The Constancy of Red Blood Cell Lipids in Man During Extreme Variations of Dietary Fat Intake[1]

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Abstract

The nature and extent of red blood cell lipid changes were determined in four controlled dietary experiments of 8–16 days' duration involving the use of fat-free diets, and diets supplemented with high levels of corn oil and butter fat. In the first experiment, changes in red blood cell cholesterol ester, glyceride, and phospholipid fatty acids were detected within eight days of modifications in diet. In the second experiment, constant red blood cell cholesterol levels of 136 ± 6 mg/100 ml packed cells were observed in 49 individuals who had consumed fat-free diets, or diets providing 45% of calories in the form of corn oil. The effects of diets rich in corn oil or butter fat on red cell phospholipid phosphorus levels were assessed in the third experiment, and values were found to range only from 12.5 ± 0.4 to 12.7 ± 0.4 mg/100 ml packed cells. In the fourth experiment, total red cell lipid and the proportions of the various lipid classes were found to be unchanged under several dietary regimens. Calculations based on data from this investigation provide independent confirmation of earlier conclusions that the amount of lipid present in the red blood cell can be exactly accommodated in a bimolecular layer extending over the entire cell surface.

Introduction

MODERN CONCEPTS of the red blood cell membrane represent it as a bimolecular layer of lipid, covered on both sides with a layer of protein. Indirect evidence supporting this idea has come from several disciplines (1), but the fundamental question of whether the amount of lipid present is compatible with this concept has been almost completely ignored.

In several monographs and reviews (1,2,3) the only direct chemical evidence quoted in support of this model is the work of Gorter and Grendel (4), published in 1925. These investigators compared the surface area of the red cells of various species with the area occupied by the cell lipids when spread as a monomolecular film on water. For all species tested, the amount of lipid found was sufficient to provide almost exactly a layer of lipid two molecules thick for each cell. However, the data obtained in this ingenious experiment can be questioned on two counts: the red cell lipids were extracted with acetone, a solvent particularly poorly adapted to this task; and the red cell surface areas as determined by Gorter and Grendel differ by as much as 50% from presently accepted values (5). Thus, although their conclusion that the red cell membrane contains a bimolecular layer of lipid may actually be correct, the data on which it was based were incorrect.

[1] Presented in part at the AOCS meeting in Toronto, 1962.

In recent years dietary fats have been shown to affect the nature and distribution of plasma lipids, and in turn, there is evidence that certain plasma lipids exchange freely with red cell lipids (6,7). On this basis it seemed reasonable to expect that dietary fats would affect red cell lipids, and indeed it has been shown that modifications in red cell fatty acids can be brought about by this means (8,9). Although no real variation in the total amount of lipid in normal red cells has ever been demonstrated, the extent and significance of the lipid exchange between plasma and red cells has not been ascertained. The potential importance of these observations lies in the fact that in the few diseases in which qualitative or quantitative changes in red cell lipids have been well authenticated (10), it is well known that the red cells are abnormally fragile. It seems clear that an important relationship exists between the lipid content of the cell and its structural integrity.

The present study was conducted to provide detailed qualitative and quantitative information on the red blood cell lipid membrane of normal subjects under controlled dietary conditions. This report demonstrates the fact that red cell lipid levels remain constant during extreme variations in dietary fat intake, and provides independent confirmation of the conclusions of Gorter and Grendel, based on modern techniques for lipid analysis and a revised estimate of the surface area of the red blood cell.

Experimental

The data to be presented have been obtained in four dietary experiments, all of the same general plan. Male and female university students consumed various homogenized formula type diets of precisely known composition for periods of up to 16 days. The composition of the essentially fat-free basal diet shows in Table I. The other diets were modifications of the basal diet in which either corn oil or butter fat was substituted isocalorically, at a level of 45% of calories, for part of the dextri-maltose. For con-

TABLE I

Composition of Basal Diet: Amounts Required to Make a 950-Calorie Sample[a]

Ingredient	Weight in g	Composition, in g		
		Protein	Fat	Carbohydrate
Mil-ko[b]	28.1	10.0	0.1	14.6
Casec[c]	28.4	25.0	0.57	.....
Sucrose	20.0	.....	.....	20.0
Dextri-Maltose[d]	169.8	.....	.....	166.4
Total		35.0	0.67	201.0
Calories	950.0	140.0	6.03	804.0
% Calories		14.74	0.63	84.63

[a] Each 950-calorie sample was supplemented with 2 g iodized salt, 0.6 mg thiamine, 0.6 mg riboflavin, 5.0 mg niacin, 5.0 mg pyridoxine, 5.0 mg calcium pantothenate, 25 mg ascorbic acid, and 1700 I.U. Vitamin A.

[b] Skim milk preparation from Mil-ko Products, Hamilton, Ontario.

[c] Soluble form of calcium caseinate produced by Mead Johnson of Canada, Ltd., Belleville, Ontario.

[d] Mixture of maltose and dextrins from corn flour, produced by Mead Johnson of Canada, Ltd., Belleville, Ontario.

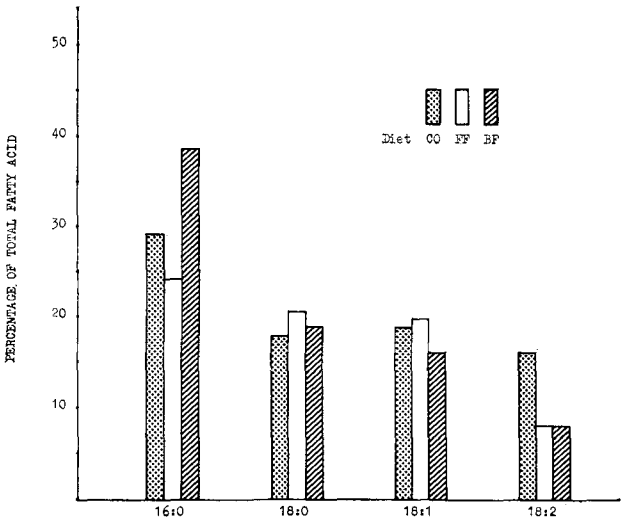

FIG. 1. Effect on the cholesterol ester fatty acids of changing from the basal diet (FF) to a diet rich in corn oil (CO) or butter fat (BF).

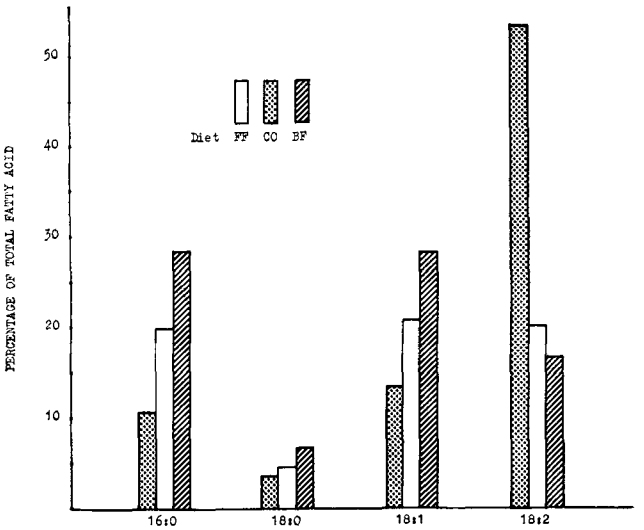

FIG. 2. Effect on the phospholipid fatty acids of changing from the basal diet (FF) to a diet rich in corn oil (CO) or butter fat (BF).

venience, these modified diets will subsequently be referred to as diets CO and BF, respectively. In addition, the diet BF of one of the groups of subjects in Experiment IV was supplemented with 1000 mg sitosterol/950 calories.

Outline of Experiments. Experiment I was designed to learn if changes in red cell fatty acids could be detected in a period as short as eight days after a change in diet. Two groups, each consisting of eight male subjects, consumed the basal diet for eight days. One group was then transferred to diet BF, and the other to diet CO. Red blood cell samples obtained on days 8 and 16 were pooled, according to group, prior to lipid fractionation and analysis of the fatty acid composition of the steryl ester, glyceride, and phospholipid fractions.

Experiment II was designed to compare the effect of corn oil and a fat-free diet on the cholesterol levels of red blood cells. In the first part of the experiment 12 women and 11 men were maintained on the basal diet for 16 days. In the second part, 15 women and 11 men consumed the basal diet for eight days, then changed to diet CO. Samples of blood were obtained from each subject on days 0,8 and 16, and the red blood cell cholesterol determined directly.

Experiment III, in which 10 male students served as subjects, was planned to permit assessment of the effect of two different diets on red cell phospholipids. Five of the subjects consumed diet BF for 16 days, and the other five consumed diet CO for a similar period. Red blood cell phospholipid levels were determined on samples from each subject prior to, and at the end of the experiment.

Experiment IV measured the effect of diet on the levels of the major red blood cell lipid classes. Cells were obtained from four groups, each composed of 8 male subjects, after they had been maintained for eight days on one or other of the following diets: 1) the basal diet; 2) diet CO; 3) diet BF; and 4) diet BF supplemented with sitosterol. After blood specimens from all members of each group had been pooled to provide 4 large samples, representative of the four dietary regimens, the red cell lipids were extracted and fractionated as described below.

In all experiments, the blood samples were 10 to 20 ml in volume, and were taken while the subjects were in the fasting state. Heparin was used as the anticoagulant.

Isolation of Red Blood Cell Lipids. As soon as possible after the blood samples had been obtained, they were centrifuged at 2000 rpm for 30 min. The plasma and buffy coat were removed by aspiration, and the red cells resuspended in an equal vol of isotonic saline. They were again centrifuged at 2000 rpm for 30 min, after which the supernatant and any remaining buffy coat were removed. It can be shown that this procedure will yield a sample essentially free from leucocytes and platelets, and containing less than 0.5% trapped plasma (12). Thus there is no need for repeated washing of the red cells, and in fact, the contradictory evidence about the effect of such washing on the red cell lipids suggests that the practice should be avoided (13,14).

Lipid extracts were prepared from the washed cells by a method similar to that of Folch et al. (15). To ensure complete recovery of the lipid, each sample had to be extracted with a total of 36 vol chloroform and 18 vol methanol. Non-lipid contaminants were removed by washing the extract with 0.3 vol 0.05 N KCl (16), and allowing the phases to separate overnight. The lipid-rich phase was concn in a rotary film evaporator, and after a suitable aliquot had been removed for the gravimetric determination of total lipid, the remaining material was separated quantitatively into cholesterol ester, glyceride, free cholesterol, and phospholipid fractions by silicic acid column chromatography (17).

Analytical Methods. Cholesterol determinations were made directly on the washed red cells of individual donors (18). Phospholipid phosphorus was determined by the Hurst modification (19) of the method of Beveridge and Johnson (20). Glyceride glycerol was measured by a chromotropic acid procedure which combined the saponification technique of Carlson and Wadstrom (21) with the color development method of Hanahan and Olley (22). The fatty acids from each of the three ester groups were obtained by alkaline hydrolysis followed by ether extraction of the acidified reaction mixture (23). Fatty acid esters were prepared with diazomethane (24), and were characterized on a Beckman GC-2A gas chromatograph equipped with a hot wire detector. The stainless steel column, six ft in length and $\frac{3}{16}$ in. inside diam, was packed with 20 g firebrick, 60–80 mesh, coated with 20% by wt of diethylene glycol succinate. The column temp was maintained at 220C,

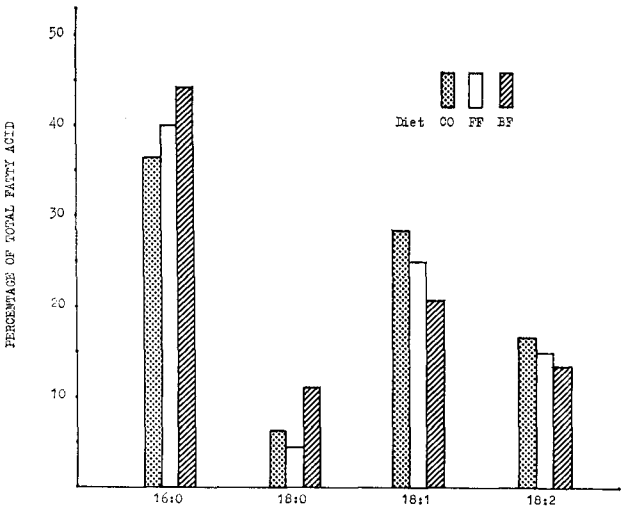

FIG. 3. Effect on the glyceride fatty acids of changing from the basal diet (FF) to a diet rich in corn oil (CO) or butter fat (BF).

and the temp of the injection port at 280C. The carrier gas was helium, and it was used at a flow rate of approximately 125 ml/min. Reference fatty acid methyl esters were obtained from Applied Science Laboratories, Inc., State College, Pa.

Results and Discussion

The first modern evidence that dietary fat could influence the red blood cell fatty acids of man was presented by Horwitt et al. (8) in 1959. They examined the red cells of subjects who had for two years consumed a diet providing 60 g corn oil each day, and found the linoleic acid content to be almost double that found in the red cells of subjects on an unrestricted diet. Leibetseder and Ahrens (9) supplemented this work by partially fractionating the ester group prior to fatty acid analysis. In a single subject they observed that a diet supplying 90 g corn oil/day led, within 10 days, to a marked increase in linoleic acid in both the acetone-soluble and the acetone-insoluble fractions of red cell lipids. In Experiment I of the present study, changes in red blood cell fatty acids were seen within eight days of a change in diet (FF to CO, or BF). A summary of the results, in which only data for the major fatty acids is included, shows in Figures 1–3. The change from the basal diet to the high butter fat diet led to a considerable increase in the palmitic acid content of both the phospholipid and cholesterol ester fractions. Similarly, the change to the diet high in corn oil led to marked increases in the amount of linoleic acid in the same lipid classes. The glyceride fatty acid spectrum varied little in response to changes in dietary fatty acids, although the trend observed was in the direction anticipated assuming that glyceride fatty acids are influenced by dietary fatty acids. Thus, butter fat, high in palmitic acid and low in linoleic acid, led to an increase in palmitic acid and a decrease in linoleic acid in this fraction. The corn oil, low in palmitic and high in linoleic acid, had the opposite effect. Direct comparison of these results with those of others is not feasible, because previous investigators have not attempted the isolation of the three red cell lipid ester groups. Qualitatively, however, the results of the present study are similar to those Horwitt et al. (8) and Leibetseder and Ahrens (9) obtained for the total and the partially resolved red cell esters, respectively. In view of the rapidity

TABLE II

The Effect of Diet on the Cholesterol Content of Red Blood Cells and Plasma

Diet	Subjects		Cholesterol, mg/100 ml		
			Day 0	Day 8	Day 16
Fat Free (days 0–16)	Women (12)	Red cells	134 ± 6	137 ± 6	134 ± 7
		Plasma	185 ± 33	141 ± 30	145 ± 31
	Men (11)	Red cells	136 ± 4	137 ± 8	132 ± 6
		Plasma	187 ± 28	128 ± 18	131 ± 22
Fat Free (days 0–8), Corn Oil (days 9–16)	Women (15)	Red cells	138 ± 7	138 ± 5	138 ± 7
		Plasma	187 ± 28	133 ± 25	123 ± 25
	Men (11)	Red cells	139 ± 4	135 ± 6	139 ± 6
		Plasma	190 ± 36	134 ± 17	116 ± 23

and extent of these changes, the constancy of total lipid and the individual lipid classes (see below), and the known inability of the mature red cell to synthesize lipid (25,26), these changes in fatty acid spectra must represent an exchange between the plasma and red cell fatty acids as suggested by Leibetseder and Ahrens (9). The discovery of such a rapid effect of dietary fat on red cell lipids indicated that the selected diets and the chosen experimental period were suitable for the remainder of the proposed investigation.

The results of the second experiment show in Table II. As anticipated, plasma cholesterol levels fell significantly during the first eight days of fat deprivation and remained low for the duration of the experiment. In contrast, red blood cell cholesterol levels stayed virtually unchanged throughout the entire period of the fat free diet. Similar observations regarding the cholesterol levels of red blood cells were made in the second part of this experiment, in which the subjects changed from a fat free diet to a diet providing 45% of calories from corn oil. The red blood cell cholesterol levels remained constant despite a significant decrease in plasma cholesterol during the initial 8 days on the fat free diet and the final 8 days on diet CO. The average red cell cholesterol level for the female subjects, based on 81 determinations on 27 women, was 136 ±6 mg/100 ml packed red blood cells. The corresponding value for male subjects, derived from 66 observations on 22 men was also 136 ± 6 mg/100 ml. These values agree closely with the values of 139 ± 5 mg/100 ml reported by Brun (27), 137 ± 4 mg/100 ml found by Reed et al. (13), and 137 ± 7 mg/100 ml observed by Mancini and Keys (28), and support the thesis advanced by Brun (27) that under most circumstances, including marked changes in plasma cholesterol levels, the cholesterol content of the red cells remains within relatively narrow limits. Further support is found in reports by a number of other investigators. For example, London and Schwartz (29) observed a nephrotic patient with plasma and red cell cholesterol concn of 636 and 146 mg/100 ml, respectively, and Olson (30) has commented on two cases of xanthomatous biliary cirrhosis in which serum cholesterol was 1200–2000 mg/100 ml, but red cell cholesterol was within normal range. In the experiment of Mancini and Keys (28), involving 6 hospitalized males, it was found that alterations in diet resulting in average changes of from −64 to +52 mg cholesterol/100 ml serum led to no significant change in the cholesterol content of the red cells. Fels et al. (10) made comparable observations in an experiment in which hypercholesterolemia was induced in rabbits by diet or

TABLE III

The Effect of Variation in Dietary Fat on Red Blood Cell Phospholipid Phosphorous Content

Subjects	Dietary Regimen	Phospholipid phosphorus mg/100 ml of red cells
10	unrestricted	12.5 ± 0.4
5	CO	12.6 ± 0.4
5	BF	12.7 ± 0.4

drugs. It appears therefore that the absence of any correlation between the cholesterol levels in plasma and in the red blood cells is well established.

Table III summarizes the data on the effect of unrestricted, CO, and BF diets on the phospholipid phosphorus contents of red blood cells determined in the third experiment. The observed values range from 12.5 ± 0.4–12.7 ± 0.4 mg/100 ml red cells. It is of interest that Reed et al. (13), and Farquhar (31) have recently reported values for red blood cell phospholipid phosphorus of 13.7 + 0.7 and 11.9 ± 0.8 mg/100 ml red blood cells, respectively.

Detailed analysis of the fatty acid composition of the individual phospholipid classes (11) permitted us to estimate that the average mol wt for the group was 766, and that the average phosphorus content was 4.03%. This value is in good agreement with classical estimates, although somewhat higher than that recently reported by Reed et al. (13). Based on this value, the total phospholipid content of the red cells in these experiments ranged from 310 ± 10–315 ± 10 mg/100 ml red cells. The absence of variation between subjects, and groups of subjects on different diets, indicates that marked variations in dietary fat intake had no discernible effect on the total red cell phospholipid concentration, in which respect these observations closely paralleled those noted for cholesterol.

In the fourth experiment (Table IV), the total red cell lipid, the proportions of the various red cell lipid classes, and the plasma cholesterol levels were determined under several different dietary regimens. The total lipid of the erythrocyte averaged 1.68% of the dry wt of the cell, and in all cases consisted principally (over 80%) of unesterified cholesterol and phospholipid. In agreement with Reed et al. (13), cholesterol esters and glycerides represented only 3–5% of the total lipid, and an uncharacterized fraction, presumably glycolipid (32), accounted for the remaining 9–14%. Noting the constancy of the percentage composition of the various lipid components (Table IV), and recalling the previous data with respect to the constancy of red cell cholesterol and phospholipid content, it becomes apparent that extreme variations in dietary fat intake have, at least within 8–16 days, no significant effect on the total amount of any of the red blood cell lipid classes.

Combining the results reported above on the cholesterol and phospholipid content of the red blood cell

TABLE IV

Lipid Composition of Red Blood Cells of Human Subjects Following Eight Days on Various Dietary Regimens (results expressed as % of total lipid)

	Dietary regimen			
	Basal	CO	BF	BFS[a]
Free cholesterol	22.2	23.0	21.6	21.8
Phospholipid	61.1	63.0	61.0	61.2
Cholesterol esters	0.2	1.5	1.4	1.3
Glycerides	2.5	2.8	3.1	2.8
Other	14.0	9.7	12.9	12.9
Plasma cholesterol, mg/ 100 ml	146	123	186	151

[a] BFS = BF + 1000 mg sitosterol/950 calories.

with the data provided by de Bernard (33) on the area occupied by cholesterol and phospholipid molecules in tightly packed films, it is possible to calculate that the area occupied by all the sterol and phospholipid from one human red cell would be 219 square microns.[2] However, cholesterol and phospolipid account for only 84% of the total red cell lipid. Using the simplifying assumption that the remaining 16% of the lipid would occupy 16% more space, it can be shown that the min area required to accommodate all of the lipid from one red cell would be 262 square microns. Houchin et al. (34) have shown that the total surface area of the red cell of man is 134 square microns, and from this it can be calculated that the amount of lipid found represents 97% of the amount required for two complete layers.

In view of this close fit for the red blood cell lipids, and the evidence available from other fields (1), it is reasonable to conclude that the red cell membrane does in fact include a bimolecular layer of lipid. Furthermore, the presence of a constant and specific amount of lipid implies that the function of this lipid is primarily structural, and is essential for the maintenance of the integrity of the cell.

Thus it is reasonable to relate the shortened life span of red cells in sickle cell anemia and untreated pernicious anemia (35) to the fact that in both of these diseases red blood cell cholesterol levels are abnormally high (10). Similarly the increased red cell fragility, and the increased reticulocyte count (and by implication the shortened life span of the red cells) observed in rats following treatment with Triparanol can be related to the abnormally low red cell cholesterol levels reported (36). Simple and short term exchange of red blood cell lipids resulting in no net gain or loss in any one component would appear to be of little physiological significance. However, a change in the total amount of at least one lipid, i.e., cholesterol, has to date invairably been associated with changes in red cell fragility or life span. Long term, or possibly permanent change in the conen of one fatty acid, even though compensated for by

[2] Cell counts, made with a Coulter electronic cell counter, revealed an average of 12.1×10^9 red cells/ml in samples prepared as described above. Using experimentally determined values, and common constants, the following calculations were made:

Cholesterol (mol wt 387), mg/ml = 1.36 (experimental)

Number of cholesterol molecules/cell $= \frac{1.36}{12.1 \times 10^9} \times \frac{1}{387 \times 10^3} \times 6.06 \times 10^{23}$
$= 1.76 \times 10^8$

Phospholipid (mol wt 766), mg/ml = 3.10 (experimental)

Number of phospholipid molecules/ cell $= \frac{3.10}{12.1 \times 10^9} \times \frac{1}{766 \times 10^3} \times 6.06 \times 10^{23}$
$= 2.03 \times 10^8$

Mol fraction phospholipid $= \frac{2.03}{1.76 + 2.03}$
$= 0.53$

Mean area occupied/molecule of phospholipid and cholesterol = 58×10^{-8} square microns (Vandenheuvel)

Total number of molecules of phospholipid and cholesterol/cell $= (1.76 + 2.03) \times 10^8$
$= 3.79 \times 10^8$

Total area occupied by phospholipid and cholesterol from one cell $= 3.79 \times 10^8 \times 58 \times 10^{-8}$
$= 219$ square microns

replacement with another fatty acid, may have an equally important effect on the physiological function of the cell. This possibility obviously justifies further investigation.

ACKNOWLEDGMENT

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REFERENCES

1. Robertson, J. D., Progr. Biophys. Chem. *10*, 343 (1960).
2. Davson, H., Circulation *26*, 1022 (1962).
3. Prankerd, T. A. J., "The Red Blood Cell," Chas. C Thomas, Springfield, Ill., 1961.
4. Gorter, E., and F. Grendel, J. Exptl. Med. *41*, 439 (1925).
5. Ponder, E., "Hemolysis and Related Phenomena," Grune and Stratton, New York, 1948.
6. Hagerman, J. S., and R. G. Gould, Proc. Soc. Exptl. Biol. Med. *78*, 329 (1951).
7. Reed, C. F., J. Clin. Invest. *38*, 1032 (1959).
8. Horwitt, M. K., G. C. Harvey and B. Century, Science *130*, 917 (1959).
9. Leibetseder, F., and E. H. Ahrens, Jr., Brit. J. Haematology *5*, 356 (1959).
10. Fels, G., E. Kanabrocki and E. Kaplan, Clin. Chem. *7*, 16 (1961).
11. Hill. J. G., in preparation.
12. Chaplin, H., and P. L. Mollison, Blood *7*, 1227 (1952).
13. Reed, C. F., S. N. Swisher, G. V. Marinetti and E. G. Eden, J. Lab. Clin. Med. *56*, 281 (1960).
14. Lovelock, J. E., Biochem. J. *60*, 692 (1955).
15. Folch, J., M Lees and G. H. S. Stanley, J. Biol. Chem. *226*, 497 (1957).
16. Entenman, C., JAOCS *38*, 534 (1961).
17. Hirsch, J., and E. H. Ahrens, Jr., J. Biol. Chem. *233*, 213 (1958).
18. Abel, L. L., B. B. Levy, B. B. Brodie and F. E. Kendall, *Ibid.* *195*, 357 (1962).
19. Hurst, R. O., Can. J. Biochem. Physiol. *36*, 1251 (1958).
20. Beveridge, J. M. R., and S. E. Johnson, Canad. J. Res., Sec. E, *27*, 159 (1949).
21. Carlson, L. A., and L. B. Wadstrom, Clin. Chim. Acta. *4*, 1 (1959).
22. Hanahan, D. J., and J. N. Olley, J. Biol. Chem. *231*, 813 (1958).
23. Official and Tentative Methods of Analysis of the A.O.A.C., Sixth Ed., 1945, method 36–3.
24. Schlenk, H., and J. L. Gellerman, Analytical Chem. *32*, 1412 (1960).
25. Buchanan, A. A., Biochem. J. *74*, 25P (1960).
26. Marks, P. A., A. Gellhorn and C. Kidson, J. Biol. Chem. *235*, 2579 (1960).
27. Brun, G. C., "Cholesterol Content of the Red Blood Cells in Man." H. K. Lewis, London, 1939.
28. Mancini, M., and A. Keys, Proc. Soc. Exptl. Biol. Med. *104*, 371 (1960).
29. London, I. M., and H. Schwartz, J. Clin. Investigation *32*, 1248 (1953).
30. "Chemistry of Lipides as Related to Atherosclerosis," ed. I. H. Page, Chas. C Thomas, Springfield, Ill. 1958, p. 130–2.
31. Farquhar, J. W., Biochem. Biophys. Acta *60*, 80 (1962).
32. Klenk, E., and K. Lauenstein, Z. Physiol. Chem. *291*, 249 (1952).
33. de Bernard, L., cited by F. A. Vandenheuvel, Canad. J. Biochem. Physiol. *40*, 1299 (1962).
34. Houchin, D. N., J. I. Munn and B. L. Parnell, Blood *13*, 1185 (1958).
35. Berlin, N. I., T. A Waldmann and S. M. Weissman, Physiol. Rev. *39*, 577 (1959).
36. Blohm, T. R., T. Kariya and M. W. Laughlin, Arch. Biochem. Biophys. *82*, 250 (1959).

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Preparation of 9,15-Octadecadienoate Isomers[1]

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Abstract

Linolenic acid was reduced with hydrazine to produce a mixture containing a max of dienoic acids. After methylation this mixture was separated into trienoic, dienoic, monoenoic, and saturated esters by countercurrent distribution (CCD) with acetonitrile and hexane. The dienoic ester was further fractionated by CCD with methanolic silver nitrate and hexane to separate pure *cis,cis*-9,15-octadecadienoate and the equimixture of *cis,cis*-9,12- and 12,15-octadecadienoates.

Following isomerization of the *cis,cis*-9,15-octadecadienoate with selenium, the geometric isomers were fractionated by CCD with methanolic silver nitrate and hexane. Pure *trans,trans* and pure *cis,cis* isomers were isolated, as well as an unresolved mixture of *cis,trans* and *trans,cis* isomers. The characteristics of these isomers and related compounds are compared as determined by CCD, IR absorption, and capillary gas-liquid chromatography (GLC).

Introduction

THE HYDRAZINE REDUCTION of linolenic acid produces seven fatty acids of which *cis,cis*-9,15-octadecadienoic acid is one (6). In this comparatively simple reaction neither *trans* isomers nor positional isomers are formed as they are in a catalytic reduction (4,7). If the reduction is stopped at max concn of dienoic acids, the *cis,cis*-9,15-octadecadienoic acid is one-third of the dienoic acids or ca. 16%. After methylation the dienoic esters can be fractionated from saturates, monoenes, and trienes by CCD in an acetonitrile-hexane system (6,8). Subsequently, by using an argentation system of hexane and 0.2*N* silver nitrite in 90% methanol, the 9,15-dienoate can be separated by CCD from the 9,12 and 12,15 isomers. This latter system separates compounds by degree of unsaturation, by geometric configuration of double bonds, and by the number of methylene groups between double bonds for polyunsaturated esters (5). In the present work, the preparation of pure *cis,cis*-9,15-octadecadienoate is described, together with its isomerization by selenium to produce geometric isomers. This mixture of methyl esters is separated by using CCD with the argentation system to give pure *trans,trans* and *cis,cis*-9,15-octadecadienoates, as well as an unresolved mixture of mono *trans* isomers (*cis, trans* and *trans, cis*) 9,15-octadecadienoates.

Experimental

Hydrazine Reduction. A mixture of 87.3% linolenic, 9.8% linoleic, and 2.9% oleic acids obtained from a urea crystallization of linseed oil fatty acids was reduced as follows: to 204 g was added 2 liters 95% ethanol; the solution was warmed to 50C before adding 174 ml hydrazine hydrate. Compressed air was bubbled through the mixture to provide stirring and the necessary oxygen (6). The reaction was stopped after 6 hr and 20 min by shutting off air flow and adding 2.4 liters dilute HCl (1:5). The sample was extracted with pentane-hexane, washed, and dried. After solvent evaporation, the sample was esterified with 1,040 ml methanol and 2 ml H_2SO_4. Methyl esters were extracted with pentane-hexane after refluxing for 7.5 hr, washed, dried, and distilled under vacuum to yield 183.3 g of the following composition: 21.3% triene, 47.3% diene, 25.8% monoene, and 5.6% stearate.

Countercurrent Distribution Procedure. A 200-tube automatic CCD apparatus in which each tube contained 40 ml of lower solvent layer was used. The distributions were made according to the single-

[1] Presented at the AOCS meeting in Minneapolis, 1963.
[2] A laboratory of the No. Utiliz. Res. and Dev. Div., ARS, USDA.