deficient in arginine for optimum chick growth, addition of this amino acid to a mixture of essential amino acids simulating casein gave a very favorable response in weight gain and lowered serum cholesterol values. These results are in agreement with those reported by Johnson et al [99] on an intact casein diet. However, the addition of arginine to a mixture of crystalline amino acids did not produce a marked change in serum cholesterol level. Similar results were obtained with graded levels of glycine. Therefore, the requirement for a specific amino acid for normal growth and a "normal" serum cholesterol level may depend on the right proportion and proper balance of all the essential amino acids in protein.

The specific amino acid involved in either the depression or elevation of serum cholesterol levels did not necessarily have to be an amino acid previously classified as indispensable. For example, addition of a mixture of non-essential amino acids which simulated casein generally showed a marked hypocholesterolemic effect although no advantage in weight gain was noted. Individual amino acids in the group of nonessential amino acids were without effect, but in various combinations some amino acids were observed to depress serum cholesterol levels. Thus, the addition of serine and aspartic acid or serine, aspartic acid and alanine lowered the serum cholesterol level significantly. Addition of proline alone or in combination with other nonessential amino acids seemed to elevate slightly the serum cholesterol level. Casein a milk protein raises serum cholesterol while soy protein lowers serum cholesterol levels [100]. This is a complex issue. The complete protein in eggs, meat and dairy products may be the best source of essential amino acids.

I agree with food choices of the NCEP to decrease whipped toppings made from hydrogenated fat and to decrease doughnuts, potato chips, corn chips, french fries, deep fat fried foods, and candy bars. I see no reason to decrease natural cheeses or egg yolk. The natural cheeses are excellent sources of protein and repeated studies [101-105], the last one by Flynn et al [64], have shown that eggs do not increase plasma cholesterol levels when used in conjunction with a balanced diet. Ice cream is moderately high in fat but is preferable to sherbert because ice cream contains more protein and Ca and less sugar. One hundred g of ice cream contains 202 calories, 3.6 g protein, 23.5 g carbohydrate and 132 mg Ca as compared to 140 calories, 1.1 g protein, 30.4 g carbohydrate, and 54 mg Ca in sherbert [106]. The difference of 62 calories between ice cream and sherbert is approximately one-half of that in a 10-oz soft drink. Furthermore, the NCEP made no recommendations on soft drinks, which is now a \$40 billion industry.

The consumption of soft drinks has increased from 19.8 gal/capita/year in 1968 to 32.0 gal/capita/year in 1989 and total alcohol beverage consumption increased from 33.6 to 38.9 gal/capita/year during the same time period [4]. On the other hand, milk consumption has decreased from 31.3 to 25.5 gal/capita/year since 1968. Although some soft drinks are sweetened with non-calorie aspartame, the total consumption of "soft drinks" and alcohol provides a substantial amount of calories to convert to SFA in vivo [9] and to contribute to the excess calorie intake leading to obesity [2]. Of the prestigious organizations listed in *Eat for Life: The Food and Nutrition Board's Guide to Reducing Your Risk of Chronic Disease* that was recently published by the Institute of Medicine National Academy of Science [107], only the American Diabetes Association specifically recommended limiting simple sugar intake.

IMPORTANCE OF TRACE MINERALS

The NCEB recommends elimination of "fatty beef." Beef provides trace minerals and 25.6 g of protein/capita/day to the American diet [4,107,108], which have made it possible to date for Americans to "get by" on a high-fat, high-soft drink, "empty-calorie" diet. Beef is a rich source of trace elements such as zinc (Zn), copper (Cu), chromium, cobalt, manganese, selenium, and molybdenum, all of which are necessary to the enzymes involved in lipid metabolism [109]. In countries with less heart disease, diets contain higher levels of these trace minerals [110] than in the US diet. Zn has recently been shown to be a component of Zn protein (parathymosin), a key in the enzyme involved in carbohydrate metabolism [111]. Both Mn and

Zn confer stability to arginase, which catalyzes the first committed step in the hydrolysis of L-arginine to L-ornithine and urea [112]. One Zn and one Mg ion, as well as three Cu ions, are components of cytochrome c oxidase, a key enzyme involved in electron transfer activity [113]. The last three studies were carried out in 1991, and there will undoubtedly be more studies to emphasize the role of trace elements in the metabolism of nutrients. (For more information on trace elements see [108]).

APPLICATION

Atherosclerosis, the underlying cause for heart disease and cerebral strokes, occurs at low and high plasma cholesterol levels. Atherosclerosis occurs in arterial cells and not in the plasma, but there are probably factors in the plasma which predispose arterial cells to develop atherosclerosis. High plasma cholesterol levels accentuate these conditions.

Current emphasis on reducing the consumption of eggs, meat and dairy products has resulted in an increase in fat consumption and more, rather than less, fat in the diet through the consumption of high-calorie, often deep-fat-fried snack foods. Furthermore, the inattention to Mg and the trace minerals that the animal-derived products, vegetables, grains and fruits supply has resulted in less than optimum intake of Mg and trace minerals, all of which are involved in lipid metabolism. The NCEP recommendation to increase fruits and vegetables is laudable. The most important factor for good health is still a balanced diet containing the proper number of calories.

ACKNOWLEDGMENT

I thank Dr. C. N. Davis, North Carolina Medical School, Department of Biostatistics, Chapel Hill, who was responsible for statistical analysis of the LRC-CPPT and who sent me data that were collected at the 12 centers. I also thank Dr. Ed Ahrens for supplying data on the Mg content of the test diet [46]; Dr. Claire Zizza, Human Nutrition Information Service, USDA, for consumption data; Dottie Slavik for typing; and Collin Van Ulchen and Julie Thiel for statistical analysis.

REFERENCES

1. The Expert Panel: Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. *Arch Intern Med* 148:39-69, 1988.

Viewpoint on NCEP Report

2. Grundy SM, Denke MA: Dietary influences on serum lipids and lipoproteins. *J Lipid Res* 31:1149-1172, 1990.
3. Du Pont J, White PJ, Feldman EB: Saturated and hydrogenated fats in food in relation to health. *J Am Coll Nutr* 10:577-592, 1991.
4. Putnam JJ, Allhouse JE: "Food Consumption, Prices, and Expenditures, 1968-89." Washington, DC: US Dept of Agriculture, Econ. Res. Ser., Stat Bull, No. 825, p 43, 1991.
5. US Dept of Agriculture: "Food Fats and Oils: Per Capita Consumption, 1909-66." Washington, DC: USDA, Food Consumption, Prices, Expenditures, Agric. Econ. Rep. No. 138, p 64, 1967.
6. American Heart Association: "Diet and Heart Disease." New York: AHA, 1961.
7. Kummerow FA: Dietary recommendations to reduce cholesterol consumption may have undesirable consequences. *Paroi Arterielle/Arterial Wall* 7:1 3-5, 1981.
8. Intersociety Commission of Heart Disease Resources: Report of Inter-Society Commission of Heart Disease Resources. *Circulation* 42:A55-94, 1970.
9. Kummerow FA: Nutrition imbalance and angiotoxins as dietary risk factors in coronary heart disease. *Am J Clin Nutr* 32:58-83, 1979.
10. Durlach J: Recommended dietary amounts of magnesium: Mg RDA. *Magnesium Research* 2:195-203, 1989.
11. Seelig MS: Nutritional status and requirements of magnesium: with consideration on individual differences and prevention of cardiovascular disease. *Magnesium Bull* 8:171-185, 1986.
12. Seelig MS: Magnesium requirements in human nutrition. *Magnesium Bull* 3(1a):26-47, 1981.
13. Seelig MS: Requirement of magnesium by the normal adult. *Am J Clin Nutr* 14:342-390, 1964.
14. Heaton FW: Magnesium in intermediary metabolism. In Cantin M, Seelig MS (eds): "Magnesium in Health and Disease." New York: Spectrum, pp 43-55, 1980.
15. Rayssiguier Y, Gueux E, Weiser D: Effect of magnesium deficiency on lipid metabolism in rats fed a high carbohydrate diet. *J Nutr* 111:1876-1883, 1981.
16. Rayssiguier Y: New data on magnesium and lipids interrelationships in pathogenesis of vascular diseases in magnesium deficiency. In Halpern MJ, Durlach J (eds): "Physiopathology and Treatment Implications." New York: Karger, pp 122-131, 1985.
17. Rayssiguier Y, Gueux E, Cardot P, Thomas G, Robert A, Trugnan G: Variations of fatty acid composition in plasma lipids and platelet aggregation in magnesium deficient rats. *Nutr Res* 6:233-240, 1986.
18. Rayssiguier Y: Magnesium and lipid interrelationships in the pathogenesis of vascular diseases. *Magnesium Bull* 3:165-177, 1981.
19. Rayssiguier Y: Role of magnesium and potassium on cardiac and vascular smooth muscle. *Magnesium* 3:226-238, 1984.
20. Rayssiguier Y: Magnesium, lipids and vascular diseases. Experimental evidence in animal models. *Magnesium* 5:182-190, 1986.
21. Rayssiguier Y, Gueux E: Magnesium and lipids in cardiovascular diseases. *J Am Coll Nutr* 5:507-519, 1986.
22. Rayssiguier Y, Gueux E: The reduction of plasma triglyceride clearance by magnesium-deficient rats. *Magnesium* 2:132-138, 1983.
23. Spector A, Yorek M: Membrane lipid composition and cellular function. *J Lipid Res* 26:1015-1035, 1985.
24. Mahfouz MM, Kummerow FA: Effect of magnesium deficiency on desaturase activity and fatty acid composition of rat liver microsomes. *Lipids* 24:727-732, 1989.
25. Gueux E, Rayssiguier Y: The hypercholesterolemic effect of magnesium deficiency following cholesterol feeding in the rat. *Magnesium Bull* 3:126-129, 1981.
26. Tongai A, Rayssiguier Y, Motta A, Gueux E, Maurois P, Heaton F: Mechanism of increased erythrocyte membrane fluidity during magnesium deficiency in weanling rats. *Am J Physiol* 257:270-276, 1989.
27. Rayssiguier Y, Gueux E, Motta C: Magnesium deficiency effect on fluidity and function of plasma and subcellular membranes. In Lassene B, Durlach J (eds): "Magnesium: A Relevant Ion." London: John Libbey, pp 311-319, 1991.
28. Mahfouz MM, Smith TL, Kummerow FA: Changes in phospholipid composition and calcium flux in LLC-PK cells cultured at low magnesium concentrations. *Biochim Biophys Acta* 1006:75-83, 1989.
29. Mahfouz MM, Smith TL, Kummerow FA: Changes of linoleic acid metabolism and cellular phospholipid fatty acid composition in LLC-PK cells at low magnesium concentrations. *Biochim Biophys Acta* 1006:70-74, 1989.
30. Golf SW, Riediger H, Matthes S, Kuhn D, Bortz C, Graef V, Temme H, Katz N, Roka L, Cseke J: Effect of magnesium on hyperlipidaemia. *Magnesium Research* 4:238, 1991.
31. Flink EB: Magnesium deficiency in human subjects—a personal historical perspective. *J Am Coll Nutr* 4:17-31, 1985.
32. Gueux E, Mazur A, Cardot P, Rayssiguier Y: Magnesium deficiency affects plasma lipoprotein composition in rats. *J Nutr* 121:1222-1227, 1991.
33. Rayssiguier Y, Noe L, Etenne J, Gueux E, Cardot P, Mazur A: Effect of magnesium deficiency on post-heparin lipase activity and tissue lipoprotein lipase on the rat. *Lipids* 26:182-186, 1991.
34. Kummerow FA: Hypothesis: Possible role of magnesium and calcium in the development of structure and function of the plasma membrane in mammalian cells and in human disease. *J Am Coll Nutr* 11:410-425, 1992.
35. Grundy SM: Pathogenesis of hyperlipoproteinemia. *J Lipid Res* 25:1611-1618, 1984.
36. Grundy S, Vega LG: Plasma cholesterol responsiveness to saturated fatty acids. *Am J Clin Nutr* 47:822-824, 1988.
37. Vergroesen AJ: Dietary fat and cardiovascular disease: possible modes of action of linoleic acid. *Proc Nutr Soc* 31:323-329, 1972.
38. Margolis S, Dobs A: Nutritional management of plasma lipid disorders. *J Am Coll Nutr* 8:33-45, 1989.
39. McOsker DE, Mattson FH, Sweringen HB, Kligman AM: The influence of partially hydrogenated dietary fats on

- serum cholesterol levels. *JAMA* 180:380-385, 1962.
40. Horlick L: The effect of artificial modification of food on the serum cholesterol level. *Can Med Assoc J* 83:1186-1192, 1960.
 41. Greene J, Feldman E: Physician's office guide to lipid-lowering diet. *J Am Coll Nutr* 5:443-452, 1991.
 42. Vergroesen AJ, Crawford M: "The Role of Fats in Human Nutrition." New York: Academic, 1989.
 43. Grasso S, Gunning B, Imaichi K, Michaels G, Kinsell L: Effects of natural and hydrogenated fats of approximately equal dienoic acid content upon plasma lipids. *Metabolism* 11:920-924, 1962.
 44. Hashim SA, Arteaga A, van Itallie TB: Effect of saturated medium-chain triglycerides on serum lipids in man. *Lancet* 1:1105-1108, 1960.
 45. Kagan A, Harris BR, Winkelstein W, Johnson KG, Kato H, Syme SL, Rhoads GC, Gay ML, Nichaman MZ, Hamilton HB, Tillotson J: Epidemiologic studies of coronary heart disease and stroke in Japanese men living in Japan, Hawaii and California: demographic, physical, dietary and biochemical characteristics. *J Chron Dis* 27:345-364, 1974.
 46. Ahrens E, Hirsch J, Insull W, Tsaltas T, Blomstrand R, Peterson M: The influence of dietary fats on serum-lipid levels in man. *Lancet* 2:943-953, 1957.
 47. Seelig MS: "Magnesium Deficiency in the Pathogenesis of Disease." New York: Plenum, p 1, 1980.
 48. Ahrens E: The use of liquid formula diets in metabolic studies: 15 years experience. *Adv Metab Disord* 4:297-315, 1970.
 49. Ahrens EH, Hirsch J, Insull W, Peterson ML: Dietary fats and human serum lipide levels. In Page IH (ed): "Chemistry of Lipids as Related to Atherosclerosis." Springfield, IL: CC Thomas Publishing Co, pp 224-252, 1958.
 50. Keys A, Anderson JT, Grande F: "Essential" fatty acids, degree of unsaturation, and effect of corn (maize) oil on the serum-cholesterol level in man. *Lancet* 1:66-68, 1957.
 51. Keys A, Anderson JT, Grande F: Serum cholesterol in man: diet fat and intrinsic responsiveness. *Circulation* 19:201-214, 1959.
 52. Keys A, Anderson JT, Grande F: Diet-type (fats constant) and blood lipids in man. *J Nutr* 70:257-266, 1960.
 53. Grande F, Anderson JT, Keys A: The influence of chain length of the saturated fatty acids on their effect on serum cholesterol concentration in man. *J Nutr* 74:420-428, 1961.
 54. Bronte-Stewart B, Antonis A, Eales L, Brock JF: Effects of feeding different fats on serum-cholesterol level. *Lancet* 1:521-526, 1956.
 55. Seelig MS, Haddy FJ: Magnesium and the arteries: 1. Effects of magnesium deficiency on arteries and on the retention of sodium, potassium, and calcium. In Cartin M, Seelig MS (eds): "Magnesium in Health and Disease." New York: Spectrum, pp 605-638, 1980.
 56. Seelig MS, Berger AR: Range of normal serum magnesium values. *N Engl J Med* 290:974-975, 1974.
 57. Seelig MS, Heggveit HA: Magnesium interrelationships in ischemic heart disease: a review. *Am J Clin Nutr* 27:59-79, 1974.
 58. Seelig MS: Magnesium deficiency with phosphate and vitamin D excesses: role in pediatric cardiovascular disease? *Cardiovasc Med* 3:637-650, 1978.
 59. Rayssiguier Y: Magnesium and lipid interrelationships in the pathogenesis of vascular diseases. *Magnesium Bull* 3:165-177, 1981.
 60. Morgan KJ, Stampely GL, Zabik ME, Fisher DR: Magnesium and calcium dietary intakes of the US population. *J Am Coll Nutr* 4:195-206, 1985.
 61. Kummerow FA, Smith T, Mahfouz M, Pikul J: Dietary fat and plasma lipid physical properties in swine. *J Am Coll Nutr* 10:346-354, 1991.
 62. National Research Council, National Academy of Science: "Nutrient Requirements of Swine," 8th ed. Washington, DC: National Academy of Sciences, 1979.
 63. Kummerow FA, Wasowicz E, Smith T, Yoss N, Thiel J: Plasma lipid physical properties in swine fed margarine or butter in relation to the dietary magnesium intake. *J Am Coll Nutr* (in press).
 64. Flynn M, Nolph G, Sun G, Navidi M, Krause G: Effects of cholesterol and fat modification of self-selected diets on serum lipids and their specific fatty acids on normocholesterolemic and hypercholesterolemic humans. *J Am Coll Nutr* 2:93-106, 1991.
 65. Anderson TW, Le Riche WH, Mackay IS: Sudden death and ischemic heart disease. *N Engl J Med* 280:805-807, 1969.
 66. Altura BT, Altura BM: The role of magnesium in etiology of stroke and cerebrovasospasm. *Magnesium* 1:277-291, 1982.
 67. Seelig MS: Cardiovascular consequences of magnesium deficiency and loss. Pathogenesis, prevalence, and manifestation. Magnesium and chloride loss in refractory potassium repletions. *Am J Cardiol* 63:4G-21G, 1989.
 68. Haddy FJ, Seelig MS: Magnesium and the arteries: II. Physiologic effects of electrolyte abnormalities on arterial resistance. In Cantin M, Seelig MS (eds): "Magnesium in Health and Disease." New York: Spectrum, pp 639-657, 1980.
 69. Seelig MS: Increased need for magnesium with the use of combined estrogen and calcium for osteoporosis treatment. *Magnesium Res* 3:197-215, 1990.
 70. Schroeder HA: Relation between hardness of water and death rates from certain chronic and degenerative diseases in the United States. *J Chron Dis* 12:586-591, 1960.
 71. Durlach J, Bara M, Guet-Bara A: Magnesium level in drinking water and its cardiovascular risk factor: a hypothesis. *Magnesium* 4:5-15, 1985.
 72. Karppanen H: Epidemiological studies on the relationship between magnesium intake and cardiovascular diseases. *Artery* 9:190-199, 1981.
 73. Marier JR: Magnesium content of the food supply in the modern day world. *Magnesium* 5:1-8, 1986.
 74. Lipid Research Clinics Program: The Lipid Research Clinics coronary primary prevention trial results: 1. Reduction in incidence of coronary heart disease. *JAMA* 251:351-364,

Viewpoint on NCEP Report

- 1984.
75. O'Brien PC, Fleming TR: A multiple testing procedure for clinical trials. *Biometrics* 35:549-556, 1979.
 76. Chen H, Zhuang H, Han Q: Serum high density lipoprotein cholesterol and factors influencing its level in healthy Chinese. *Atherosclerosis* 48:71-79, 1983.
 77. Chen H, Han-zhong Z, Han Q: The relationship between serum lipid levels and nutrient intake in healthy inhabitants of urban and rural Shanghai. *Chin Med J* 102:60-66, 1989.
 78. Li JZ, Li PY, Wang S, Jiang L, Zhao SH, Guo HB, Gao H, Zhang ZM, Fang XZ: Serum-lipid and lipoprotein patterns of aging populations from birth to senescence. *Chin Med J* 101:659-664, 1988.
 79. Yao H, Liangshou L, Lansun L: The risk factors of coronary arteriographically defined coronary heart disease—a case control study. *J Fourth Military Med Univ* 9:380-383, 1988.
 80. Taura S, Taura M, Tokuyasu K, Kamio A, Kummerow FA, Cleveland JC: Human arterio- and atherosclerosis: identical to that in 6 and 36 month old swine fed a corn diet free of cholesterol and saturated fat. *Artery* 4:100-106, 1978.
 81. Van Blitterswijk WJ, Van Hoeven RP, Van Der Meer BW: Lipid structural order parameters (reciprocal of fluidity) in biomembranes derived from steady state fluorescence polarization measurements. *Biochim Biophys Acta* 644:323-332, 1981.
 82. Vincent M, Gallay J: Time-resolved fluorescence anisotropy study of effect of *cis* double bond in structure of lecithin and cholesterol-lecithin bilayer using n-(9-anthroxyloxy) fatty acids as probes. *Biochemistry* 23:6514-6522, 1984.
 83. Lehninger AL (ed): "Biochemistry: The Molecular Basis of Cell Structure and Function," 2nd ed. New York: Worth Publishing, pp 227-246, 1975.
 84. Enig M, Atal S, Keeney M, Sampugna J: Isomeric *trans* fatty acids in the US diet. *J AM Coll Nutr* 5:471-486, 1990.
 85. Applewhite TH: Statistical "correlations" relating *trans*-fat to cancer: a commentary. *Proc Fed Am Soc Exp Biol* 38:2435-2436, 1979.
 86. Consumer and Food Economics Institute: "Composition of Foods, Fats and Oils. Agriculture Handbook No. 8-4." Washington, DC: SEA, USDA, 1979.
 87. Bailey AE: "Industrial Oil and Fat Products." New York: Interscience Publishers, 1951.
 88. American Heart Association: "Diet and Heart Disease." Dallas: AHA, 1968.
 89. Gerst EC: "Proceedings of the Symposium on the Decline in Coronary Heart Disease Mortality. The Role of Cholesterol Change." New York: Center for Continuing Education in the Health Sciences, College of Physicians and Surgeons, Columbia University, 1983.
 90. Stallones R: The rise and fall of ischemic heart disease. *Sci Am* 5:53-59, 1980.
 91. Kinsella JE, Bruckner G, Mai J, Shimp J: Metabolism of *trans* fatty acids with emphasis on the effects of *trans*, *trans*-octadecadienoate on lipid composition, essential fatty acid and prostaglandins: an overview. *Am J Clin Nutr* 34:2307-2318, 1981.
 92. Mahfouz MM, Smith TL, Kummerow FA: Effect of dietary fats on desaturase activities and the biosynthesis of fatty acids in rat-liver microsomes. *Lipids* 19:214-222, 1984.
 93. Hill JG, Kuksis A, Beveridge JMR: The constancy of red blood cell lipids in man during extreme variations of dietary lipid intake. *J Am Oil Chem Soc* 41:393-397, 1964.
 94. Cunnane SC, Hoy S-Y, Duff P-D, Ells KR, Horrobin DF: Essential fatty acid and lipid profiles in plasma and erythrocytes in patients with multiple sclerosis. *Am J Clin Nutr* 50:801-806, 1989.
 95. Holman RT, Pusch F, Svingen B, Dutton HJ: Unusual isomeric polyunsaturated fatty acids in liver phospholipids of rats fed hydrogenated oil. *Proc Natl Acad Sci USA* 88:4830-4834, 1991.
 96. Mensink RP, Katan MB: Effect of dietary *trans* fatty acids on high-density and low density lipoprotein cholesterol levels in healthy subjects. *N Engl J Med* 323:439-445, 1990.
 97. Holman RT, Johnson SB, Ogburn PL: Deficiency of essential fatty acids and membrane fluidity during pregnancy and lactation. *Proc Natl Acad Sci USA* 88:4835-4839, 1991.
 98. Kokatnur MG, Kummerow FA: Amino acid imbalance and cholesterol levels in chicks. *J Nutr* 75:319-329, 1961.
 99. Johnson D, Leveille GA, Fisher H: Influence of amino acid deficiencies and protein level on the plasma cholesterol of the chick. *J Nutr* 66:367-376, 1958.
 100. Carroll KK: Review of clinical studies on cholesterol lowering response to soy protein. *J Am Diet Assoc* 91:820-827, 1991.
 101. Slater G, Mead J, Dhopeswarkar G, Alfin-Slater RB: Plasma cholesterol and triglycerides in men with added eggs in the diet. *Nutr Rep Int* 14:249-260, 1976.
 102. Porter MW, Yamanaka W, Carlson SD, Flynn MA: Effect of dietary egg on serum cholesterol and triglyceride of human males. *Am J Clin Nutr* 30:490-495, 1977.
 103. Kummerow FA, Kim Y, Hull MD, Pollard J, Dorossier DL, Valek J: The influence of egg consumption on the serum cholesterol level in human subjects. *Am J Clin Nutr* 30:664-673, 1977.
 104. Flynn MA, Nolph GB, Flynn TC, Kahrs R, Krause G: Effect of dietary egg on human cholesterol and triglycerides. *Am J Clin Nutr* 32:1051-1057, 1979.
 105. Flynn MA, Nolph GB, Sun GY, Lanning B, Krause G, Dally J: Serum lipids and eggs. *J Am Diet Assoc* 86:154-158, 1986.
 106. Poseti LP, Orr ML: "Composition of Foods." Washington, DC: Agriculture Handbook No. 8-1, Agricultural Research Service, US Dept of Agr, November, 1976.
 107. Woteki CE, Thomas R: "Eat for Life. The Food and Nutrition Board's Guide to Reducing Your Risk of Chronic Disease." Washington, DC: Committee on Diet and Health Food and Nutrition Board, Institute of Medicine, National Academy of Sciences Press, p 10, 1992.
 108. Simpson JR, Farris DE: "The World's Beef Business." Ames: The Iowa State University Press, 1982.
 109. Singh RB, Mori H, Kummerow FA: Macro and trace minerals metabolism in coronary disease. Trace elements in

- Medicine 9:132-144, 1992.
110. Singh RB, Mori H, Kokatnur M, Kummerow FA: "Nutrition in Coronary Heart Disease and Sudden Cardiac Death." Moradabad, India: International College of Nutrition, 1991.
111. Brand IA, Heinickel A: Key enzymes of carbohydrate metabolism as targets of the 11.5-kDa Zn²⁺-binding protein (parathymosin). *J Biol Chem* 266:20984-20989, 1991.
112. Green SM, Ginsburn A, Lewis MS, Hensley P: Roles of metal ions in the maintenance of the tertiary and quaternary structure of arginase from *Saccharomyces cerevisiae*. *J Biol Chem* 266:21474-21481, 1991.
113. Pan L, He Q, Chan SI: The nature of zinc in cytochrome c oxidase. *J Biol Chem* 266:19109-19112, 1991.

Fred A. Kummerow, PhD, FACN
205 Burnside Research Laboratory
University of Illinois
1208 W. Pennsylvania Ave.
Urbana, IL 61801

Received February 1992; revision accepted June 1992.

Plasma Lipid Physical Properties in Swine Fed Margarine or Butter in Relation to Dietary Magnesium Intake

Fred A. Kummerow, PhD, FACN, Erwin Wasowicz, PhD, Terrance Smith, PhD, FACN, Norma L. Yoss, BS, and Julie Thiel, BS

Burnsides Research Laboratory, University of Illinois, Urbana (F.A.K., E.W., T.S., J.T.), and Harlan E. Moore Heart Research Foundation, Champaign, Illinois (F.A.K., N.L.Y.)

Key words: magnesium, cholesterol, low-density lipoproteins, high-density lipoproteins

Plasma lipids obtained from swine which had been fed butter or margarine at two dietary magnesium (Mg) levels indicated that the level of dietary Mg was more significant to plasma total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) levels than was the presence of butter or margarine. At 270 mg Mg/kg, which is considered adequate for swine, there was a significant difference in the plasma TC between swine fed margarine and those fed butterfat (105 and 126 mg %, respectively). Plasma LDL-C was higher in swine fed butter than in those fed margarine (88 and 71 mg %, respectively). In swine fed an additional 247 mg Mg/kg, however, there was no significant difference in plasma TC between those fed margarine or butter. Although at 247 mg Mg/kg plasma LDL-C was higher in swine fed margarine and HDL-C was higher in those fed butter, there were no significant differences in the order parameters of LDL and HDL. Studies in which the influences of dietary fats on plasma cholesterol were first noted were carried out on liquid diets deficient in Mg. Mg, a cofactor in the enzymes involved in desaturation of saturated fatty acids, is also necessary in desaturation of linoleic to arachidonic acid.

Abbreviations: ANOVA = analysis of variance, apo B = apoprotein B, 20:4 ω 6 = arachidonic acid, Ca = calcium, DPH = diphenylhexatriene, EFA = essential fatty acid, FA = fatty acid, HDL = high-density lipoprotein, LDL = low-density lipoprotein, 18:2 ω 6 = linoleic acid, Mg = magnesium, PL = phospholipid, PUFA = polyunsaturated fatty acid, NCEP = National Cholesterol Education Program, NRC = National Research Council, S_{DPH} = order parameter (using DPH), SFA = saturated fatty acids, TC = total cholesterol, TG = triglyceride

INTRODUCTION

Du Pont et al [1], in a recent review, pointed to 30 years of research that led the National Cholesterol Education Program (NCEP) to establish guideline criteria for medical intervention and to shift the distribution of "cholesterol levels in the entire population to a low range" [2]. These guidelines are based on the assumption that hydrogenated vegetable oil sources are biologically more adaptable to such a shift than are animal fat sources such as butter. For example, the NCEP stated that "margarine represents partially hydrogenated vegetable oil and is preferable to butter." This statement was probably based on human studies of Ahrens [3,4] on liquid diets which were

deficient (i.e., 96 mg/day) in Mg [5]. The RDA for magnesium (Mg) (350 mg/day for the adult male and 280 mg/day for the adult female) represents a compromise between needs estimated by balance studies and the usual dietary Mg intake by a population in which Mg deficiency rarely appears except in pathologic conditions [6]. Furthermore, Emken [7] tabulated results from 14 studies on vegetable oils and concluded that it was difficult to compare results "because of experimental differences such as the dietary fatty acid composition, type of vegetable oil fed, *trans* fatty acid content of oils, life-style of subjects or their age, and dietary cholesterol and fat levels." Following the observations of Rayssiguier et al [8-20] that Mg actively participates in lipid metabolism, we studied the influence of

Address reprint requests to F.A. Kummerow, PhD, 205 Burnsides Research Laboratory, University of Illinois, 1208 W. Pennsylvania Avenue, Urbana, IL 61801.

Journal of the American College of Nutrition, Vol. 12, No. 2, 125-132 (1993)
Published by the American College of Nutrition

dietary Mg content on plasma lipids in swine fed butter or margarine.

MATERIALS AND METHODS

Housing and Diets

As in a previous study [21], 48 Yorkshire male piglets (2 months old, 12–18 kg) were randomly divided into six groups. Three groups were fed a basal diet of 87.25% ground yellow corn, 10% defatted soybean meal and a 2.75% multiple vitamin and mineral premix which contained 270 mg of Mg/kg of diet [22]. The other three groups were fed the same basal diet except that MgO/MgCaO₃ was added to bring the Mg level up to 517 mg of Mg/kg diet. One group on each of the two dietary Mg levels was maintained as a control, while the other two groups had either 10% butterfat or 10% margarine added to the diet. Fatty acid (FA) composition of the diets is presented in Table 1. The animals were housed in a facility with concrete slatted floors in separate pens equipped with self-feeders and water. The water contained about 2.6 mg of Mg/100 ml. All groups received this experimental dietary treatment continuously from 2 through 6 months of age.

Table 1. Fatty Acid Composition of Fats in the Diet

Fatty acid	Basal diet (wt %)	Butter (wt %)	Margarine (wt %)
4:0	—	4.2	—
6:0	—	2.8	—
8:0	—	1.4	—
10:0	—	3.6	—
12:0	—	3.9	—
14:0	—	11.2	0.1
16:0	12.5	29.5	11.7
18:0	1.9	11.3	7.7
20:0	0.3	0.1	0.3
22:0	—	—	0.3
24:0	0.1	—	0.1
16:1	0.1	1.4	0.1
18:1-cis	25.5	20.9	32.3
18:1-trans*	—	2.4	23.8
18:2n-6	58.5	1.5	20.1
18:3n-3	0.9	0.5	1.8
Others	0.2	5.3	1.7

1) Butter was obtained from Commodity Credit Corp, USDA in 68-lb cubes.

2) Margarine was purchased through Central Food Stores, University of Illinois, in 1-lb packages.

* Total *trans* was determined by infrared spectroscopy and expressed in terms of elaidic acid as noted by "Official Methods and Recommended Practices of the American Oil Chemists' Society," 3rd ed. Champaign, IL: American Oil Chemists' Society, 1984.

Blood Collection and Assays

At the end of the 4-month feeding period the animals were fasted overnight and sacrificed at a commercial facility. Blood was collected from the vena cava into heparinized tubes and kept on ice for 1 hour during transport to the laboratory. The blood was then centrifuged within 30 minutes of arrival. Centrifugation to isolate the plasma was at 2000 g for 30 minutes. Plasma triglycerides (TG), total cholesterol (TC) and protein were determined using commercially available diagnostic kits (Sigma Chemical, St. Louis, MO). Plasma calcium (Ca) and Mg levels were determined by atomic absorption (Roger Adams Laboratory, University of Illinois, Urbana, IL).

FA Analysis of the Plasma Lipids

Plasma total lipids were extracted using the method of Folch et al [23]. FA methyl esters were prepared with 14% boron trifluoride-methanol (Alltech Associates, Inc, Deerfield, IL) as outlined by Morrison and Smith [24]. FA methyl esters were analyzed on a Hewlett-Packard gas chromatograph (model 5790A) equipped with a flame ionization detector, and Supelcowax-10, 30 m × 0.25 mm ID fused silica column (Supelco, Inc., Bellefonte, PA). The oven temperature was programmed from 170 to 220°C at 2°C/minute. The injector and detector temperatures were 250 and 290°C, respectively. FA methyl esters were identified by comparing their retention times with those of commercial standards (Nu-Check Prep, Inc, Elysian, MN).

Fractionation of Lipoproteins

Plasma was fractionated into low-density lipoprotein (LDL) and high-density lipoprotein (HDL) by sequential density centrifugation [25]. For HDL, 2 ml plasma was placed in a Beckman, polyallomer 5/8" × 3" (No 326814) and filled with a 1.006 g/ml sodium chloride and potassium bromide solution. It was then centrifuged at 45,000 rpm in a Ti50 rotor (Beckman, Arlington Heights, IL) for 23 hours at 4°C. All but about 6 ml of the solution in the tube was collected (VLDL), and density was checked using a refractometer. The remainder was diluted to a density of 1.063 g/ml and centrifuged 36 hours at 45,000 rpm at 4°C. The top portion (LDL) of the contents of the tube was removed to check density, and the remainder was adjusted to a density of 1.210 g/ml. This portion was centrifuged 66 hours at 45,000 rpm at 4°C and the HDL recovered. Both LDL and HDL were then exhaustively dialyzed against phosphate-buffered saline at 4°C.

Determination of Order Parameters

Concentrated fractions of this dialyzed LDL and HDL were diluted with phosphate-buffered saline to a scattering at 365 nm of 0.200 absorbance units or less. We have

found that this dilution eliminates the need to correct fluorescent polarization readings for light scattering.

A stock solution of diphenylhexatriene (DPH) was prepared by dissolving 1 mg probe in 1 ml dimethyl sulfoxide. For fluorescent analysis the stock probe was added to diluted lipoprotein at a rate of 1 μ l/ml of sample and incubated at 37°C in a circulating water bath. Polarization measurements were made at an excitation wavelength of 365 nm and so emission wavelength of 460 nm. Both slits were set at 5 nm. Order parameter (S_{DPH}) was calculated according to the equation of Van Blitterswijk et al [26]:

$$r = (I_{||} - I_{\perp}) / (I_{||} + 2I_{\perp})$$

$$r_{\infty} = (4/3)r - 0.10$$

$$S_{DPH} = r_{\infty} / 0.365$$

Statistical analysis was performed with IBM SPSS-PC (SPSS Inc, Chicago, IL) analysis of variance (ANOVA).

RESULTS

The level of dietary Mg influenced plasma TC and LDL levels more than did vegetable or animal fat content (Table 2). With 270 mg of Mg/kg of diet (considered adequate for swine [27]), there was a notable difference in plasma TC between those fed margarine and those fed butterfat (105 and 126 mg %, respectively). Plasma LDL cholesterol (LDL-C) was higher in those fed butter as compared with margarine (88 and 71 mg %, respectively). However, the three groups fed an additional 247 mg Mg/kg of diet showed no difference in plasma TC, whether fed margarine or butter, i.e., 115 and 112 mg % respectively. LDL-C or HDL-C levels did not differ. Plasma LDL-C increased in animals fed margarine, while plasma HDL-C was increased

in animals fed butter. Total TG, HDL-TG, S_{DPH} , cholesterol-to-protein or HDL-to-protein ratios, Mg or Ca plasma levels did not differ.

The variables were analyzed for their effect on TC, LDL-C and HDL-C by two-way ANOVA. The ANOVA test determined if there was a significant difference between the mean values of the results. To determine which variable accounted for the greatest difference in the groups, the estimated ω^2 value was calculated for this statistical two-way ANOVA [28]. The index ω^2 , the proportion of variance in Y accounted for by X, has been extended to the present situation where there are two or more independent variables. ω^2 represents the strength of association between independent and dependent variables. That is, when ω^2 is zero, X does not aid in predicting the value of Y. On the other hand, when ω^2 is 1.00, the value of X lets us know Y exactly [29]. TC showed a significant response to the manipulation of dietary Mg content ($p = 0.020$) (Table 3). Dietary fat was not significant; however, the interaction of diet and Mg level was extremely significant ($p = 0.008$). Upon estimated ω^2 analysis, the interaction was found to be responsible for most variance ($\omega^2 = 0.31$) as compared to the Mg level ($\omega^2 = 0.15$) and fat content of the diet ($\omega^2 = 0.03$). From these data it can be concluded that TC content of the plasma was affected more significantly by Mg than by fat content of diet, although the greatest effect was due to interaction between fat and Mg content.

Plasma LDL-C was also significantly influenced by the Mg content of the diets ($p = 0.044$) and the interaction between fat and Mg ($p = 0.002$) (Table 3). The interaction of variables was largely responsible for the LDL-C level ($\omega^2 = 0.44$) as compared to either of the other variables (Mg $\omega^2 = 0.10$, diet $\omega^2 = 0.0$). LDL-C was, therefore dependent largely on the presence and amount of both fat and Mg. HDL-C analysis yielded a marginally significant

Table 2. Composition of the Cholesterol, Triglyceride, Order Parameters and Calcium, Magnesium Levels in Plasma From Swine Fed Different Diets

	Butter		Margarine		Basal	
	270 mg Mg/kg	517 mg Mg/kg	270 mg Mg/kg	517 mg Mg/kg	270 mg Mg/kg	517 mg Mg/kg
Ttl Chol*	126 ± 17†	112 ± 17	105 ± 2	115 ± 8	114 ± 8	100 ± 11
LDL Chol*	88 ± 13	76 ± 8	71 ± 8	88 ± 5	73 ± 8	64 ± 8
HDL Chol*	32 ± 4	29 ± 9	26 ± 4	23 ± 6	32 ± 3	29 ± 4
Total TG*	33 ± 4	33 ± 11	41 ± 21	24 ± 5	26 ± 10	32 ± 11
HDL TG*	31 ± 4	28 ± 5	26 ± 12	23 ± 9	27 ± 10	34 ± 13
LDL (S_{DPH})	0.45 ± 0.08	0.52 ± 0.05	0.51 ± 0.05	0.36 ± 0.2	0.50 ± 0.06	0.46 ± 0.05
HDL (S_{DPH})	0.42 ± 0.04	0.46 ± 0.05	0.43 ± 0.05	0.40 ± 0.09	0.41 ± 0.03	0.43 ± 0.07
CHDL/Pro.	0.60 ± 0.19	0.75 ± 0.47	0.46 ± 0.11	0.82 ± 0.03	0.33 ± 0.08	1.49 ± 0.50
Chol/Pro.	1.62 ± 0.20	2.21 ± 0.40	1.72 ± 0.18	2.43 ± 0.44	1.66 ± 0.35	3.9 ± 1.17
Ca (μ g/ml)	116 ± 9	116 ± 15	103 ± 10	122 ± 9	126 ± 8	126 ± 2
Mg (μ g/ml)	20 ± 2	21 ± 1	18 ± 2	20 ± 3	20 ± 1	22 ± 2

† Standard deviation.

* Units of mg/dl.

Table 3. Results of Two-Way Analysis of Variance¹

Source of variation	Total cholesterol			LDL cholesterol			HDL cholesterol		
	Mean square	F	ω^2	Mean square	F	ω^2	Mean square	F	ω^2
Within cells	190			159			28		
Mg ²	1301	6.84*	0.15	781	4.90*	0.10	43	1.54	0.02
Diet ³	311	1.64	0.31	85	0.53	NS	102	3.62*	0.22
Mg by diet	1323	6.96**	0.03	1589	9.97*	0.44	0.27	0.01	NS

¹ IBM software program, SPSS-PC.

² Two variables: adequate or inadequate magnesium in diet.

³ Three variables: basal, butter or margarine in diet.

* $p \leq 0.05$ ("p" denotes the significance of the F value).

** $p \leq 0.01$.

NS: Not significant.

"F" is determined by dividing the mean square of the source by the mean square of the error [27].

ANOVA result for HDL level due to fat manipulation of the diet ($p = 0.054$) (Table 3). Analysis of the amount of total variance of HDL-C level fat is responsible for an estimated $\omega^2 = 0.22$. The fat in the diet is a stronger force in determining HDL-C than is the Mg content or interaction of Mg and fat variables.

An analysis of the FA composition of the total lipids in the plasma of those fed 270 mg Mg/kg indicated that more linoleic acid (18:2 ω 6) was present in the plasma in those fed margarine than in those fed butter. However, when an adequate amount of Mg was present in the diet there did not seem to be any advantage in increasing the amount of 18:2 ω 6 in the total diet. The ground whole corn in the basal diet provided enough 18:2 ω 6 for the plasma lipids to contain as much 18:2 ω 6 and arachidonic acid (20:4 ω 6) as in the plasma of the swine fed margarine. When dietary Mg was increased to 517 mg of Mg/kg of diet, the level of 20:4 ω 6 in the plasma lipids was significantly higher in the animals fed margarine and lower in those fed butter. In contrast, in those fed 270 mg of Mg/kg of diet, the level of 20:4 ω 6 was higher in the plasma of those fed butter. Mg is, therefore, necessary to desaturation of 18:2 ω 6 to 20:4 ω 6 in fats which contain hydrogenated (*trans*) fat such as in margarine (Table 4).

DISCUSSION

The report of the NCEP [2] has not taken into consideration the dynamics involved in lipid metabolism at the molecular level. In cellular membranes, both changes in acyl unsaturated and cholesterol content can result in an altered organizational state of the lipid in the membrane, which can be detected by measurement of S_{DPH} [26]. In the plasma, lipoproteins exist as spherical micelle-like particles rather than as a rigid phospholipid (PL) and cholesterol/protein interlaced structures of the type found in cell

membranes. More saturated acyl chains in lipoproteins may increase the packing order (rigidity) while unsaturated acyl chains in the lipid portion of lipoproteins may increase the freedom of motion of the spherical micelle-like particles. In cholesterol-lecithin bilayers, cholesterol moderates both effects making unsaturated chains less random and saturated chains less rigid [30]. Since we noted no significant differences in S_{DPH} in the plasma LDL or HDL from animals fed margarine vs butter, the same may also be true for the relationship of cholesterol with saturated and unsaturated acyl chains in the lipid protein of the LDL and HDL lipoproteins.

According to the NCEP report [2], the risk of heart disease is reduced by 2% for every one mg % lowering of the plasma cholesterol level when a saturated fatty acid (SFA) is replaced by polyunsaturated fatty acid (PUFA). The present study suggests, however, that the plasma cholesterol level may be lowered by PUFA because less cholesterol is needed for the plasma to keep the microviscosity (the S_{DPH}) of the plasma LDL recognizable by the receptor sites on the endothelium. Although the explanation for enzyme action as a lock and key arrangement is no longer in vogue [31], it may be useful in explaining the lower plasma cholesterol level with increased PUFA intake. The liver must secrete LDL particles that are recognized by the receptor sites on cell membranes (the lock). The order parameter of the lipid portion of the LDL (the key) may be as important as the apoprotein portion [32] for the recognition of LDL to enable the key to work in the lock (the receptor site). As we have noted in a previous study [21], even though the plasma Mg level was higher in swine fed 517 mg of Mg/kg of diet, no significant difference in order parameter was noted when compared with the plasma Mg level of swine fed 270 mg Mg/kg. However, a difference in desaturase activity was noted in swine fed the margarine diet. The difference in desaturase activity may have been accentuated by the *trans* FA in margarine. We

Table 4. Fatty Acid Composition of the Total Plasma Lipid

Fatty acid	Basal		Butter		Margarine	
	270 mg Mg/kg	517 mg Mg/kg	270 mg Mg/kg	517 mg Mg/kg	270 mg Mg/kg	517 mg Mg/kg
16:0	14.24 ± 1.01†	14.40 ± 0.79	15.80 ± 0.47	15.93 ± 0.87	13.60 ± 1.14	14.37 ± 0.86
16:1 ω 9	0.55 ± 0.05	0.57 ± 0.07	0.59 ± 0.07	0.52 ± 0.03	0.73 ± 0.06	0.53 ± 0.10
16:1 ω 7	1.07 ± 0.23	0.94 ± 0.20	1.20 ± 0.21	1.01 ± 0.08	0.86 ± 0.18	0.98 ± 0.17
18:0	14.43 ± 0.59	16.15 ± 2.60	14.00 ± 0.56	14.44 ± 1.63	13.32 ± 1.52	15.49 ± 1.28
18:1 ω 9	23.46 ± 2.80	21.32 ± 0.64	22.27 ± 1.81	21.57 ± 0.68	20.58 ± 1.72	21.30 ± 2.25
18:1 ω 7	2.05 ± 0.34	1.96 ± 0.15	1.96 ± 0.14	1.80 ± 0.02	2.86 ± 0.32	2.50 ± 0.45
18:2 ω 6	22.75 ± 5.60	22.70 ± 2.60	22.26 ± 1.12	21.50 ± 1.36	26.17 ± 1.54*	21.83 ± 1.52*
18:3 ω 6	0.60 ± 0.05	0.42 ± 0.04	0.47 ± 0.04	0.48 ± 0.05	0.40 ± 0.04	0.55 ± 0.25
18:3 ω 3	0.38 ± 0.18	0.35 ± 0.03	0.40 ± 0.04	0.38 ± 0.03	0.66 ± 0.08	0.44 ± 0.07
20:0	0.31 ± 0.03	0.25 ± 0.08	0.28 ± 0.04	0.32 ± 0.08	0.26 ± 0.06	0.37 ± 0.10
20:2 ω 6	0.26 ± 0.03	0.38 ± 0.09	0.34 ± 0.05	0.26 ± 0.01	0.23 ± 0.01	0.22 ± 0.02
20:2 ω 3	0.66 ± 0.17	0.80 ± 0.29	0.70 ± 0.10	0.52 ± 0.09	0.46 ± 0.07	0.47 ± 0.12
20:3 ω 6	0.34 ± 0.10	0.45 ± 0.04	0.36 ± 0.06	0.44 ± 0.14	0.24 ± 0.05	0.29 ± 0.11
20:4 ω 6	11.76 ± 0.77	12.16 ± 0.54	11.15 ± 1.07	11.56 ± 1.46	10.16 ± 1.89*	12.70 ± 1.01*
20:5 ω 3	0.29 ± 0.18	0.22 ± 0.04	0.31 ± 0.02	0.38 ± 0.11	0.29 ± 0.06	0.38 ± 0.12
22:4 ω 6	0.95 ± 0.24	1.02 ± 0.11	0.76 ± 0.01	0.72 ± 0.11	0.62 ± 0.10	0.83 ± 0.18
22:5 ω 3	1.08 ± 0.09	1.10 ± 0.14	1.17 ± 0.21	1.16 ± 0.16	1.11 ± 0.32	1.43 ± 0.43
22:6 ω 6	0.79 ± 0.18	0.72 ± 0.05	0.96 ± 0.20	1.16 ± 0.29	1.14 ± 0.28	1.08 ± 0.18

† Standard deviation.

* Signifies pairs that are significant ($p < 0.05$) by a paired comparison t-test.

have noted a decrease in desaturase activity in rats fed hydrogenated fat [33]. An adequate level of dietary Mg seems more important to the plasma PUFA levels than the PUFA content of the dietary fat.

The confusing results that Emken and others [7] noted when hydrogenated fats replaced nonhydrogenated fats may have been due to the amount of Mg in the diets in which the plasma cholesterol-raising effect of SFA was first noted. Aherns et al in their human studies [3] used liquid diets composed of sugar, milk protein and the test fat. The basic formula consisted of 15% milk protein, 40% fat and 45% carbohydrate. Milk is a relatively poor source [34] of Mg (13 mg/100 ml). The test formula of 2500 cal/day contained 96 mg [4] or <25% of the National Research Council (NRC) requirement. Subjects in these studies [3] were hypercholesterolemic and were fed the test diet for 8–10-week periods. Twelve of the 14 studies listed in Emken's Table 5 [7] were carried out on liquid diets. Most of these diets contained no Mg, and some contained inadequate levels of Mg (Table 5). Our basal diet was adequate in Mg content. It satisfies the requirement for Mg in swine diets as defined by the NRC [27]. The level of fat in the diet may not have been taken into consideration when the recommendation for Mg was made. The addition of fat to a basal diet without an increase in Mg would dilute the Mg intake and therefore may influence lipid metabolism. Although the RDA for Mg is 350 mg/d for adult males and 280 mg/d for females [6], results from the present study suggest that the RDA for Mg should be higher, i.e., the more fat in the average diet, the higher the Mg RDA should be. In the present study, however, as no

change in plasma lipids was noted in the basal diet which did not contain added margarine or butter, the presence of dietary lipids may have accentuated the effect of Mg deficiency.

The NCEP statement that "margarine represents partially hydrogenated vegetable oil and is preferable to butter [2]" is probably based on the experimental studies of Ahrens using Mg deficient liquid diets, while studies of Keys et al [35–38] and others [39,40] with men on institutional diets of unknown Mg content concluded that the increase in cholesterol levels was due to SFA. It is possible that these institutional diets may also have been deficient in Mg. Seelig [34,41–43] has called attention to the low Mg content of the American diet, and a number of studies have suggested that Mg intake is inadequate in the US population [44–46]. In all of the experimental studies in which liquid diets deficient in Mg were used, it is possible that the activity of the ω^9 desaturating enzymes was reduced and not available for the conversion of the SFA in coconut oil or butter fat to oleic and palmitoleic acid. Corn oil is already desaturated and has little influence on plasma cholesterol [3].

Tongai et al [20] have found that weanling rats fed a Mg-deficient diet for only 8 days began to show signs of Mg deficiency with changes in lipid metabolism. We found in rats fed an inadequate level of Mg [47] a significant elevation in plasma TC, LDL-C, and TG and a lower HDL-C level. We also found that PL composition and FA of the cell membrane were influenced by the concentration of Mg in culture media. Cells grown on 6.3 or 2.6 μ M Mg showed a decrease in phosphatidylethanolamine, phospho-

Table 5. Effects of *Trans* Acids or Hydrogenated Oils on Serum Lipid Levels Compared to Unhydrogenated Oil*

Hydrogenated oil or isomeric fatty acid	Change in serum level			
	Triglyceride	Phospholipid	Cholesterol	Ref.
Peanut	ND ^a	ND	Increase	[87]**
Cottonseed	Increase	Increase	Increase	[88]**
Corn	Increase	Increase	Increase	[88]**
Corn	ND	ND	Increase	[89]**
Corn	Increase	Increase	Increase	[90]
Sunflower	Increase	No change	Increase	[92, 93]**
Margarines (2 brands)	ND	ND	Increase	[94]**
Soybean	ND	ND	No change	[95]**
Soybean	No change	No change	No change	[96]**
Soybean	No change	No change	No change	[97]
t,t- and c,t-18:2	Increase	ND	Increase	[99]**
44% <i>trans</i> acids	No change	ND	No change	[100]**
34% Elaidic acid	ND	ND	Increase	[101]**
37% Elaidic acid	ND	ND	Increase	[102]**

* Reprinted with permission from: Emken EA: Utilization and effects of isomeric fatty acids in humans. In Emken EA, Dutton HJ (eds): "Geometrical and Positional Fatty Acid Isomers." Champaign, IL: The American Oil Chemist's Society, p 121, 1979.

** Indicates a liquid diet [see 87-102].

^a ND = not determined.

tidylserine, sphingomyelin, phosphatidylinositol, and an increase in phosphatidylcholine compared to those grown on 480 μ M Mg [48]. FA composition of the cellular PL showed a significant decrease in 20:4 ω 6 and docosahexaenoic (22:4 ω 6) acids and a significant increase in 18:2 ω 6, linolenic (18:3 ω 6) and eicosatrienoic (2:3 ω 6) FA in Mg-deficient cells compared to Mg-sufficient cells [49]. Other essential minerals may also be involved in lipid metabolism and plasma LDL and HDL levels [5,50,51]

Fluorescence polarization has been used to compare fluidity of membrane preparation from Mg-deficient and control rats [20]. Rayssiguier et al [8] recently found that erythrocyte membranes, lymphocytes and hepatocyte plasma membranes from Mg-deficient animals were more fluid than were those of control rats, and Mg deficiency induced a significant decrease in anisotropy of both intact hepatic mitochondria and inner mitochondrial membranes. Several functional alterations in the membrane occurred in parallel to the physical changes measured by the increase in fluidity. The loss of Mg from membranes may contribute to the increased fluidity owing to the direct binding of the cation to PL head groups, but metabolic alterations of the lipid composition are also involved in the modification of membrane fluidity that occurs during Mg deficiency. Recent experiments by Gueux et al [9] indicate that apolipoprotein B (apo B) messenger ribonucleic acid (mRNA) in the liver of Mg-deficient rats is substantially greater than in control rats. These results showing a stimulatory effect of Mg deficiency on apo B gene expression [5] are of interest since Gueux et al [9] believe secretion of very-low-density lipoprotein is totally dependent upon apo B. Since both dietary Mg and *trans*

FA levels play a role in the desaturation of SFA and of 18:2 ω 6 to 20:4 ω 6 in vivo, recommendations for solid fat consumption should take these factors into consideration.

ACKNOWLEDGMENTS

The authors acknowledge Steve Dodge for the care of the swine, Tom McCarthy for Mg and Ca analysis at Rogers Adam Laboratory, Dottie Slavik, Kate Sours, and Jennifer Nevius for typing, Virginia K. Lukas for technical assistance, Collin van Uchelen for statistical consulting, the C.C.C. USDA for butter, the USDA International Res. Dir. Office of International Cooperation and Development, Washington, DC, Agr. #58-319R and the Wallace Genetic Foundation for support of this study.

REFERENCES

1. Dupont J, White PJ, Feldman EB: Saturated and hydrogenated fats in food in relation to health. *J Am Coll Nutr* 10:577-592, 1991.
2. NCEP: Report of the National Cholesterol Education Program expert panel on detection, evaluation and treatment of high blood cholesterol in adults. *Arch Intern Med* 148:36-69, 1988.
3. Ahrens E, Hirsch J, Insull W, Tsaltas T, Blomstrand R, Peterson M: The influence of dietary fats on serum-lipid levels in man. *Lancet* 2:944-953, 1957.
4. Ahrens, E: The use of liquid formula diets in metabolic studies: 15 years experience. *Adv Metab Dis* 4:297-315, 1970.

5. Kummerow FA: Viewpoint on the Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in adults. *J Am Coll Nutr* 11:---1992.
6. The National Research Council: "Recommended Dietary Allowance," 10th ed. Washington, DC: National Academy Press, 1989.
7. Emken EA: Utilization and effect of isomeric fatty acids in humans. In Emken EA, Dutton HJ (eds): "Geometric and Positional Fatty Acid Isomers." Champaign, IL: The American Oil Chemists' Society, pp 99-123, 1979.
8. Rayssiguier Y, Gueux E, Motta C: Magnesium deficiency effect on fluidity and function of plasma and subcellular membranes. In Lassene B, Durlach J (eds): "Magnesium: A Relevant Ion." London: John Libbey, pp 311-319, 1991.
9. Gueux E, Mazur A, Cardot P, Rayssiguier Y: Magnesium deficiency affects plasma lipoprotein composition in rats. *J Nutr* 121:1222-1227, 1991.
10. Rayssiguier Y, Gueux E, Weiser D: Effect of magnesium deficiency on lipid metabolism in rats fed a high carbohydrate diet. *J Nutr* 111:1876-1883, 1981.
11. Rayssiguier Y: New data on magnesium and lipids interrelationships in pathogenesis of vascular diseases in magnesium deficiency. In Halpern MJ, Durlach J (eds): "Physiopathology and Treatment Implications." New York: Karger, pp 122-131, 1985.
12. Rayssiguier Y, Gueux E, Cordot P, Thomas G, Robert A, Trugnan G: Variations of fatty acid composition in plasma lipids and platelet aggregation in magnesium deficient rats. *Nutr Res* 6:233-240, 1986.
13. Rayssiguier Y: Magnesium and lipid interrelationships in the pathogenesis of vascular diseases. *Magnesium Bull* 3:165-177, 1981.
14. Rayssiguier Y: Role on magnesium and potassium on cardiac and vascular smooth muscle. *Magnesium* 3:165-238, 1984.
15. Rayssiguier Y: Magnesium, lipids and vascular diseases. Experimental evidence in animal models. *Magnesium* 5:182-190, 1986.
16. Rayssiguier Y, Gueux E: Magnesium and lipids in cardiovascular diseases. *J Am Coll Nutr* 5:507-519, 1986.
17. Rayssiguier Y, Gueux E: The reduction of plasma triglyceride clearance by magnesium-deficient rats. *Magnesium* 2:132-138, 1983.
18. Rayssiguier Y, Noe L, Etienne J, Gueux E, Cardot P, Mazur A: Effect of magnesium deficiency on post-heparin lipase activity and tissue lipoprotein lipases on the rat. *Lipids* 26:182-186, 1991.
19. Gueux E, Rayssiguier Y: The hypercholesterolemic effect on magnesium deficiency following cholesterol feeding in the rat. *Magnesium Bull* 3:126-129, 1981.
20. Tongai A, Rayssiguier Y, Motta A, Gueux E, Maurois P, Heaton F: The hypercholesterolemic effect on magnesium deficiency in weanling rats. *Am J Physiol* 257:270-276, 1989.
21. Kummerow FA, Smith TL, Mahfouz MM, Pikul J: Dietary fat and plasma lipid physical properties in swine. *J Am Coll Nutr* 10:346-354, 1991.
22. Ito M, Cho BHS, Kummerow FA: Effects of a dietary magnesium deficiency and excess vitamin D₃ on swine coronary arteries. *J Am Coll Nutr* 9:155-163, 1990.
23. Folch J, Lees M, Sloane SGH: A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* 226:497-509, 1957.
24. Morrison WR, Smith LM: Preparation of fatty acid methyl esters and dimethylacetals from lipids with boronfluoride-methanol. *J Lipid Res* 5:600-608, 1961.
25. Ferreri LF: Fractionation of plasma lipoproteins: evaluation of preparative methods. *Lipid Res Methods* 10:133-156, 1984.
26. Van Blitterswijk WJ, Van Hoeven RP, Van Der Meer BW: Lipid structural order parameters (reciprocal of fluidity) in biomembranes derived from steady-state fluorescence polarization measurements. *Biochim Biophys Acta* 644:323-332, 1981.
27. National Research Council: "National Academy of Sciences Nutrient Requirement of Swine," 8th ed. Washington DC: National Academy of Sciences, 1979.
28. Moore DS, McCabe GP: "Introduction to the Practice of Statistics." New York: WH Freeman and Co, pp 756-771, 1989.
29. Hays WL: "Statistics." New York: CBS College Publishing, pp 290-291, 365-366, 1981.
30. Vincent M, Galley J: Time resolved fluorescence anisotropy study of effect of cis double bond in structure of lecithin and cholesterol lecithin bilayers using n/9-anthroxyloxy fatty acid as probes. *Biochemistry* 22:6514-6522, 1989.
31. Kornberg A, Horecker BL, Cornudella L, Oro J: Prebiological chemistry and the origin of life. In Kornberg A, Horecker BL, Cornudella L, Oro J (eds): "Reflections in Biochemistry." New York: Pergamon Press, pp 423-444, 1976.
32. Goldstein JL, Basu SK, Brown MS: Receptor mediated endocytosis of low density lipoproteins in cultured cells. In Fleischer S, Fleischer B (eds): "Methods in Enzymology." New York: Academic, pp. 241-257, 1983.
33. Mahfouz MM, Smith TL, Kummerow FA: Effect of dietary fats on desaturase activities and the biosynthesis of fatty acids in rat-liver microsomes. *Lipids* 19:214-222, 1984.
34. Seelig MS: "Magnesium Deficiency in the Pathogenesis of Disease." New York: Plenum Medical Book Co, p 1, 1980.
35. Keys A: Commentary on "Dietary Fats and Human Serum Lipid Levels." In Page IH (ed): "Chemistry of Lipids as related to Atherosclerosis." Springfield, IL: CC Thomas, pp 248-252, 1958.
36. Keys A, Anderson JT, Grande F: "Essential" fatty acids, degree of unsaturation, and effect of corn (maize) oil on the serum-cholesterol level in man. *Lancet* 1:66-76, 1957a.
37. Keys A, Anderson JT, Grande F: Serum cholesterol in man: diet fat and intrinsic responsiveness. *Circulation* 19:201-210, 1959.
38. Keys A, Anderson JT, Grande F: Diet-type (fats constant) and blood lipids in man. *J Nutr* 70:257-267, 1960.
39. Grande F, Anderson JT, Keys A: The influence of chain length of the saturated fatty acids on their effect on serum cholesterol concentration in man. *J Nutr* 74:420-428, 1961.
40. Bronte-Stewart B, Antonis A, Eales L, Brock JF: Effects of feeding different fats on serum-cholesterol levels. *Lancet* 1:521-526, 1956.

Plasma Lipid Response to Butter/Margarine, Mg

41. Seelig MS, Haddy FJ: Magnesium and the arteries: 1. Effect of magnesium deficiency on arteries and on the retention of sodium, or potassium, and calcium. In Cartin M, Seelig MS (eds): "Magnesium in Health and Disease." New York: Spectrum, pp 603-638, 1980.
42. Seelig MS, Heggtveit HA: Magnesium interrelationships in ischemic heart disease: a review. *Am J Clin Nutr* 27:59-79, 1974.
43. Seelig MS: Magnesium deficiency with phosphate and vitamin D excesses: role in pediatric cardiovascular disease? *Cardiovasc Med* 3:637-650, 1978.
44. Seelig MS: Human requirements in human nutrition. *Magnesium Bull* 3(1a): 26-47, 1981.
45. Rayssiguier Y: magnesium and lipid interrelationships in the pathogenesis of vascular diseases. Magnesium and lipid interrelationships in the pathogenesis of vascular disease. *Magnesium Bull* 3:165-177, 1981.
46. Morgan KJ, Stampely GL, Zabik HE, Fisher DR: Magnesium and calcium dietary intakes of the US population. *J Am Coll Nutr* 4:195-206, 1985.
47. Mahfouz MM, Kummerow FA: Effect of magnesium deficiency on desaturase activity and fatty acid composition of rat liver microsomes. *Lipids* 24:727-732, 1989.
48. Mahfouz MM, Smith TL, Kummerow FA: Changes in phospholipid composition and calcium flux in LLC-PK cells cultured at low magnesium concentrations. *Biochim Biophys Acta* 1006:75-83, 1989.
49. Mahfouz MM, Smith TL, Kummerow FA: Changes of linoleic acid metabolism and cellular phospholipid fatty acid composition in LLC-PK cells at low magnesium concentrations. *Biochim Biophys Acta* 1006:70-74, 1989.
50. Kummerow FA: Hypothesis: Possible role of magnesium and calcium in the development of structure and function of the plasma membrane in mammalian cells and in human diseases. *J Am Coll Nutr* 11:410-425, 1992.
51. Singh RB, Mori H, Kummerow FA: Macro and trace mineral metabolism in coronary heart disease. *Trace Elements in Medicine* 9:240-255, 1992.

Received March 1992; revision accepted September 1992.

Occurrence of *trans* Fatty Acids in Human Tissue

Except for small amounts of *trans* fatty acids in animal fats, dietary fats are composed of unsaturated fatty acids of *cis* geometric configuration. In 1928, Bertram (1) found small amounts of *trans* Δ 11-octadecenoic acid in ox, sheep, and butterfat; more recently (2), the presence of 4- to 11-percent *trans* fatty acids has been reported in deer, ox, and sheep depot fats. Although *trans* fatty acids do not seem to be normally present in non-ruminants, they are found in the depot fats of rats which have been fed *trans* fatty acids (3).

Considerable amounts of *trans* fatty acids are formed during the commercial hydrogenation of vegetable oils (4); the shortenings and margarines which include these hydrogenated oils have been reported to contain as much as 23 to 42 percent of *trans* fatty acids (5). Furthermore, the isomers formed during selective hydrogenation are composed of a complex mixture of both geometric and positional isomers (6). The consumption of such fats would presumably lead to the deposition of *trans* fatty acids in depot fats.

In the present study, autopsy and biopsy material from 24 human subjects

(7) was examined for the presence of *trans* fatty acids. The tissues were extracted in a Soxhlet apparatus for 24 hours with acetone and petroleum ether (Skellysolve F) as solvents, the extracts were dried over anhydrous sodium sulfate and filtered, and the solvent was removed under vacuum. The amounts of *trans* isomers in the lipid extracts were determined by the Jackson and Callen baseline method (8), in which a Beckman IR-2A spectrophotometer was used.

All the samples of tissue contained *trans* fatty acids. Adipose tissue contained from 2.4 to 12.2 percent, liver, 4.0 to 14.4 percent, heart, 4.6 to 9.3 percent, aortic tissue, 2.3 to 8.8 percent, and atheroma from subjects who had died of atherosclerosis, 2.3 to 8.8 percent of *trans* fatty acids. It has been pointed out that *trans* linoleic acid does not function efficiently as an essential fatty acid (9), although *trans* fatty acids seem to be metabolized (10). Furthermore, it has recently been reported that *trans* crotonyl CoA is the preferred substrate for the unsaturated acyl CoA hydratase from beef liver (11). Presumably, therefore, long-chain *trans* fatty acids may be metabolized as readily as the *cis* fatty acids. However, in view of the current controversy on the relationship of "hard" vs. "soft" fats (12), it would seem necessary

to determine what effect, if any, *trans* fatty acids have on the normal metabolic process.

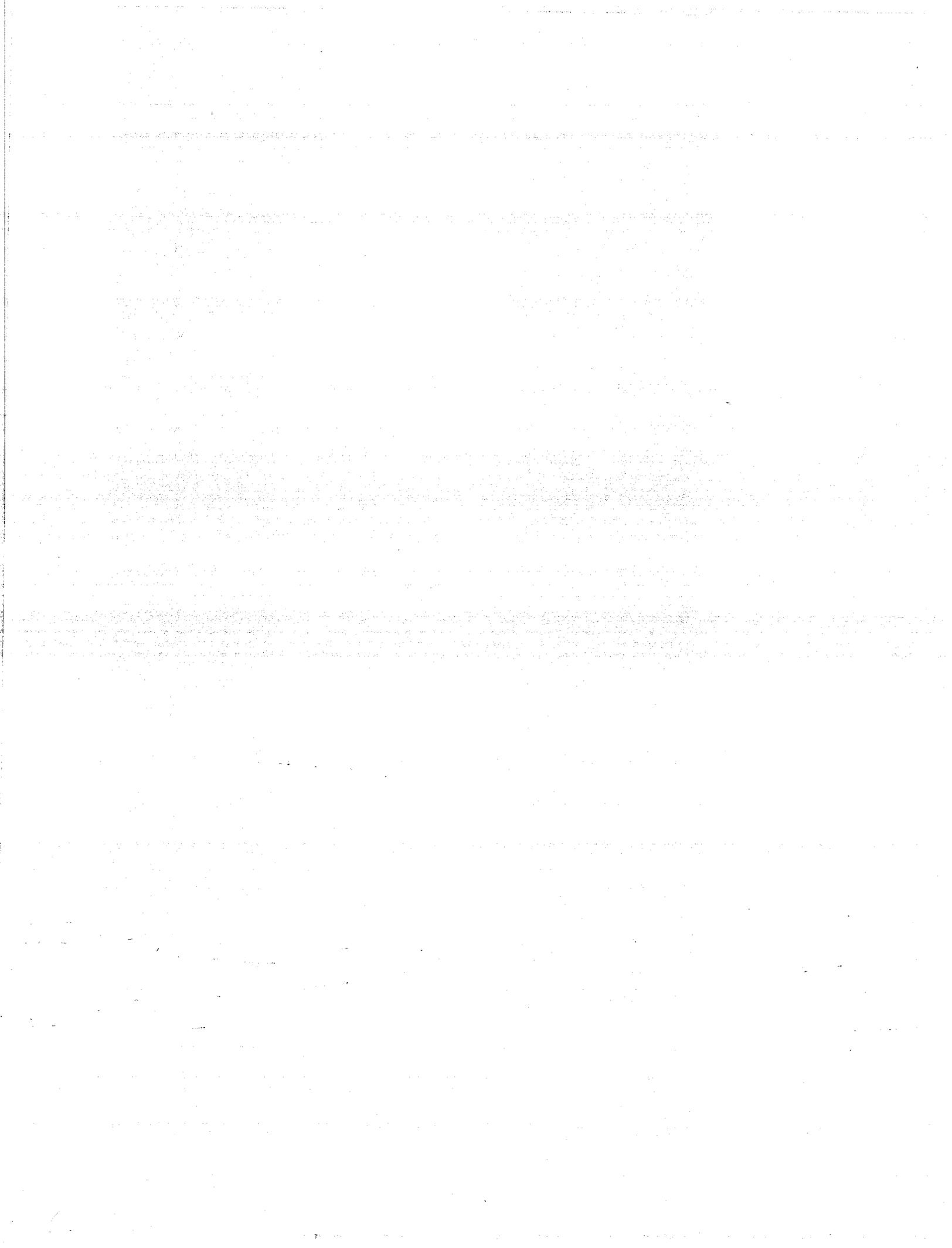
PATRICIA V. JOHNSTON
OGDEN C. JOHNSON
FRED A. KUMMEROW

Department of Food Technology,
University of Illinois, Urbana

References and Notes

1. S. H. Bertram, *Biochem. Z.* 197, 433 (1928).
2. L. Hartman, F. B. Shorland, I. R. C. McDonald, *Nature* 174, 185 (1954); R. Reiser, *Federation Proc.* 10, 236 (1951).
3. R. G. Sinclair, *J. Biol. Chem.* 115, 211 (1936); R. R. Allen *et al.*, *J. Am. Oil Chemists' Soc.*, in press.
4. T. P. Hilditch and N. L. Vidvarthi, *Proc. Roy. Soc. (London)* A122, 522 (1929).
5. A. F. Mabrouk and J. B. Brown, *J. Am. Oil Chemists' Soc.* 33, 98 (1956).
6. R. R. Allen and A. A. Kiess, *ibid.* 32, 400 (1955).
7. We wish to express our appreciation to Clarence Walton, Carle Hospital, Champaign, Ill., and to Cecil A. Krakower, University of Illinois College of Medicine, Chicago, for supplying the samples of human tissue used in this study.
8. F. L. Jackson and J. E. Callen, *J. Am. Oil Chemists' Soc.* 28, 61 (1951).
9. R. T. Holman, *Proc. Conf. on Research, Council on Research, Am. Meat Inst. Univ. Chicago, 3rd Conf. 1951*, p. 1.
10. A. D. Barbour, *J. Biol. Chem.* 101, 63 (1933).
11. S. J. Wakil, *Biochim. et Biophys. Acta.* 19, 497 (1956); J. R. Stern, A. del Campillo, A. L. Lehninger, *J. Am. Chem. Soc.* 77, 1073 (1955).
12. H. M. Sinclair, *Lancet* 270, 381 (1956).

8 August 1957



DEPOSITION IN TISSUES AND FECAL
EXCRETION OF *TRANS* FATTY
ACIDS IN THE RAT^{1,2}

PATRICIA V. JOHNSTON, OGDEN C. JOHNSON AND
FRED A. KUMMEROW

Department of Food Technology, University of Illinois, Urbana

(Received for publication August 8, 1957)

INTRODUCTION

The unsaturated fatty acids in natural fats, with few exceptions, are of the *cis* configuration, while those in hydrogenated fats are to a considerable extent of the *trans* configuration (Hilditch and Vidyarthi, '29). It has been suggested (Sinclair, '56) that there may be a relationship between the consumption of hydrogenated fats and the increased incidence of atherosclerosis and that the unnatural *trans* fatty acids in hydrogenated fats may contribute to the formation of atheroma. On the other hand, it has been assumed that the metabolism of *trans* fatty acids proceeds normally (Barbour, '33).

Recently Allen et al. ('56) have shown that rats fed *trans* fatty acids for a two-week period are capable of metabolizing the *trans* fatty acids from margarine stock as well as those from synthetic triglycerides which had the *trans* double bond in either the Δ 8 or 9 position. However, this study merely showed that the rat is capable of metabolizing *trans* fatty acids. In the present study specific amounts of *trans* fatty acids were fed for a longer period of time and the amounts of *trans* fatty

¹This study was supported by a grant-in-aid from the National Livestock and Meat Board.

²Portion of a thesis presented by P. V. Johnston as partial fulfillment of the requirements for the degree of Doctor of Philosophy in Food Technology.

acids deposited in the carcass measured in order to determine: (1) whether a relatively constant or a maximum level of *trans* fatty acids was deposited in the tissue, (2) if the *trans* fatty acids which were deposited in the carcass fat disappeared when *trans* fatty acids were removed from the diet.

EXPERIMENTAL

A basal diet which consisted of 64% glucose,³ 31% casein and 5% Wesson ('32) salt mix was used in all the feeding experiments. A margarine stock which contained 40% of *trans* fatty acids provided the source of *trans*, and was added at the expense of the glucose. Two percent of soybean oil served as a source of essential fatty acids and the test fat was added at a 10% level. Four grams of a water-soluble vitamin mix were added to each kilogram of food. This mixture was composed of choline 93.5 mg, thiamine 1.24 mg, riboflavin 1.24 mg, pyridoxine 1.24 mg, calcium pantothenate 2.48 mg, and folic acid 0.30 mg per 100 mg. The fat-soluble vitamins were given by drop per once each week.⁴

One hundred and three weanling female rats were used; three were sacrificed and all but 10 of the remainder were divided into two major groups. The diet for group I contained 10% margarine stock; for group II, 5% margarine stock and 5% olive oil. Each major group was divided into 5 subgroups. The animals in subgroup A were fed the test diet for a period of one month, those in subgroup B for two months and those in subgroup C for three months (table 1). The animals in subgroups D and E were fed 10% margarine stock for one month and then transferred to a diet free of *trans* fatty acids. This diet contained 2% of soybean oil as the only fat source (table 2). The animals in subgroup D were sacrificed after being on the fat-free diet for one month and those in subgroup E after

³ Cerelese.

⁴ One drop per rat of the following vitamin mixture was administered once each week. Five grams vitamin A (200,000 U.S.P. units, courtesy of Distillation Products), 0.0054 gm vitamin D₂ and 2.535 gm vitamin E (mixed tocopherols) in 100 ml of olive oil.

TABLE 1

The percentage of trans fatty acids in the carcass fat of rats fed dietary fat in the form of 10% margarine stock or 5% margarine stock and 5% olive oil for one to three months

GROUPS	NO. OF RATS	TOTAL FAT	TRANS FATTY ACIDS	TRANS IN LIVER FAT ¹	DIET. TRANS FOUND IN LIVER ¹	MEAN BODY WT. GAIN AFTER		
						3 Weeks	7 Weeks	12 Weeks
		gm	%	%	%	gm	gm	gm
I. 10% Margarine stock								
Total content in:								
A. 1 month	5	16.5 ± 2.5 ²	16.7 ± 1.4 ²	18.0	0.57	29.6 ± 3.1 ²	—	—
B. 2 months	10	29.2 ± 3.7	18.9 ± 1.3	17.8	0.18	26.9 ± 6.0	164.2 ± 15.5	—
C. 3 months	10	47.8 ± 5.6	18.0 ± 0.9	16.8	0.09	31.1 ± 4.3	167.2 ± 14.2	209.8 ± 14.9
II. 5% Margarine + 5% olive oil								
Total content in:								
A. 1 month	5	19.7 ± 1.9	10.5 ± 0.6	10.8	0.82	30.2 ± 3.5	—	—
B. 2 months	10	30.8 ± 3.9	11.3 ± 0.7	9.8	0.13	29.6 ± 3.5	164.0 ± 8.3	—
C. 3 months	10	44.3 ± 5.7	10.8 ± 0.9	12.5	0.12	27.6 ± 2.6	167.0 ± 10.4	194.4 ± 11.4
Control	10	40.7 ± 6.5	0	0	—	29.2 ± 2.3	172.6 ± 12.1	204.8 ± 14.3

¹ Data for the liver fat are calculated from the pooled samples for each group.

² Standard deviation of the mean.

two months. The remaining 10 rats were fed the basal diet plus 10% of olive oil and served as the *cis* or "natural" fat group.

All animals were individually housed, the food consumption was noted, feces samples collected, and the animals weighed at least once each week. The rats were fasted for 24 hours and anesthetized. The livers were removed immediately after death and weighed. All samples were stored at -20°C while awaiting analysis.

The carcass was transferred to a large beaker and digested with the aid of concentrated hydrochloric acid. The beaker and contents were heated in a steam cabinet at 60°C for 24 hours; the fat floated to the surface and was readily removed with petroleum ether (Skellysolve F). The remaining solution was extracted three times by shaking with Skellysolve F in a separatory funnel and the combined extracts washed free of acid with water, dried over anhydrous sodium sulfate and freed from solvent under vacuum. All extractions were carried out quantitatively.

The fat was extracted from the livers by grinding them in a mortar with anhydrous sodium sulfate and then extracting the mixture in a Soxhlet apparatus for 24 hours with acetone followed by Skellysolve F. The solvents were removed under vacuum after drying over anhydrous sodium sulfate.

The feces were treated with 30% hydrochloric acid before extraction in order to break down any soaps present, and the solution extracted with Skellysolve F. The combined extracts were dried over anhydrous sodium sulfate and freed from solvent under vacuum.

The analysis for *trans* double bonds was carried out with the aid of a Beckman IR2A infrared spectrophotometer. The Jackson and Callen ('51) baseline method was used for calculating percentage of *trans* double bonds in the fat. A 5% solution of the samples in carbon disulfide was used for the analyses, and all results were based on a trielaidin standard.

RESULTS

Trans fatty acids were only detected in the tissues of the animals which had received a diet containing *trans* fatty acid. No *trans* fatty acids were found in the tissues or feces of the three animals sacrificed at the beginning of the experiment. Only traces of absorption at 10.3 μ in the animals which had received 10% olive oil as a source of dietary fat were noted. These traces of *trans* fatty acid may have been due to the limitation of the method of analysis or may have been due to the transfer of *trans* fatty acids from the breeding colony⁵ during the weanling period. At the end of the first month of feeding the animals contained between 15.5 and 18.8% of *trans* fatty acids in the carcass fat; at the end of the second and third month the *trans* fatty acid content of the carcass increased less than 2 and 1% respectively (table 1).

The total amount of carcass fat increased considerably during the three-month period. After one month the animals contained approximately 16 gm, after two months 29 gm, and after three months 48 gm, of total carcass fat respectively. However, despite the increase in total fat, the percentage of *trans* fatty acids in this fat remained virtually unaltered. During this period the animals had consumed 12.5 to 15 gm of diet per day or 0.5 to 0.6 gm of *trans* fatty acids per day.

In order to maintain the percentage of fat existing in the *trans* form at a constant level, the actual weight of *trans* fatty acids in the body naturally increased in proportion to the increase in total carcass fat. For example, a rat having 14 gm of total carcass fat which contained 18.8% of *trans*, had an actual weight of 2.6 gm of *trans* fatty acids in its carcass; whereas an older animal having 43.2 gm of total fat which contained 18.7% of *trans* had 8.0 gm of *trans* fatty acids in its carcass. Hence, the rat organism selected a specific amount of the dietary *trans* acids to deposit in the fat stores and thus maintain a constant concentration of these acids in its body fat. The

⁵ Holtzman Rat Company, Madison, Wisconsin.

amount of dietary *trans* fatty acids found in the tissues seemed to depend on three major factors:

1. The amount of dietary *trans* fatty acids which were immediately metabolized.
2. The rate at which the already deposited *trans* fatty acids were mobilized from the fat stores and metabolized.
3. The amount of dietary *trans* fatty acids which were excreted in the feces.

Since the food consumption per rat was noted every day, a balance study could be made to determine the efficiency of metabolism of *trans* fatty acids by the rat. As the larger portion of the deposited *trans* fatty acids was found in the carcass body fat the individual figures for total body fat and percentage of *trans* fatty acids were determined and used in the calculations. The total fat content and percentage of *trans* fatty acids of both the feces and liver were based on pooled samples, since only small amounts of the ingested *trans* fatty acids were found in these samples. The analysis of the liver fat for each group is reported in tables 1 and 2. The calculations revealed that during the first, second and third months respectively the animals which received 10% of margarine stock deposited between 25.6 and 19.0, 24.5 and 14.2, and 22.0 and 15.4% of the dietary *trans* fatty acids in their carcass fat; 0.54, 0.18 and 0.09% in their liver fat, and throughout the entire feeding period excreted between 1 and 4.5% of the ingested *trans* fatty acids. The remainder of the *trans* fatty acids were apparently metabolized.

Trans fatty acids seemed to be metabolized in essentially the same manner as a labeled fatty acid (Lovern, '55). That is, a certain percentage of the labeled fat was selected to make up the total fat composition. The percentage of labeled fat was maintained at a constant level provided that the food intake remained unaltered. The remainder was metabolized and a small amount excreted.

The animals which had received 10% of dietary fat in the form of 5% margarine stock and 5% olive oil contained approximately 20 gm of carcass fat after one month, 31 gm after

TABLE 2

Decrease of trans fatty acid in carcass fat of rats which had been transferred for one to two months to a diet free of trans fatty acids

GROUPS	NO. OF RATS	TOTAL FAT	TRANS FATTY ACIDS	TRANS IN LIVER FAT ¹	DIET. TRANS FOUND IN LIVER ¹	MEAN BODY WT. GAIN AFTER 3 WEEKS
		<i>gm</i>	<i>%</i>	<i>%</i>	<i>%</i>	<i>gm</i>
I. From 10% margarine stock, Decrease to in:						
D. 1 month	10	27.4 ± 5.1 ²	6.5 ± 0.68 ²	9.1	0.17	30.3 ± 4.0 ²
E. 2 months	10	30.3 ± 4.8	4.4 ± 0.87	8.3	0.11	28.1 ± 2.3
II. From 5% margarine + 5% olive oil, decrease to in:						
D. 1 month	10	28.3 ± 4.3	4.9 ± 0.57	10.3	0.19	27.2 ± 4.1
E. 2 months	10	32.5 ± 4.2	2.8 ± 0.98	6.4	0.22	26.0 ± 3.5
Control	10	40.7 ± 6.5	0	0	—	29.2 ± 2.3

¹ Data for the liver fat are calculated from the pooled samples for each group.

² Standard deviation of the mean.

TRANS FATTY ACIDS IN THE FAT

two months, and 44 gm after three months (table 1). They utilized the ingested *trans* fatty acids in a similar manner to those fed 10% of margarine stock. That is, a constant level of *trans* fatty acids was maintained in the carcass fat (table 1). However, the percentage of *trans* fatty acids in the carcass fat was slightly higher than half of that found in the carcass fat of animals which had received 10% of margarine stock. This discrepancy can be explained by the fact that the inclusion of olive oil in the diet appeared to facilitate the absorption of the *trans* fatty acids. This observation was confirmed by the fact that the animals which had received olive oil and margarine stock excreted only small quantities of the dietary *trans* fatty acids. In no case did this amount exceed 1% and in many cases the amount of absorption at 10.3 μ was too small for the calculation of *trans* fatty acids with any degree of accuracy. The higher absorption of *trans* fatty acids from the gastrointestinal tract evidently led to a higher percentage deposition of *trans* fatty acids in the depot fat.

The balance studies on the animals receiving 5% of olive oil and 5% of margarine stock showed that during the first month 39.6 to 29.7% of the dietary *trans* fatty acids were deposited in the carcass fat and 0.82 in the liver fat. These figures seem strikingly higher than those found for the animals receiving 10% of margarine stock. However, this is merely a reflection of the higher absorption by the animals of the *trans* fatty acids due to the inclusion of olive oil. During the second and third months when the concentration of *trans* fatty acids did not appreciably increase, 27.9 to 19.9 and 22.5 to 17.9% of the dietary *trans* fatty acids were deposited in the carcass fat and 0.12 to 0.22% in the liver fat. The amount of dietary *trans* fatty acids excreted by the animals in this group was extremely small and in most cases below 1%. The remainder of the dietary *trans* fatty acids were apparently metabolised.

The percentage of *trans* fatty acids in the carcass fat decreased when *trans* fatty acids were removed from the diet. They did not completely disappear from the tissues even at the end of two months on a diet free of *trans* fatty acid (table 2).

After one month on the diet free of *trans* fatty acid, the carcass fat of the rats which had received 10% of margarine stock (table 1) had decreased to 6.5% and after two months to 4.4% of *trans* fatty acids (table 2). The carcass fat of the animals which had received margarine stock and olive oil contained approximately 11% of *trans* fatty acids (table 1). After one month on a diet free of *trans* fatty acid, this had decreased to 4.9% and after two months to 2.8% of *trans* fatty acids (table 2).

The results are again in agreement with the behavior of labeled fat. That is, once the labeled fat is removed from the diet, it gradually disappears from the tissues. The depletion of *trans* fatty acids appeared to be somewhat slower than may be expected. However, the present study is in apparent agreement with the work of Kohl ('38) who found that over 30 days were required to deplete animals which had been fed elaidic acid for only three days. The latter study was made before the perfection of the infrared method of analysis for *trans* double bonds. As recent reports have pointed to the relative inaccuracy of the older technique (lead salt-alcohol method) for the determination of *trans* acids (Jackson and Callen, '51), a direct comparison between the study of Kohl and the present one cannot be made.

The depletion of *trans* fatty acids from the liver was slower than from the carcass. This slower rate of depletion was probably due to the fact that the *trans* fatty acids in the depot fat were mobilized and entered the metabolic pool. Hence, the amount of *trans* fatty acids in the liver fat remained higher than in the carcass while this mobilization was in progress.

Holman and Aaes-Jorgensen ('56) indicated that the isomers of *trans* fatty acids inhibit growth. However, in the present study, no evidence of such inhibition was observed. The mean body weight gains of each group after three weeks on the diets are reported in tables 1 and 2. After 7 weeks the animals in groups IB IC, IIB and IIC showed an average mean body weight gain of 166 gm while the animals in the control group which received olive oil as the fat source showed

an average gain of 172.6 gm. The animals in groups IC and IIC which received the diet for a further 5 weeks showed an additional average gain of 35 gm during this time while the animals in the control group gained another 32.2 gm (table 1). Application of the "t" test to the weight data indicated that the differences were not significant (Snedecor, '55). This conflict with the results of Holman and Aaes-Jorgensen may be explained by the fact that in the present study the rats received an adequate amount of essential fatty acids whereas those used by Holman et al. had been depleted of essential fatty acids.

It can be concluded from the present study that rats are capable of efficiently metabolizing *trans* fatty acids over a three-month feeding period. However, the hypothesis of Sinclair ('56) cannot be rejected solely on the basis of the efficiency of metabolism of these fatty acids by the rat. A recent report of Johnston et al. ('57) has established that *trans* fatty acids are present in human tissues. The *trans* fatty acids found in human tissue are no doubt of dietary origin. The results of the present study indicate that if *trans* fatty acids do contribute to the onset of any degenerative disease, this apparently does not occur due to an inability of the animal to metabolize *trans* fatty acids. The effect of the *trans* fatty acids on other metabolites remains unknown. Before Sinclair's hypothesis is considered completely unfounded, further investigations appear essential.

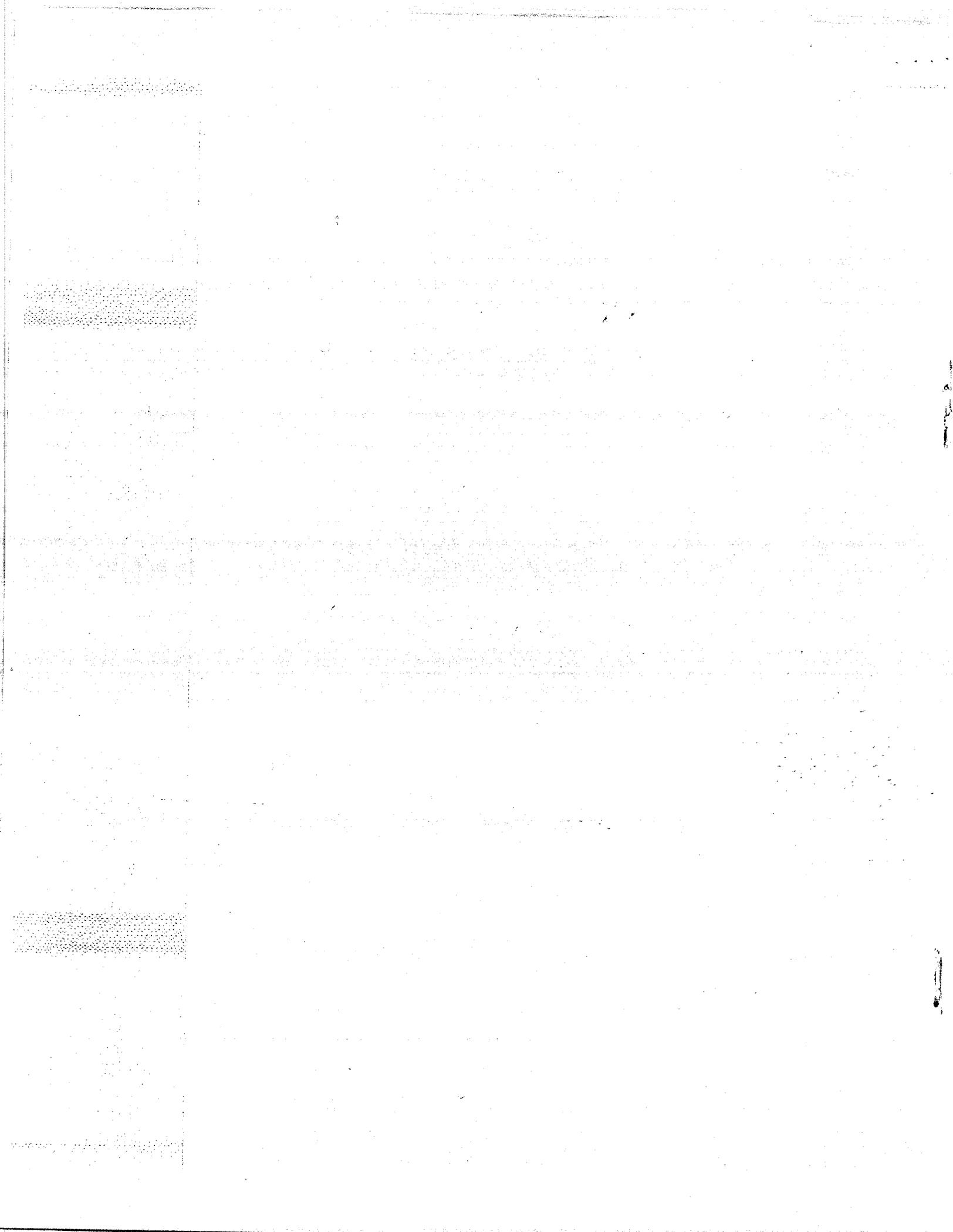
SUMMARY

Trans fatty acids in the form of hydrogenated margarine stock were fed to rats. *Trans* fatty acids were found to be deposited in the tissues only when they were present in the diet. The largest amounts of the deposited *trans* fatty acids were found in the carcass fat, smaller amounts in the liver, and only small quantities were excreted in the feces; the major portion of the ingested *trans* fatty acids were metabolized. When the *trans* fatty acids were removed from the diet

they gradually decreased in amount in the tissues. The presence of *trans* fatty acids in the diet did not appear to inhibit growth.

LITERATURE CITED

- ALLEN, R. R., A. A. KIESS, P. V. JOHNSTON AND F. A. KUMMEROW 1956 The metabolism of triglycerides containing *cis* and *trans* fatty acids. Presented at the Fall Meeting of the American Oil Chemists' Society, September 1956, J. Am. Oil Chem. Soc., (In press).
- BARBOUR, A. D., 1933 The deposition and utilization of hydrogenation iso-oleic acid in the animal body. J. Biol. Chem., 101: 63.
- HILDITCH, T. P., AND N. L. VIDYARTHI 1929 Products of partial hydrogenation of higher monoethylenic esters. Proc. Roy. Soc. London, A122: 522.
- HOLMAN, R. T., AND E. AAES-JORGENSEN 1956 Effect of *trans* fatty acid isomers upon essential fatty acid deficiency in rats. Proc. Soc. Exp. Biol. Med., 93: 175.
- JACKSON, F. L., AND J. E. CALLEN 1951 Evaluation of the Twitchell iso-oleic method: Comparison with the infrared *trans*-iso-oleic method. J. Am. Oil Chem. Soc., 28: 61.
- JOHNSTON, P. V., O. C. JOHNSON AND F. A. KUMMEROW 1957 The occurrence of *trans* fatty acids in human tissue. Science, 126: 698.
- KOHL, M. F. F. 1938 A balance sheet of fat absorption. III. The disappearance of elaidic acid from the tissues of the rat. J. Biol. Chem., 126: 731.
- LOVERN, J. A. 1955 The Chemistry of the Lipids of Biochemical Significance. John Wiley and Sons, Inc. New York, N. Y., p. 90.
- SINCLAIR, H. M. 1956 Letter to the editor: Deficiency of essential fatty acids and atherosclerosis. Lancet, 270: 381.
- SNEDECOR, G. W. 1955 Statistical Methods. The Iowa State College Press.
- WESSON, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Science, 75: 339.



Non-Transfer of *Trans* Fatty Acids from Mother to Young.*† (23601)

PATRICIA V. JOHNSTON, OGDEN C. JOHNSON AND FRED A. KUMMEROW
(Introduced by C. S. Vestling)

Department of Food Technology, University of Illinois, Urbana, Ill.

Bertram(1) first reported the presence of traces of *trans* Δ^{11} octadecenoic acid in oxen, sheep and butterfat. More recently Swern *et al.*(2) reported that beef fat contained not only traces but between 5 and 10% *trans* fatty acids. Hartman and his coworkers extended these investigations to the depot fats of various animals(3) and reported that whereas ruminants contained considerable amounts of *trans* fatty acids, non-ruminants and birds contained little or none. Reiser(4) has suggested that the occurrence of *trans* fatty acids in ruminants is due to the activity of the intestinal flora. Although *trans* fatty acids do not normally appear in the depot fats of non-ruminants *trans* fatty acids are deposited in the tissues whenever they are fed as a dietary component(5-7). It therefore, appeared of interest to note whether *trans* fatty acids were transferred to the tissues of non-ruminants by other than dietary means.

In the present study considerable amounts of *trans* fatty acids were deposited in the

tissues of female rats by feeding these acids in the form of hydrogenated margarine stock. The rats were then mated and both mother and young analyzed for their *trans* fatty acid content.

Methods. One kg of the basal diet was prepared from glucose 640 g, casein 310 g, and Wesson salt mix(8) 50 g. Four g of a water soluble vitamin mix was added to each kg of feed(9) and the fat soluble vitamins were given by dropper twice each week.† Fifteen % of hydrogenated margarine stock containing 40.7% *trans* fatty acids was added to the feed at the expense of the glucose. Two % of corn oil was also added at the expense of glucose to serve as a source of essential fatty acids. Three rats were sacrificed at the beginning of the experiment, and 10 rats were placed on the experimental diet for 73 days and then mated. Eight mothers and their litters were sacrificed by anaesthesia with ether immediately after birth of the young. The remaining 2 litters were allowed to suckle the maternal milk for 9

* This study was supported by grant-in-aid from National Livestock and Meat Board, Chicago, Ill.

† Portion of thesis presented by P. V. Johnston in partial fulfillment of requirements for Ph.D. degree in Food Technology, University of Illinois, Urbana.

‡ One drop/rat of the following vitamin mixture administered twice each week. Five g vit. A (200,000 U.S.P. units, courtesy Distillation Products), .0054 g vit. D₂ and 2.535 g vit. E (mixed tocopherols) in 100 ml of olive oil.

NON TRANSFER OF *Trans* ACIDS

TABLE I. Percentage of *Trans* Fatty Acids Passed on by a Mother Rat to Her Young.

No.	Mother		Young		No. of young in litter†	No. of young born alive
	Total carcass fat, g	<i>Trans</i> fatty acid in carcass fat, %	Total carcass fat, g	<i>Trans</i> fatty acid in carcass fat, %		
1	41.8	23.5	.68	<.5	12	9
2	38.8	26.2	.76	.5	12	10
3	38.6	26.8	.55	.5	10	9
4	37.5	25.1	.42	.5	9	9
5	43.2	24.0	.82	.5	12	11
6	40.8	26.6	.32	.5	7	7
7	37.6	26.6	.75	.5	11	11
8	38.6	25.7	.40	.5	10	9
9	27.9*	26.1	3.3	24.3	9	8
10	20.1*	23.8	3.3	24.8	10	8

* Last 2 litters allowed to suckle 9 days.

† This number represents the number of young analyzed except for No. 9 and 10 in which cases only those rats surviving suckling period were analyzed. These numbers were 7 and 6 respectively.

days before the young were sacrificed. The fat was extracted from the carcasses by a method previously described(9). The percentage of *trans* fatty acid was determined by use of a Beckman IR2A infra red spectrophotometer and the Jackson and Callen baseline method(10). All the analyses were done in carbon disulfide solution at a 5% concentration and the results based on a tri-laidin standard.

Results. Although the carcass fat of female rats which had been fed margarine stock contained between 23.5-26.8% of *trans* fatty acids, less than 0.5% of *trans* fatty acids was found in the carcass fats of their young at birth (Table I). On the other hand, the carcass fat of the young which were allowed to suckle the maternal milk for 9 days contained approximately the same percentage of *trans* fatty acids as their mothers.

No *trans* fatty acids have been detected in adult rats unless they had received a dietary source of *trans* fatty acids(9). Fats extracted from rats fed olive oil have been shown to contain only traces of absorption at 10.3 μ . This absorption represented less than 0.5% of *trans* fatty acid and may have been due to the limitation of the method of analysis or may have been due to the transfer of *trans* fatty acids from the breeding colony§ during the weanling period. The amount of total fat extracted from young which were allowed to suckle increased substantially, as

is normally the case in suckling rats(11). It was observed that the yield of total fat from the mothers of these young was lower than that of the mothers sacrificed on the birth of their young (Table I). It would thus appear that the mothers mobilized considerable quantities of their depot fat, some of which was used in the production of milk fat. A marked increase in the *trans* fatty acid content of young therefore resulted.

The observation that the *trans* fatty acid content of the young increased on suckling is in agreement with a study by McConnell and Sinclair(12). They also found a large increase in the *trans* fatty acid content in the fat extracted from rats which had suckled the milk of a mother fed a diet rich in elaidic acid. However, these authors reported the finding of 10% *trans* fatty acids in the depot fat of the young immediately after birth. The present study does not confirm this finding. There are 2 possible explanations for this conflict. As has been previously pointed out, the method of analysis used by these workers has recently been shown to be unreliable(10). Furthermore, the rats used in the study by McConnell and Sinclair received a much higher quantity of dietary *trans* fatty acids than the rats in the present study. The passage of considerable quantities of *trans* fatty acids to the young may have been a result of overloading the mother rat with *trans* fatty acids.

The number of young in each litter and

§ Holtzman Co., Madison, Wis.

NON TRANSFER OF *Trans* ACIDS

the number born alive is reported in Table I. Parturition was uncomplicated in all cases and 90% of the young were born alive. A diet which contains 5% hydrogenated fat has been shown to be too deficient in essential fatty acids to allow normal lactation(11). However, in the present study the diet was supplemented with enough corn oil to successfully overcome any essential fatty acid deficiency.

A recent report by Alfin-Slater *et al.*(13) that *trans* fatty acids are harmless was based on the results of feeding *trans* fatty acids to rats for 46 generations. The present results indicate that only traces of *trans* acids would be passed on to the fetus and each generation of rats would therefore start to accumulate them only after birth.

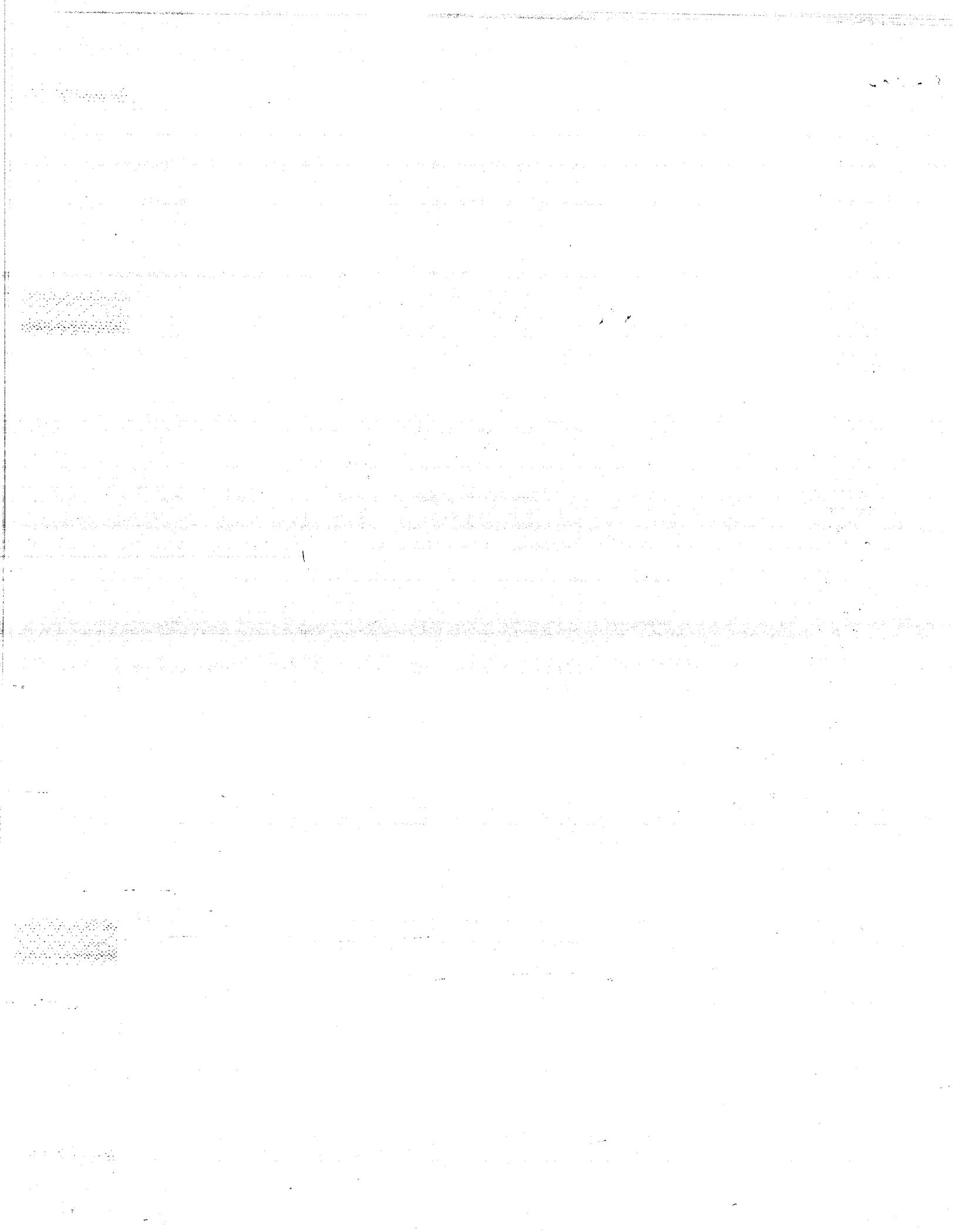
Johnston *et al.*(14) found human tissue to contain 2.0-14% of *trans* fatty acids. It would be of interest to know whether these *trans* fatty acids originated exclusively from dietary fat or were transferred through the placental wall, from a mother to her baby. Further studies in this regard are in progress.

Summary. Less than 0.5% of *trans* fatty acids were found in fat extracted from young born to mother rats which contained between

23.5 and 26.8% *trans* fatty acids in their carcass fats. The amount of *trans* fatty acids in the carcass fats of the young was markedly increased when they were allowed to suckle the maternal milk for 9 days.

1. Bertram, S. H., *Biochem. Z.* 1928, v197, 433.
2. Swern, D., Knight, H. B., and Eddy, R. C., *J. Am. Oil Chemists' Soc.*, 1952, v29, 44.
3. Hartman, L., Shorland, F. B., and McDonald, I. R. C., *Nature*, 1954, v174, 185.
4. Reiser, R., and Reddy, H. G. R., *J. Am. Oil Chemists' Soc.*, 1956, v33, 155.
5. Sinclair, R. G., *J. Biol. Chem.*, 1936, v115, 211.
6. Barbour, A. D., *ibid.*, 1933, v101, 63.
7. Kohl, M. F., *ibid.*, 1938, v126, 709.
8. Wesson, L. G., *Science*, 1932, v75, 339.
9. Johnston, Patricia V., Johnson, O. C., and Kummerow, F. A., 1957, to be published.
10. Jackson, F. L., and Callen, J. E., *J. Am. Oil Chem. Soc.*, 1951, v28, 61.
11. Kummerow, F. A., Pan, H. P., and Hickman, H., *J. Nutrition*, 1952, v46, 489.
12. McConnell, K., and Sinclair, R. G., *J. Biol. Chem.*, 1937, v118, 123.
13. Alfin-Slater, R. B., Wells, A. F., Aftergood, L., and Deuel, H. J., Jr., *J. Nutrition*, 1957, v63, 241.
14. Johnston, Patricia V., Johnson, O. C., and Kummerow, F. A., *Science*, 1957, v126, 698.

Received August 6, 1957. P.S.E.B.M., 1957, v96.



THE EFFECT OF DIETARY FAT ON THE
REPRODUCTIVE PERFORMANCE AND THE MIXED
FATTY ACID COMPOSITION OF
FAT-DEFICIENT RATS¹

F. A. KUMMEROW, H. P. PAN AND H. HICKMAN
Department of Food Technology, University of Illinois, Urbana

(Received for publication September 5, 1951)

It has been shown in previous studies (Evans et al., '34; Maeder, '37; Mackenzie et al., '39; Quackenbush et al., '42) that reproduction failure always ensues in female rats which have grown to maturity on a fat-free diet. After a prolonged gestation period and excessive vaginal bleeding, the young are born dead or die soon after birth. However, if the diet is supplemented with linoleic or arachidonic acid or natural oils containing these fatty acids, normal reproduction ensues. For example, when female rats which had grown to maturity on a fat-free diet were supplemented daily with as little as 100 mg ethyl linoleate three weeks before they were bred, the gestation period was normal and up to 83% of the young were weaned (Quackenbush et al., '42).

The role which dietary sources of essential fatty acids play in reproduction was not elucidated by fat analyses of mother and young. Quackenbush et al. ('42) stated that a remarkable constancy was noted in the iodine values and the percentages of total fat extracted from the animals which had been kept on the fat-free and from those kept on the fat-supplemented diets.

¹ This investigation was supported by research grant No. RG-1171 (R) from the National Institute of Health, Public Health Service. A preliminary report of this paper was given at the XIIth International Congress of Pure and Applied Chemistry, New York, N. Y., September 11, 1951.

It has recently been shown that iodine values do not necessarily reflect changes in the percentage composition of a fat produced by the feeding of dietary fats (Hite et al., '49; Kummerow et al., '49). In the present study, therefore, the percentage composition of the fats extracted from mother and young were determined with the aid of spectrophotometric analyses and the results correlated with reproduction performance. Furthermore, young were removed from representative pregnant females with the aid of Caesarean section during the latter part of the gestation period in order to determine possible causes of intra-uterine death.

EXPERIMENTAL

Preparation of animals

To minimize the pre-experimental storage of essential unsaturated fatty acids, 122 female weanling rats were fed for 17 weeks a fat-free ration which consisted of crude casein, glucose,² salts (Wesson, '32) and all of the known vitamins in adequate amounts (table 1). The animals were then divided into 6 groups; two groups were fed the known B complex vitamins in crystalline form, and the remainder were fed rice bran concentrate³ fortified with choline chloride, riboflavin, *p*-aminobenzoic acid and folic acid. In addition, two groups were fed 5% corn oil and one group 5% hydrogenated fat.

At 17 weeks of age the animals weighed 172 to 186 gm. They seemed normal in appearance except for a slight scalliness of the hind paws and tail. The animals were mated with normal males. Eighteen days after sperm were found in the vaginal smear, the animals were segregated in individual cages and weighed daily. From the 18th to the 22nd day of the gestation period, a 4th of the pregnant animals which had been kept on the fat-free rations were anesthetized

² Cerelose.

³ Vitab.

and the young removed from the uterus by means of Caesarean section.

Fat analyses of the carcass

Two different methods were used for carcass fat analyses; one involved a saponification and the other a solvent ex-

TABLE I
Composition of rations

COMPONENT	FED TO GROUP NUMBER					
	1	2	3	4	5	6
	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>
Casein ¹	180	180	180	180	180	180
Wesson salts	40	40	40	40	40	40
Cerelose	780	730	750	750	700	700
Corn oil	..	50	50	..
Hydrogenated fat ²	50
Rice bran conc. ³	30	30	30	30
Vitamin supplement	4	4	5	6	5	5

¹ Plus 0.33 gm cystine.

² Crisco, Proctor and Gamble Co., Ivorydale, Ohio.

³ Vitab, Nopco Chemical Co., Harrison, N. J.

⁴ Inositol 99.0 mg, choline chloride 308 mg, niacin 26 mg, riboflavin 1.0 mg, thiamine 3.0 mg, *p*-aminobenzoic acid 3.0 mg, folic acid 0.8 mg, calcium pantothenate 7.0 mg, pyridoxine hydrochloride 2.5 mg, and biotin 0.04 mg added per kilogram of basal ration.

⁵ Choline chloride 120 mg, riboflavin 0.8 mg, *p*-aminobenzoic acid 3.0 mg, and folic acid 0.7 mg added to every 30 gm of Vitab.

⁶ Cystine 2.2 gm, inositol 860 mg, choline chloride 2.0 gm, niacin 54 mg, riboflavin 8.7 mg, thiamine 0.96 mg, calcium pantothenate 3.7 mg, pyridoxine hydrochloride 1.6 mg, *p*-aminoazobenzoic acid 3.0 mg, folic acid 0.7 mg and biotin 0.015 mg added to every 30 gm of Vitab. The Vitab was increased from 3 to 6% and the concentration of vitamins added to ration 1 and 2 was doubled after the rats had been bred.

traction procedure. In the former, three representative animals and their newly born young were saponified in 30% aqueous potassium hydroxide, freed of non-saponifiable material, and the fatty acids obtained as previously described (Quackenbush and Steenbock, '42). In the latter, the intestinal tract was removed from the mature animals and

the remaining carcass extracted with Skelly-solve F. The weighed carcass was placed in a Waring Blendor and an equal weight of anhydrous sodium sulfate and Skelly-solve F added and blended for two minutes. The tissues were allowed to settle, the solvent decanted and the extraction repeated three times. The tissues were kept in contact with the solvent for at least 8 hours during the 4th extraction period.

The combined Skelly-solve F extracts were dried with sodium sulfate, transferred to a tared round bottom flask, freed from solvent under vacuum and weighed. The residue was separated into neutral fat and phospholipids by precipitation from acetone. Both the acetone-soluble or neutral fat and the acetone-insoluble or phospholipid fractions were freed from solvent under vacuum, weighed and subjected to spectrophotometric analysis as previously described (Brice et al., '45; Potter and Kummerow, '50). The young were put through a similar procedure.

RESULTS

Reproduction and lactation

A failure of normal parturition always ensued unless corn oil or hydrogenated fat was included in the diets. Labor began at term in the animals which had been kept on the fat-free diets, but parturition was accompanied by excessive hemorrhage. Parturition was not completed for two or three days, while the animals lost weight and became extremely weak and anemic. Approximately 14% of the females died before giving birth to their young; 80% of the latter were born dead, some in an advanced stage of decomposition. The dead young weighed approximately the same as normal living young. All of the young which were born alive died within 24 hours after birth. On Caesarean section, living young were found in every animal examined at the 20th and 21st days of gestation. However, dead young began to appear in the uterus after the 21st day of the gestation period.

When corn oil or hydrogenated fat was included in the diets, normal parturition was noted. The animals on the diet which contained 5% corn oil lactated normally and weaned 85% of their young. However, all of the young from the animals on the diet which contained 5% hydrogenated fat died within 72 hours after birth.

Fat analyses

The results obtained in total fat analyses indicated that the animals which had been kept on a fat-free diet were

TABLE 2

The yield of total fat extracted from mother and young, its iodine value and mixed fatty acid composition

CATEGORY OF INTEREST	MOTHER		YOUNG	
	Fat-deficient diet	Corn oil diet	Fat-deficient diet	Corn oil diet
	%	%	%	%
Yield of total fat	12.2	14.7	1.2	1.3
Iodine value	75.0	84.8	110.6	116.0
<i>Mixed fatty acid composition of total fat</i>				
Linoleic acid	2.6	16.2	2.7	7.6
Trienoic acid	0.0	0.0	2.4	0.0
Arachidonic acid	2.5	8.8	10.2	17.2
Oleic acid	68.3	29.4	72.2	49.6
Saturated acids	26.5	45.5	12.4	25.4

deficient in arachidonic acid and not deficient in fat per se. The animals which had been kept on the fat-free diet contained approximately the same percentage of total fat as those which had received supplements of corn oil.

The mature animals and young on the former diet contained 12.2 and 1.2%, and those on the latter diet contained 14.7 and 1.3% fat, respectively (table 2). However, the data obtained on spectrophotometric analyses indicated that the fatty acid composition of these fats differed markedly. The animals which had received corn oil contained almost 4

times more arachidonic acid than those on the fat-free diet, or 8.8 and 2.5%, respectively. This difference in arachidonic acid content was also reflected in the offspring from these animals. The young from the animals which had received corn oil contained almost twice as much arachidonic acid as those from the animals on the fat-free diet, or 17.2 and 10.2%, respectively.

TABLE 3

The percentage composition of the mixed fatty acids of the neutral fat and the phospholipid from adult female rats

GROUP	RATION	LINO- LEIC	TRIE- NOIC	ARACHI- DONIC	OLEIC ACID	SATURATED FATTY ACIDS
		%	%	%	%	%
<i>Neutral fat</i>						
1	Cryst. vitamins	3.2	0.0	1.0	71.3	24.2
2	Cryst. vitamins + corn oil	6.4	0.0	1.4	75.7	16.5
3	Vitab	0.7	0.3	0.2	68.6	30.1
4	Vitab + cryst. vitamins	0.6	0.5	0.3	68.9	29.7
5	Vitab + corn oil	11.3	0.0	1.2	60.0	27.5
6	Vitab + hydrog. fat	1.6	0.3	0.5	74.2	23.4
<i>Phospholipid</i>						
1	Cryst. vitamins	2.3	0.0	3.8	64.0	29.9
2	Cryst. vitamins + corn oil	13.1	0.0	3.4	39.1	44.4
3	Vitab	1.7	0.4	2.2	53.8	41.9
4	Vitab + cryst. vitamins	1.3	0.3	1.2	59.4	37.9
5	Vitab + corn oil	11.4	0.0	5.0	42.1	41.5
6	Vitab + hydrog. fat	6.5	0.0	4.9	40.7	52.1

The fat-deficient animals seemed to synthesize oleic and a trienoic acid in a vain attempt to increase the percentage of unsaturated fatty acids in the carcass fat. The mature animals and young on the fat-deficient diet contained 38.9 and 22.6% more oleic acid than those on corn oil, or 68.3 and 29.4, and 72.2 and 49.6%, respectively.

The arachidonic acid seemed to be concentrated in the phospholipid fraction in both the mature animals and young (table 3). The most striking differences were noted in the phospholipid fraction of the fat extracted from the young

(table 4). The young from the animals which had received corn oil contained from 5 to 10 times more arachidonic acid than those from the animals on the fat-free diet, and twice as much arachidonic acid as those from the animals on hydrogenated fat. It was interesting to note that the trienoic acid was not present in the phospholipid fraction of the animals which had received dietary sources of preformed fat.

TABLE 4

The percentage composition of the mixed fatty acids of the neutral fat and the phospholipid from newly born young

GROUP	RATION	LINO- LEIC	TRIE- NOIC	ARACHI- DONIC	OLEIC AND SAT- URATED ACIDS
		%	%	%	%
<i>Neutral fat</i>					
1	Cryst. vitamins	6.4	4.7	1.1	87.8
2	Cryst. vitamins + corn oil	9.4	0.0	1.5	89.1
3	Vitab	3.2	4.4	2.0	90.4
4	Vitab + cryst. vitamins	3.4	5.0	2.7	88.9
5	Vitab + corn oil	9.5	0.0	1.9	88.6
6	Vitab + hydrog. fat	4.6	0.2	0.9	94.3
<i>Phospholipid</i>					
1	Cryst. vitamins	6.4	5.1	4.7	83.8
2	Cryst. vitamins + corn oil	12.1	0.0	24.8	63.1
3	Vitab	8.2	6.5	2.1	83.2
4	Vitab + cryst. vitamins	9.6	4.7	1.9	83.8
5	Vitab + corn oil	11.9	0.0	21.9	66.2
6	Vitab + hydrog. fat	11.0	0.0	11.6	77.4

DISCUSSION

The present results indicate that a dietary source of unsaturated fat, such as corn oil, is necessary for normal reproduction and lactation. Furthermore, a commercially available hydrogenated fat did not furnish a sufficient amount of unsaturated fat to meet the requirements for normal lactation. The analytical data presented in this paper, and the results of Quackenbush et al. ('42) indicate that the linoleic acid in corn oil, or linoleic acid per se, is

the limiting factor involved in normal reproduction. The linoleic acid served as a source of arachidonic acid; the latter could not be synthesized without it. The newly synthesized arachidonic acid seemed to be concentrated in the phospholipid fraction and must have played a vital role in the reproduction process. In this connection, it has been noted that the degree of unsaturation of the lipids in human blood gradually increases until term (Muhlbock, '37). This increase in unsaturation may have been due to an increased synthesis of arachidonic acid. The spectrophotometric data indicate that animals which did not receive a dietary source of arachidonic acid synthesized a trienoic acid.

Nunn and MacLean ('38) isolated a similar trienoic acid, from the fat of rats which had been kept on a fat-free diet. These workers found this trienoic acid to be a C_{20} acid, and named it dihydroarachidonic acid. It is interesting to note that an acid of this type has not been found in the phospholipid fraction of rat (Kummerow et al., '49) or poultry fat, although it has been found in the neutral rat fraction (Chu and Kummerow, '50) and in the fat extracted from heart tissue (Rieckehoff et al., '49). The dihydroarachidonic acid could represent a vain effort of the body to synthesize the essential arachidonic acid.

Whether the young which contained the trienoic acid were normal could not be determined. They were alive in the uterus of the mother, indicating that this fatty acid did not interfere with some vital function during intra-uterine life, nor did it seem to interfere in the metabolic functions of the mature animals.

The difficulty noted during parturition could have been due to the poor muscle tone of the uterine wall and may have involved a fundamental change in the mixed fatty acid composition of the lipoproteins in the muscle fiber of the uterus. Szent-Gyorgyi ('47) has pointed out that myosin which has been repeatedly recrystallized still contains 3% lipid material. Part of the latter seemed to be an alcohol-soluble

cephalin capable of functioning as a powerful thrombokinase. The excessive loss of blood in our female rats which had been kept on the fat-free diet may therefore have been due to a failure of the animals to build lipoproteins which contained the cephalin necessary for proper thrombokinase activity.

No excessive hemorrhage was noted in the animals which had received hydrogenated fat. Therefore, the hydrogenated fat seemed to contain enough linoleic acid to furnish a sufficient amount of this fatty acid to synthesize the necessary cephalin. However, linoleic acid was not present in sufficient quantities to satisfy the requirements for normal lactation.

The B complex vitamins did not seem to be involved as adding extra vitamins and increasing the rice bran concentrate from 3 to 6% did not influence reproduction or lactation.

SUMMARY

Female rats which had grown to maturity on a fat-free diet, and bred, gave birth to young which were born dead or died soon after birth. When this diet was supplemented with 5% hydrogenated fat, the animals gave birth to living young which did not live more than 72 hours, while those fed 5% corn oil weaned 85% of their young.

Total fat analyses of representative female rats and their newly born young indicated that the animals which had been kept on the fat-free diet were deficient in arachidonic acid and not deficient in fat per se. The largest differences occurred in the phospholipid fraction of the fat extracted from the young. The phospholipids of the young from animals which had received corn oil contained from 5 to 10, and those from animals on hydrogenated fat two, times more arachidonic acid than those from rats on the fat-free diet.

LITERATURE CITED

- BRICE, B. A., M. L. SWAIN, B. B. SCHAEFFER AND W. C. AULT 1945 Spectrophotometric determination of small proportions of polyunsaturated constituents in fatty materials. *Oil and Soap*, 22: 219.

- CHU, T. K., AND F. A. KUMMEROW 1950 The deposition of linolenic acid in chickens fed linseed oil. *Poultry Sci.*, *29*: 846.
- EVANS, H. M., S. LEPKOVSKY AND E. A. MURPHY 1934 Vital need of the body for certain unsaturated fatty acids. *J. Biol. Chem.*, *106*: 431.
- HITE, J. P., S. E. KLOXIN AND F. A. KUMMEROW 1949 Fat rancidity in eviscerated poultry. *Poultry Sci.*, *28*: 249.
- KUMMEROW, F. A., E. OTTO, G. JACOBSON AND P. RANDOLPH 1949 The effect of antioxidants on the metabolism of linolenic acid by acrodynic rats. *Biol. Antioxidants, Trans. of the 4th Conf., Josiah Macy, Jr., Foundation, New York, N. Y.*, p. 148.
- MACKENZIE, C. G., J. B. MACKENZIE AND E. V. MCCOLLUM 1939 Growth and reproduction on a low-fat diet. *Biochem. J.*, *33*: 935.
- MAEDER, E. C. 1937 The effect of fat in simplified diets on the reproductive organs of the female albino rat during gestation. *Anat. Rec.*, *70*: 73.
- MUHLBOCK, O. 1937 Über den gehalt an ungesathigten fettsauren in blut bei gesunden frauen, in der schwangerschaft und im Wochenbett. *Klin. Wochschr.*, *16*: 853.
- NUNN, L. C. A., AND I. S. MACLEAN 1938 The nature of the fatty acids stored by the liver in the fat deficiency disease of rats. *Biochem. J.*, *32*: 2178.
- POTTER, G. C., AND F. A. KUMMEROW 1950 Factors involved in the spectrophotometric analyses of fats. *J. Am. Oil. Chem. Soc.*, *27*: 190.
- QUACKENBUSH, F. W., F. A. KUMMEROW AND H. STEENBOCK 1942 The effectiveness of linoleic, arachidonic and linolenic acids in reproduction and lactation. *J. Nutrition*, *24*: 213.
- QUACKENBUSH, F. W., AND H. STEENBOCK 1942 Body fats in rat acrodynia. *Ibid.*, *24*: 393.
- RIECKEHOFF, I. G., R. T. HOLMAN AND G. O. BURR 1949 Polyethenoid fatty acid metabolism. Effect of dietary fat on polyethenoid fatty acids of rat tissues. *Arch. Biochem.*, *20*: 331.
- SZENT-GYORGYI, A. 1947 *The Chemistry of Muscle Contraction*. Academic Press, Inc., New York, N. Y.
- WESSON, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. *Science*, *73*: 339.

Reprinted from ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, Volume 63, No. 1, July, 1956
Academic Press Inc. *Printed in U.S.A.*

**The Biological Utilization of Various Fat-Soluble
Esters of Pyridoxine by Rats**

Taketami Sakuragi and Fred A. Kummerow

The Biological Utilization of Various Fat-Soluble Esters of Pyridoxine by Rats^{1, 2}

Taketami Sakuragi and Fred A. Kummerow

From the Department of Food Technology, University of Illinois,
Urbana, Illinois

Received October 27, 1955

INTRODUCTION

The biological activity of some of the so called "fat-soluble" derivatives of pyridoxine has been tested with rats (1), microorganisms (2-4), and tomato roots (5). In the present study, an attempt has been made to elucidate the biological utilization of various fat-soluble esters of pyridoxine in comparison with free pyridoxine hydrochloride.

EXPERIMENTAL

Paper-Disk Method for the Detection of Biological Activity of Pyridoxine Derivatives

The composition of the medium was essentially the same as that used for the microbiological assay for vitamin B₆ using *Saccharomyces carlsbergensis* (A.T.C.C. 4228) except that 2% of agar was incorporated into the medium (6). The sterilized agar medium was inoculated with one loopful of young 24-hr. culture of the yeast, and 10-ml. portions of the medium were poured into sterile Petri dishes and allowed to solidify. Sterilized paper disks of 1/2 in. (12.7 mm.) diameter were placed on the solidified agar, and 0.01 and 0.02 ml. of the test solutions were applied to them. The test solution was composed of an aqueous solution containing 1 µg. pyridoxine hydrochloride/ml. and an ether solution containing an equivalent level of pyridoxine in the form of pyridoxine trilinoleate, an acid-treated pyridoxine trilinoleate, or a blank solution. The latter was prepared by dispersing 1 g. of Tween 85 (polyoxyethylene sorbitan trioleate) in 50 ml. of 0.1 N hydrochloric acid (pH 1.6-1.7) and autoclaving at 20 lb. pressure for 1 hr. After cooling, the solution was neutralized to pH 5.0 with sodium hydroxide, and finally diluted

¹ This work was supported by research grant No. A-257 from the National Institutes of Health, U. S. Public Health Service, Department of Health, Education and Welfare.

² Portion of a thesis presented by T. Sakuragi as partial fulfillment of the requirement for the degree of Doctor of Philosophy in Food Technology.

TABLE I

Composition of the Vitamin B₆-Free Ration

Fat-soluble vitamins were dissolved in a highly hydrogenated coconut oil and administered by a medicine dropper weekly.

	Per 100 lb. ration
Glucose (cerelose)	68 lbs.
Corn oil	10 lbs.
Vitamin-free casein	18 lbs.
Salts (Wesson)	4 lbs.
Inositol	45.5 g.
Choline chloride	91.0 g.
Niacin	4.5 g.
Thiamine hydrochloride	200 mg.
Riboflavin	400 mg.
<i>p</i> -Aminobenzoic acid	136 mg.
Calcium pantothenate	450 mg.
Folic acid	36 mg.
Biotin	190 µg.
Vitamin A	160 I.U./week
Vitamin D	1.6 µg./week
Vitamin E	295 µg./week

to 100 ml.; 0.02 ml. of this solution was applied to each of the paper disks. The acid-treated pyridoxine trilinoleate was prepared by dispersing 100 µg. in 100 ml. of the blank solution and autoclaving for 1 hr. The Petri dishes were incubated at 30°C. for 16 hr.

Vitamin B₆ Level in the Livers of Vitamin B₆-Depleted and Normal Rats after Administration of Pyridoxine Hydrochloride and of Pyridoxine Trilinoleate

Vitamin B₆-depleted rats weighing between 108 and 146 g. were used. The rats had been kept for 11 weeks on a pyridoxine-deficient diet and then divided into various groups (Table I). Before supplementation with the pyridoxine preparations, two of the animals were sacrificed and the livers removed for analysis. Five milligrams of pyridoxine hydrochloride was administered to each of four rats orally as an aqueous solution. Twenty-three milligrams of pyridoxine trilinoleate was fed to each of the remaining four rats; this amount was equivalent to 5 mg. of pyridoxine hydrochloride. Two hours and 6 hr. after administration, two of the animals in each group were killed, and the fresh livers were saved for assay.

The normal male rats, approximately 240 g. in weight, had been kept on a synthetic diet (Table I) which had been supplemented with 1.5 µg. pyridoxine hydrochloride per gram of ration. The rats were supplemented six times with 10-mg. quantities at exact 12-hr. intervals for a total of 60 mg. of pyridoxine hydrochloride or an equivalent amount of pyridoxine trilinoleate. Four hours after the last sup-

plementation, the rats were killed and the livers, average weight approximately 10 g., from three rats in each group were pooled and assayed for vitamin B₆. The microbiological assay used throughout this work was the one reported by Atkin *et al.* (6). To test for per cent recovery, a known amount of pyridoxine hydrochloride was added to a portion of one of the homogenate samples and treated in a similar manner. A part of the same sample was also added to a known amount of the ether solution of pyridoxine trilinoleate with vigorous agitation, and the ether was removed by slightly warming the homogenate and with continuous shaking. A microbiological assay was run with this preparation after autoclaving with acid.

Excretion of Vitamin B₆ in the Urine Following the Administration of Pyridoxine Hydrochloride and of Various Esters of Pyridoxine

Four groups each of four normal male rats weighing between 205 and 225 g. were used in this assay. These rats had been kept on a synthetic diet supplemented with 1.5 µg. pyridoxine hydrochloride per gram of ration (Table I). The same diet was also fed *ad libitum* to the rats during the course of urine collection. Individual rats in the first group were fed an aqueous solution of 10 mg. pyridoxine hydrochloride, the second, an equivalent amount of pyridoxine triacetate hydrochloride, and the third and fourth groups an amount of pyridoxine trioctanoate or pyridoxine trilinoleate equivalent to 10 mg. of pyridoxine hydrochloride. The urine was collected under toluene over specified periods of time, namely (a) from 0 to 10 hr. after administration, (b) for the following 15 hr., and (c) for the next 10 hr., or a total period of 35 hr.

The urine sample collected during the first 10-hr. period following the administration of pyridoxine triacetate hydrochloride was diluted 1:50. This dilution gave a vitamin B₆ level of about 10 µg./ml. of the solution; the first 5-ml. portion was taken directly for the assay. Three hundred milligrams of vitamin-free casein hydrolyzate was dissolved in each of two 5-ml. portions of the solution, and autoclaved at 15 lb. pressure for 1 hr. after adjusting the pH to 6.9 and the volume to 10 ml.

In vitro Enzymatic Digestion of Pyridoxine Trilinoleate

Each digestion mixture consisted of 10 ml. of a 1 M phosphate buffer ranging from pH 6.0 to 8.0, 150 mg. of a commercial steapsin preparation³ or a commercial pancreatin preparation³ suspended in 3 ml. of water, 1.0 ml. of 10% sodium taurocholate solution, and 1.0 ml. of 5% egg albumin solution. Approximately 100 mg. of pyridoxine trilinoleate, synthetic trilinolein, or olive oil was weighed accurately and placed in the digestion flask. The system was incubated at 37°C. for 2 hr. The flasks were shaken every 20 min. The digestion mixture was then acidified, and the fatty materials were extracted with neutral benzene for the titration of free fatty acid following the procedures reported by Davis (7).

RESULTS AND DISCUSSION

The pyridoxine moiety in pyridoxine trilinoleate was found to be available to rats but not to *Saccharomyces carlsbergensis*. It was shown

³ Purchased from the Nutritional Biochemicals Corporation, Cleveland, Ohio.

by the paper-disk method that the latter could not utilize pyridoxine trilinoleate as a source of vitamin B₆ and that acid digestion at pH 1.6–1.7 at 20 lb. pressure for 1 hr. did not free the pyridoxine moiety. When an aqueous solution of pyridoxine hydrochloride was added to the test solution which contained pyridoxine trilinoleate or the ester which had been subjected to acid digestion, growth of *Saccharomyces carlsbergensis* was noted. Therefore, absence of growth could not have been due to the mere presence of a fatty acid or the presence of Tween 85.

The pyridoxine moiety in pyridoxine trilinoleate was found to be available to vitamin B₆-depleted as well as to normal rats. Furthermore, the depleted rats accumulated substantial amounts of pyridoxine as rapidly on supplements of pyridoxine trilinoleate as those on pyridoxine hydrochloride (Table II). Two hours after supplementation with 5 mg. of pyridoxine hydrochloride or an equivalent amount of pyridoxine trilinoleate, the fresh liver was found to contain 12–15 µg. vitamin B₆ as pyridoxine hydrochloride/g. tissue. In comparison, the livers from two unsupplemented vitamin B₆-depleted rats contained only 4.2 and 4.4 µg. vitamin B₆/g. tissue, which agreed with data previously reported (8). Six hours after supplementation, two rats which had been fed pyridoxine trilinoleate contained 11.1 and 16.0 µg., and two fed pyridoxine hydrochloride contained 7.9 and 9.3 µg./g. liver tissue. Supplements of pyri-

TABLE II

Vitamin B₆ Level^a in the Livers of the Vitamin B₆-Depleted Male Rats after the Administration of Pyridoxine Hydrochloride and of Pyridoxine Trilinoleate

Supplement ^b	Hours, after administration								
	0 hr.			2 hr.			6 hr.		
	Body wt.	Fresh liver wt.	Vitamin B ₆ /g. liver	Body wt.	Fresh liver wt.	Vitamin B ₆ /g. liver	Body wt.	Fresh liver wt.	Vitamin B ₆ /g. liver
Pyridoxine hydrochloride	g.	g.	µg.	g.	g.	µg.	g.	g.	µg.
	138	6.6	4.2	146	7.1	13.2	129	6.1	9.3
				116	5.8	15.0	123	5.4	7.9
Pyridoxine trilinoleate	108	5.6	4.4	140	6.3	14.3	132	6.5	11.1
				117	5.9	12.3	121	4.8	16.0

^a The amount of vitamin B₆ is indicated as pyridoxine hydrochloride.

^b Supplementation was made so as to supply 5 mg. of pyridoxine hydrochloride or an equivalent amount of pyridoxine trilinoleate per rat.

doxine trilinoleate, therefore, sustained a higher level of liver vitamin B₆ over a longer period of time than pyridoxine hydrochloride.

Normal male rats killed 4 hr. after supplementation showed a similar trend. The levels of those supplemented with 60 mg. of pyridoxine hydrochloride or an equivalent amount of pyridoxine trilinoleate contained 10.0 and 15.6 μ g. of pyridoxine/g. tissue, respectively. In comparison, the unsupplemented rats contained only 7.0 μ g. pyridoxine/g. tissue. It therefore seems evident that a greater retention of pyridoxine was obtained by supplementation with pyridoxine trilinoleate than with pyridoxine hydrochloride.

A known amount of pyridoxine hydrochloride which had been added to a liver homogenate was recovered completely within the range of experimental error. On the other hand, none of the pyridoxine trilinoleate incorporated into the homogenate was recovered, indicating that autoclaving in an acid medium did not make the pyridoxine fragment in pyridoxine trilinoleate available to *S. carlsbergensis*.

The urinary excretion tests also indicated that pyridoxine in the form of the long-chain fatty acid ester was retained longer than the hydrochloride (Table III). Furthermore, the rate of excretion of vitamin B₆ during the first 10 hr. after supplementation was approximately two to three times less with the trioctanoate and the trilinoleate than with the hydrochloride or 215, 140, and 400 μ g., respectively. Whether the low excretion of vitamin B₆ after feeding pyridoxine triacetate into the urine

TABLE III

Urinary Excretion of Vitamin B₆^a by Normal Male Rats Following the Administration of Various Pyridoxine Preparations
Average hourly output per rat.

Supplement ^b	Hours, after administration			Average body wt. g.	No. or rats
	0-10	10-25	25-35		
	μ g.	μ g.	μ g.		
Pyridoxine hydrochloride	400	35	7	213	4
Pyridoxine triacetate hydrochloride	170	40	14	223	4
Pyridoxine trioctanoate	215	80	24	219	4
Pyridoxine trilinoleate	140	75	40	214	4

^a The amount of vitamin B₆ is indicated as pyridoxine hydrochloride.

^b Supplemented so as to supply a preparation equivalent to 10 mg. of pyridoxine hydrochloride.

TABLE IV

Activity of Vitamin B₆ and Related Compounds for the Growth of *Saccharomyces carlsbergensis* and the Effect of Various Treatments

No.	Compound	Treatment	Activity ^a
1.	Pyridoxine triacetate.HCl	None	0.25-0.45 ^b
2.	Pyridoxine triacetate.HCl	A. D. ^c	1.0
3.	Mixture A ^d	None	0.65 ^b
4.	Mixture A	A. D.	1.0
5.	Pyridoxal.HCl	C. H. ^e followed by A. D.	1.0
6.	Pyridoxine triacetate.HCl	C. H.	0.65
7.	Pyridoxine triacetate.HCl	C. H. followed by A. D.	1.0
8.	Mixture B ^c	None	0.85 ^b
9.	Mixture B	A. D.	0.70
10.	Mixture B	C. H.	0.87
11.	Mixture B	C. H. followed by A. D.	1.0
12.	Mixture B (5 ml.) plus normal rat urine (0.1 ml.) ^d	C. H. (high) ^e	0.86
13.	Mixture B (5 ml.) plus normal rat urine (0.1 ml.)	C. H. (high) followed by A. D.	1.0

^a The activity of pyridoxine.HCl was considered to be 1.0. Pyridoxal.HCl and pyridoxamine.2HCl also showed an equal activity on a molar basis.

^b An equimolar mixture of pyridoxine triacetate.HCl and pyridoxine.HCl.

^c A mixture of 2 moles of pyridoxine triacetate.HCl and 8 moles of pyridoxal.HCl.

^d The vitamin B₆ level present in the urine became negligible, since 0.1 ml. of the urine supplied only 0.07 μ g. as pyridoxine.HCl, while the 5 ml. of mixture B contained 50 μ g. as pyridoxine.HCl.

^e A. D.: Alkali digestion: an aqueous solution containing the preparation at a level of ca. 10 μ g./ml. was mixed with an equal volume of 4 N aqueous sodium hydroxide solution (final concentration was 2 N), and refluxed for 30 min. The solution was then neutralized and used for the assay.

^f C. H.: Casein hydrolyzate treatment; 100 mg. of vitamin-free casein hydrolyzate was added to 5 ml. of an aqueous solution containing about 10 μ g. of a preparation/ml. The solution was adjusted to pH 6.9 with a pH meter, and the volume made up to 10 ml. It was then autoclaved at 15 lb. pressure for 1 hr.

^g C. H. (high): This indicates the use of 300 mg. of vitamin-free casein hydrolyzate instead of 100 mg. for 5 ml. of a sample solution.

^h Care was taken not to apply any pressure during sterilization, since it was known that autoclaving caused secondary changes on pyridoxine triacetate (2, 3).

was due to actual excretion or due to the presence of unchanged pyridoxine triacetate, to which the assay organism did not respond as well as to free vitamin B₆, was shown by differential assay (Table IV). This assay was based on the fact that the activity of pyridoxine triacetate

was 25-45% of that of free vitamin B₆ for *S. carlsbergensis*. When the acetate was hydrolyzed with alkali, it revealed 100% activity as vitamin B₆. Thus the difference in the activity of the preparation containing pyridoxine triacetate before and after the alkali treatment was an indication of the presence of pyridoxine triacetate. This assay procedure was also applicable to the mixture of the triacetate and alkali-labile pyridoxal. Prior to the assay, the mixture was autoclaved with vitamin-free casein hydrolyzate, which brought about the conversion of pyridoxal to alkali-stable pyridoxamine (9). The condition of this treatment did not alter the activity of pyridoxine and pyridoxamine (Table IV). A vitamin B₆ assay was carried out on the urine sample before treating with casein hydrolyzate and also after the treatment with and without subsequent alkali digestion. The readings of the assay on these three samples were exactly identical. These results, therefore, indicated that no unchanged pyridoxine triacetate was excreted into the urine of rats even when a large dose of the triacetate was administered.

The results from the *in vitro* enzymatic digestion indicated that no hydrolysis of pyridoxine trilinoleate resulted by a commercial "lipase" preparation or by a commercial "pancreatin" at 37°C. after a 2-hr. incubation period. The digestions were attempted at pH's 6.0, 6.4, 6.8, 7.2, 7.4, and 8.0. Under the same conditions, the hydrolysis of olive oil by the "lipase" proceeded up to 25, 28, and 36% at pH's 6.0, 6.8, and 8.0, respectively. The hydrolysis of synthetic trilinolein also occurred in the presence of the "pancreatin"; the reading reached 11, 11, and 35% at pH's 6.0, 6.8, and 8.0, respectively.

ACKNOWLEDGMENTS

The authors wish to thank I. J. Aves for his assistance in the animal experiments, N. T. Rand for his assistance in the microbiological assay, and Miss A. F. Schmidt for her help throughout this work.

SUMMARY

It was demonstrated that in rats a longer retention of vitamin B₆ could be maintained when pyridoxine trilinoleate was fed than when an equivalent amount of pyridoxine triacetate or pyridoxine hydrochloride was fed. Unchanged pyridoxine triacetate did not appear to be excreted in the urine even after the administration of a large dose of the triacetate. A commercial "steapsin" or "pancreatin" failed to hydrolyze pyridoxine trilinoleate at 37°C. in a 2-hr. period.

REFERENCES

1. UNNA, K., *Proc. Soc. Exptl. Biol. Med.* **43**, 122 (1940).
2. BOHONOS, N., HUTCHINGS, B. L., AND PETERSON, W. H., *J. Bacteriol.* **44**, 479 (1942).
3. SNELL, E. E., *J. Am. Chem. Soc.* **66**, 2082 (1944).
4. CARPENTER, L. E., AND STRONG, F. M., *Arch. Biochem.* **3**, 375 (1944).
5. ROBBINS, W. J., *Am. J. Botany* **29**, 241 (1942).
6. ATKIN, L., SCHULTZ, A. S., WILLIAMS, W. L., AND FREY, C. N., *Ind. Eng. Chem., Anal. Ed.* **15**, 141 (1943).
7. DAVIS, B. D., *Arch. Biochem.* **15**, 351 (1947).
8. SCHWEIGERT, B. S., SAUBERLICH, H. E., ELVEHJEM, C. A., AND BAUMANN, C. A., *J. Biol. Chem.* **165**, 187 (1946).
9. SNELL, E. E., *J. Biol. Chem.* **157**, 491 (1945).