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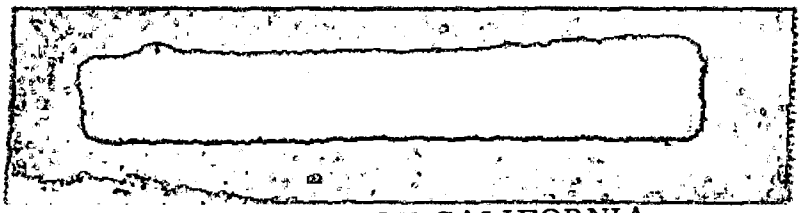

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ABSTRACT

Dialysis of three major lipoprotein fractions from human serum, S_f 20-10^5, S_f 0-20, and -$S_{1.20}$ 0-16 against low concentrations of cupric ion is associated with accelerated chemical alteration of polyunsaturated fatty acids and cholesterol. Less extensive changes in these acids were observed during dialysis against ion-free water. Reaction products of these chemical alterations were irreversibly retained on succinic acid-diethylene glycol-polyester gas-chromatographic columns. Present evidence suggests that these reaction products are relatively polar compounds probably in the chemical class of lipoperoxides.

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INTRODUCTION

During dialysis of serum lipoproteins against cupric ion, Ray and co-workers observed marked changes in analytic ultracentrifugal patterns.[1] In these studies cupric ion was established as a catalyst for reactions leading to lipoprotein degradation. It was suggested that this reaction was oxidative in nature and that unsaturated compounds, such as unsaturated fatty acids, were involved. Degradative effects have also been observed during isolation and storage of lipoprotein fractions.[2] Gurd has associated such degradative or aging effects in lipoprotein solutions with shifts in ultraviolet absorption spectra that are indicative of lipoperoxide formation.[3]

Gas chromatographic techniques have provided a sensitive method for the determination of biologic fatty acids. In this report we have applied these techniques to the measurement of fatty acids from lipoprotein fractions exposed to specific dialysis conditions. It was of especial interest to establish which fatty acids are involved in the cupric ion reaction and what their elution properties are on a succinic acid - diethylene glycol polyester chromatographic column before and after dialysis. The relationship of the fatty acid changes to the gross macromolecular degradation is discussed.

METHODS

Ultracentrifugal Isolation and Analysis of Lipoprotein Fractions. Previously reported ultracentrifugal flotation techniques were used for the isolation and analysis of three lipoprotein fractions from human serum.[4] The specific lipoprotein fractions isolated were the S_f 20-10^5, S_f 0-20, and -$S_{1.20}$0-16. These ultracentrifugal designations and their relationship to the physical properties of these lipoprotein fractions have been reported in previous communications.[5] Two individual sera were used in this investigation. Serum from a nonfasting 52-year-old male subject was used for the isolation of S_f 20-10^5 lipoproteins. The S_f 0-20 and -$S_{1.20}$0-16 lipoproteins were isolated from a nonfasting 22-year-old female subject. No precautions against the presence of metal ions were taken during the ultracentrifugal isolation procedures. Upon isolation the lipoprotein fractions were immediately subjected to dialysis.

Dialysis Procedures. Lipoprotein fractions were dialysed in Visking cellulose casing (18/32 inch diameter). The tubing was previously washed and tested with ion-free water. Ion-free water was prepared by passing laboratory distilled water through the following large capacity ion-exchange colums: first, Dowex-50, then, Dowex-1, and, finally, an equal part mixture of Dowex-50 and Dowex-1. Water prepared by this procedure was considered ion-free and was used in the preparation of cupric ion solutions for dialysis.

The lipoprotein fractions were dialysed against ion-free water and ion-free water containing approximately 1.5×10^{-5} moles/l of cupric ion. In the dialysis procedure 1 ml of lipoprotein solution was dialysed against approximately 200 ml of solution. A small amount of air was present in the dialysis bag. Dialysis was carried out on a gently rocking platform in a refrigerator maintained between 0 - 4°C. Dialysis solutions were routinely

changed approximately every 12 hours. Upon sampling, the lipoprotein fractions were carefully removed from the dialysis tubing, the tubing was washed with a small amount of ion-free water, and this wash was pooled together with the lipoprotein solution for further extraction procedures.

The schedule for sampling of the S_f 20-10^5 lipoprotein fraction was as follows: 0, 25.5, 29.25, 44.0, and 138.75 hours after beginning of dialysis. Both S_f 0-20 and -$S_{1.20}$ 0-16 fractions were sampled at: 0, 11.0, 39.25, 56.5, and 128.75 hours after dialysis. Notation was made of the physical appearance of the lipoprotein fractions throughout the course of the dialysis.

Lipid Procedures. Lipids from control and dialysed lipoprotein fractions were extracted by procedures based on the method of Sperry.[6] Lipid extracts were methylated by transesterification with methanol according to the method of Stoffel.[7] Cholesterol present in the lipid extract was not removed from the fatty acid methyl esters prior to their injection into the chromatographic column.

Gas Chromatographic Procedures. Gas chromatographic analyses were performed on a 52-inch column (6 mm. inner diameter) unit reported by Upham.[8] Analyses were made at 195°C utilizing a strontium-90 ionization detection system.[9] Packing material consisted of Chromosorb (48-65 mesh) coated (30% wt/wt) with succinic acid-diethylene glycol polyester (LAC-2R-728 .)[1] Argon was used as the carrier gas. Peak heights and elution time values were tabulated for every fatty acid ester peak on the chromatograms. This information was put on punched cards and the calculation of fatty acid composition was performed by computer.[10] The known major fatty acids are reported according to the nomenclature proposed by Dole.[11] Minor components have been grouped into four separate classes. The classification

[1] Cambridge Industries Company Inc., 101 Potter Street, Cambridge 42, Mass.

of these components is based on their elution times relative to known methyl esters under the above conditions. Thus, these four classes have been designated: class A, methyl esters eluting before 16:0 (methyl palmitate); class B, methyl esters eluting between 16:0 and 18:0 (methyl stearate); class C, methyl esters eluting between 18:2 (methyl linoleate) and 20:4 (methyl arachidonate); and class D, methyl esters eluting after 20:4. In this report the identified components are: 16:0 (methyl palmitate), 16:1 (methyl palmitoleate), 18:0 (methyl stearate), 18:1 (methyl oleate and methyl elaidate), 18:2 (methyl linoleate), and 20:4 (methyl arachidonate). A reference chromatogram indicating the above classification system is presented in Fig. 1.

RESULTS AND DISCUSSION

Physical Changes in Lipoproteins during Dialysis. After 25.5 hours of dialysis against ion-free water lipoproteins of the S_f 20-10^5 fraction showed only a very slight turbidity. There was no further detectable aggregation or color change during the dialysis. Upon dialysis against cupric ion there was a slight increase in turbidity and loss of color (yellow to white) after 25.5 hours. At approximately 29.25 hours a gross turbidity with marked aggregation was observed. The turbidity and loss of color persisted to termination of sampling.

Dialysis of the S_f 0-20 lipoprotein fraction resulted in slight turbidity and color change at 56.5 hr, followed by marked aggregation at approximately 69.5 hr. Upon dialysis against cupric ion, gross turbidity and loss of color were observed at approximately 11 hr. Considerable aggregation and color loss were observed throughout the remainder of the experiment.

During dialysis against ion-free water, the -$S_{1.20}$ 0-16 lipoprotein solution remained clear and showed little color change throughout the sampling period. Upon dialysis against cupric ion slight turbidity and color loss were

observed within 11 hr. Marked turbidity was noted around 39.25 hr. Turbidity and color changes persisted to the end of the experiment.

The above observations show a definite similarity between S_f 20-10^5 and $-S_{1.20}$ 0-16 lipoproteins in their responses to cupric ion and ion-free water dialysis. On the other hand, the physical changes in S_f 0-20 lipoprotein solutions occurred sooner during cupric ion dialysis than was observed with the other lipoprotein fractions. Moreover, dialysis of S_f 0-20 lipoproteins against ion-free water led to gross physical changes absent in the other fractions under the same conditions. These observations suggest that the S_f 0-20 lipoproteins are more sensitive to dialysis procedures.

Analytic Ultracentrifugal Results. Appropriate analytic ultracentrifugal analyses were performed on the S_f20-10^5, S_f 0-20, and $-S_{1.20}$ 0-16 lipoprotein fractions at 47.5, 40.25, and 40.25 hr respectively, after cupric ion dialysis. At the above times all of these fractions were markedly turbid. In all three cases the ultracentrifugal patterns showed no detectable flotation of intact lipoproteins. The aggregated material was rapidly packed at the meniscus during the acceleration of the ultracentrifuge to full speed (52, 640 rpm). It is thus apparent that the cupric ion dialysis leads to gross lipoprotein aggregation which is not reversed by suspension in aqueous solutions of sodium bromide. In this experiment no sedimenting material was noted in the lipoprotein solutions under analytic ultracentrifugation.

Gas Chromatographic Analyses. The results of the gas chromatographic analyses are presented in Tables I-III. Fatty acid methyl esters are tabulated for lipoprotein fractions exposed to the following conditions: (1) no dialysis, (2) dialysis against ion-free water, and (3) dialysis against 1.5×10^{-5} moles/l. cupric ion solution. The content of the various fatty acid methyl esters is expressed as weight per cent of the total methyl esters injected onto the column.

Significant changes occur in the fatty acid composition of each lipoprotein fraction after dialysis against cupric ion. Dialysis against ion-free water is associated with some alterations in composition but considerably less than are observed with cupric ion. In particular, during dialysis against cupric ion, significant decreases in the polyunsaturated fatty acids, linoleate and arachidonate, are observed. The content of oleic acid is reduced in all fractions and shows a pronounced drop in the S_f 0-20 lipoprotein fraction. In these gas chromatograms no new major peaks appear which might indicate the elution of chemical products arising from the alteration of the polyunsaturated fatty acids. On the contrary, there is evidence that the products of polyunsaturated fatty acids alteration are irreversibly retained on the succinic acid-diethylene glycol polyester coating. This evidence is presented at the bottom of Tables I - III, where for the same weight of methylated lipid injected onto the column there is a significant reduction, especially after cupric ion dialysis, in the total fatty acid methyl esters detected with the chromatographic analysis. Such differences are also observed for the lipoprotein fractions dialysed against ion-free water, but to a much lesser extent than in the cupric ion dialysis.

At the present time our knowledge of the chemical structure and properties of the reaction products is only indirect. They are apparently retained on the relatively polar succinic acid-diethylene glycol polyester resin in a manner similar to other relatively polar organic compounds which we have studied. Thus, on this resin, certain lipid and steroid compounds containing hydroxyl or ketone groups (e.g., dihydroxy stearic acid and cholesten -3-one) have exhibited either exceedingly long retention times or were not eluted at all.[2] This similarity in elution behavior leads us to suspect that polar oxygen-containing compounds may be formed during cupric ion dialysis. Published studies on copper catalysed oxidation of linoleic acid indicate the formation of hydroperoxides resulting from the action of copper on peroxides present in the acid.[12] If hydroperoxides are being formed during lipoprotein dialysis against cupric ion, then, from the above, it would be reasonable to expect that such compounds would be retained either irreversibly or appreciably longer than known unaltered fatty acids. It is, however, entirely possible that the new compounds may not be hydroperoxides but are some other altered forms of polyunsaturated fatty acids which can be retained on the succinic acid polyester resin.

In gas chromatographic analyses of methylated extracts of serum lipids we have noted peaks associated with cholesterol which elute at approximately six stearate times.[13] During the course of cupric ion dialysis these peaks fall significantly indicating a removal of some cholesterol by the chromatographic system. Again, it is reasonable to suspect that polar products of cholesterol are being formed which are irreversibly retained on the succinic acid polyester column.

The marked aggregation of the lipoprotein fractions upon cupric ion dialysis implies an alteration in the surface properties of the lipoprotein macromolecules associated with chemical changes in polyunsaturated fatty acids and cholesterol. A similar phenomenon, under oxidative conditions, has been observed during the fixation of lipoprotein molecules by acid solutions of osmium tetroxide for electron microscopy.[14] In this case the lipoprotein aggregates are redispersed by adjusting the pH of the solution to approximately 8. The ability to redisperse these aggregates and the general agreement between the resulting electron micrograph data on lipoprotein size and shape, and ultracentrifugal or light scattering measurements for the same variables suggest that oxidative changes primarily alter the surface properties of lipoproteins without significantly disrupting their macromolecular structure. This may also be the case with lipoprotein molecules after dialysis against cupric ion. The influence of pH and ionic strength on the lipoprotein aggregates from cupric ion dialysis will be reported in a future communication.

Recent investigations in animals on the pharmacologic action of fatty acid oxidation products indicate considerable alterations in metabolism and physiologic status after administration of these products.[15] The incorporation of these products into lipoprotein structures may so alter the surface properties, as was noted above, that the usual metabolic reactions involving lipoprotein macromolecules may be significantly affected. Thus, it may be of considerable importance to ascertain whether in states on high plasma copper or in instances of continual ingestion of oxidized lipids there appear discernible alterations in lipoprotein stability and metabolism.[16]

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[2] Unpublished experiments.

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Table I. The percentage fatty acid composition of S_f 20-10^5 lipoprotein fraction as a function of conditions and duration of dialysis

Fatty Acids	Lipoprotein Fatty Acid Composition							
	After no dialysis*		After dialysis against ion-free water		After dialysis against 1.5×10^{-5} moles/l. Cu^{++}			
	0 hr.	138.75 hr.	25.5 hr.	138.75 hr.	25.5 hr.	29.25 hr.	44.0 hr.	138.75 hr.
Class A	4.0	3.5	3.8	3.0	3.6	2.8	3.1	4.4
16:0	25.7	26.6	25.6	24.1	24.4	23.0	24.4	24.5
16:1	6.8	7.1	7.1	6.2	6.5	6.0	5.9	5.8
Class B	0.5	0.5	0.5	0.6	0.6	0.5	0.5	1.1
18:0	6.4	6.3	6.1	6.2	6.0	5.7	5.9	6.0
18:1	30.6	31.4	29.9	29.2	29.3	27.5	27.7	25.2
18:2	18.3	18.8	18.8	16.2	15.3	14.1	9.6	3.0
Class C	4.7	3.5	3.6	3.3	3.3	2.5	2.4	2.4
20:4	2.9	2.8	2.8	2.2	1.3	1.0	0.6	0.4
Class D								
Altered fatty acids irreversibly retained on column†	0	0	2.2	9.3	9.5	17.2	20.0	27.5

*Nondialysed control samples were stored at 0-4°C prior to extraction.

†Values for the percentage of altered fatty acids present were estimated from a comparison of the injected weight of methylated lipid sample with the amount detected on its gas chromatogram. It was assumed that in the control samples effectively all of the injected methyl esters were eluted.

Table II. The percentage fatty acid composition of S_f 0-20 lipoprotein fraction as a function of conditions and duration of dialysis

Fatty Acids	Lipoprotein Fatty Acid Composition					
	After no dialysis	After dialysis against ion-free water		After dialysis against 1.5×10^{-5} moles/l. Cu^{++}		
	0 hr.	11 hr.	128.75 hr.	11 hr.	56.5 hr.	128.75 hr.
Class A	1.4	1.2	1.5	1.3	4.0	3.1
16:0	18.6	15.7	17.3	16.1	21.5	17.1
16:1	3.2	2.8	3.1	2.8	3.7	1.9
Class B	1.1	0.9	1.1	1.0	2.9	2.7
18:0	6.1	5.0	5.4	5.1	6.8	5.6
18:1	21.5	19.9	19.6	19.4	18.5	7.0
18:2	36.9	30.8	29.8	17.9	2.1	0.5
Class C	3.0	2.4	2.5	1.7	2.3	2.6
20:4	6.2	4.7	3.2	1.3		
Class D	2.2	1.4	1.2	0.8	1.7	1.2
Altered fatty acids irreversibly retained on column*	0	15.3	15.3	32.8	36.5	58.3

*Values for the percentage of altered fatty acids present were estimated from a comparison of the injected weight of methylated lipid sample with the amount detected on its gas chromatogram. It was assumed that in the control samples effectively all of the injected methyl esters were eluted.

Table III. The percentage fatty acid composition of $-S_{1.20}$ 0-16 lipoprotein fraction as a function of conditions and duration of dialysis

Fatty Acids	Lipoprotein Fatty Acid Composition						
	After no dialysis	After dialysis against Ion-free water		After dialysis against 1.5×10^{-5} moles/l. Cu^{++}			
	0 hr	11 hr.	128.75 hr.	11 hr.	39.25 hr.	56.5 hr.	128.75 hr.
Class A	1.7	1.7	1.5	1.4	2.4	1.8	1.5
16:0	20.6	19.8	16.9	17.6	21.1	17.8	18.9
16:1	2.8	2.7	2.4	2.6	2.7	2.5	2.0
Class B	1.2	0.8	1.0	1.1	2.3	2.2	1.7
18:0	8.3	7.8	6.9	7.8	7.9	6.9	7.8
18:1	17.8	18.3	14.8	15.8	13.8	11.0	11.4
18:2	33.9	33.6	25.5	26.0	1.7	1.0	0.7
Class C	4.4	3.4	2.5	2.1	1.3	1.5	1.7
20:4	9.4	9.3	5.3	5.2			
Class D		2.0	1.5	0.7	1.1	1.0	1.1
Altered fatty acids irreversibly retained on column*	0	0.8	21.7	20.0	45.6	54.2	53.3

*Values for the percentage of altered fatty acids present were estimated from a comparison of the injected weight of methylated lipid sample with the amount detected on its gas chromatogram. It was assumed that in the control samples effectively all of the injected methyl esters were eluted.

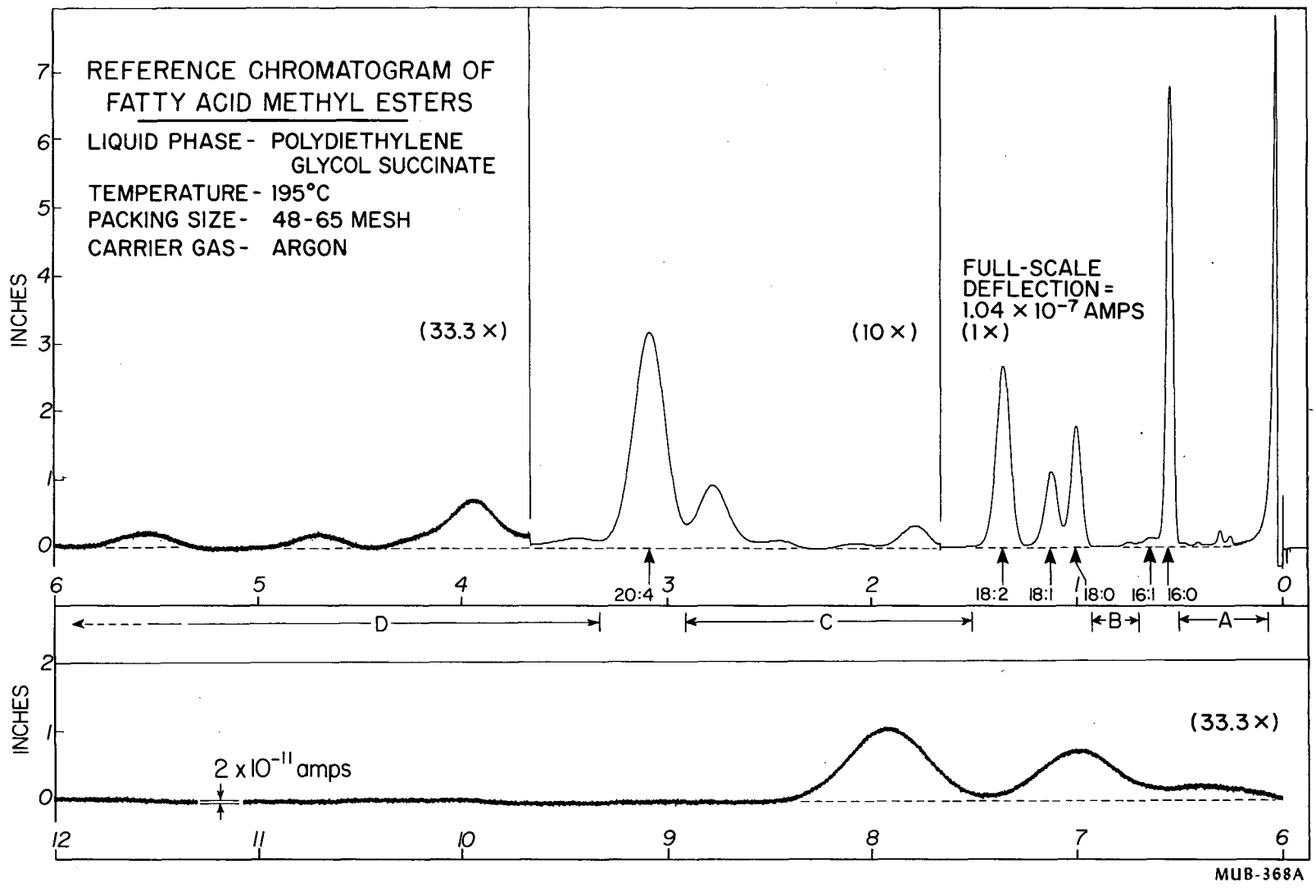

Fig. 1. Reference chromatogram of fatty acid methyl esters showing elution properties of known major components. Relatively minor components are grouped into four classes A, B, C, and D as indicated on the elution time scale. The elution time scale is marked off in units of methyl stearate elution times.

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