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# Interaction of serum lipoproteins with the hydroperoxide of methyl linoleate*

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## SUMMARY

The interaction of serum lipoproteins with the hydroperoxide of methyl linoleate was studied by means of analytical ultracentrifugal or paper electrophoretic analysis. The results indicated that lipohydroperoxide seemed to have a marked effect on the stability of low density or β-lipoproteins. The hydroperoxide of methyl linoleate extensively denatured the isolated low density lipoproteins. An initial or primary association of the hydroperoxide of methyl linoleate with low density lipoproteins was noted prior to its denaturation. Furthermore, the results indicated that the hydroperoxide of methyl linoleate selectively or preferentially denatured β-lipoproteins and not other serum lipoproteins or protein constituents. *In vivo* studies indicated that the hydroperoxide of methyl linoleate inhibited lipid absorption. Although some lipohydroperoxide was absorbed from the intestinal tract when diluted with methyl linoleate, it is not known whether an exogeneous source of lipohydroperoxide can contribute to the *in vivo* denaturation or degradation of β-lipoproteins.

Deposition of lipids in the aorta and its branches has been a dominant factor in the development of atherosclerosis (1, 2, 3). Kayahan (4) has claimed that denatured protein in the intima of atherosclerotic subjects is responsible for an increase in lipid-binding capacity of the aorta. On the other hand, the accumulation of lipid might be caused by an abnormality in lipid metabolism which may or may not eventually cause denaturation of the protein.

A sudanophilic and acid-fast pigment has been demonstrated to occur in variable amounts, frequently in abundant quantities, in the atheromatous lesions in man (5). Burt (6) has claimed that the incidence of acid-fast material or "ceroid" pigment in the vessel is directly proportional to the degree of intimal thickening. Although this material is often intensely sudanophilic, it was not extracted by fat solvents (7). The ceroid pigment was believed to form by autoxidation or peroxidation of unsaturated fat *in vitro* (8). The bound lipid may form *in situ* or may represent abnormal end products of lipoprotein metabolism. It has been shown that atherosclerotic aortas contain lipoperoxides and that the peroxide content parallels the degree of severity of the atherosclerosis, while normal aortas are free from lipoperoxides (9). Recently, *in vitro* nonenzymatic formation of lipoperoxide in heart muscle and liver mitochondria fractions has been reported (10, 11). Furthermore, the presence of lipoperoxides in the liver and muscle of rabbits kept on a vitamin E-deficient diet has been observed by various workers (12, 13). It may be possible that, as a result of certain disturbances of lipid metabolism, lipoperoxides may be formed in the aorta *in vivo* and may denature β-lipoproteins, facilitating the deposition of lipid materials in the aorta.

The present experiments were designed to study the interaction of lipoperoxide with low density or β-lipoproteins and with other serum proteins and lipoproteins. In addition, the extent of absorption of lipoperoxide in lymph-cannulated rats and the effects of intraperitoneal injection on serum protein and lipoprotein constituents were studied in order to determine the origin of lipoperoxide *in vivo*.

## METHOD

The methyl linoleate used in this investigation was prepared from safflower oil by the bromination and debromination method as described by Rollett (14)

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and modified by Frankel and Brown (15). The hydroperoxide of methyl linoleate was prepared at 0° by a modification of the method of Holman and Greenberg (16) and Privett *et al.* (17, 18). Soybean lipoxidase was obtained by the extraction of soybean flour[1] in acetate buffer at pH 4.5. Colloidal linoleate substrate was prepared by emulsification of methyl linoleate in phosphate buffer (pH 7.0) which contained 1% albumin.[2] The hydroperoxide of methyl linoleate was purified by a countercurrent extraction method as described by Privett *et al.* (19); the peroxide value of the separated fraction was 5820 meq per kg. Methyl palmitate was prepared from palmitic acid[3] (20).

Pooled samples of chicken serum or human serum obtained from normal male volunteers were subjected to an initial centrifugation at 40,000 × *g* for 30 minutes to remove coarse material. The solution density of the sera was then adjusted to 1.063 with saline solution, and a 1 ml portion of the low density lipoprotein fraction isolated from every 5 ml of original serum according to the method of Gofman *et al.* (21) at 30,000 rpm in a Spinco Model L centrifuge. Exactly 1.2 ml of the lipoprotein fraction thus obtained and 10 mg of methyl linoleate or the hydroperoxide of methyl linoleate were placed in a Warburg flask and shaken in a Warburg apparatus under nitrogen at 20° for 0, 2, 5, and 10 hours. The lipoprotein fraction was then centrifuged at 12,000 × *g* for 20 minutes and the clear solution subjected to ultracentrifugal analysis in a Spinco Model E ultracentrifuge at a bar angle of 45° and at a speed of 52,640 rpm with an acceleration time of 5 minutes, 30 seconds. The first picture was taken 8 minutes after obtaining maximum speed, and pictures were taken at 8-minute intervals.

In a study on the interaction of lipohydroperoxide with serum protein and lipoprotein constituents, 2.4 ml of human serum and 20 mg of the hydroperoxide of methyl linoleate or fresh methyl linoleate were placed in a Warburg flask and shaken in a Warburg apparatus under nitrogen at 20°. After 0, 2, 4, 6, and 10 hours of shaking, approximately 0.15 ml of the serum was transferred with the aid of a syringe into a polyethylene capillary tube and centrifuged at 12,100 × *g* for 20 minutes. The denatured lipoproteins or lipoprotein-lipohydroperoxide complex and unreacted methyl ester which floated on top were removed by cutting off this portion of the tube. The clear solution which remained was subjected to quantitative electrophoresis with the aid of a Spinco Model R-Series D paper electrophoresis cell and a Heathkit voltage regulated power supply. Veronal buffer, pH 8.6, 0.075 ionic strength, was permitted to wet the paper strips (Schleicher and Schuell 2043 A mgl), and 6 or 20 lambda of sample was applied with the aid of a Spinco sample applicator for the electrophoretic fractionation of serum proteins or of serum lipoproteins, respectively. A current of 5 milliamperes was passed through the papers for 15 hours. The filter papers were stained with bromophenol blue for serum proteins according to the Spinco Procedure B (22), and with Ciba red 7B for serum lipoproteins according to the procedure of Straus and Wurm (23). After staining, the strips were scanned with a Spinco Model RB Analytrol.

In order to observe an initial or primary association of the hydroperoxide of methyl linoleate with low density lipoprotein molecules prior to the denaturation of the lipoproteins, the low density lipoprotein fraction of chick serum was shaken with the hydroperoxide of methyl linoleate for 2 hours under the same condition as employed for the ultracentrifugation studies. After removal of the floating material at 50,000 × *g* for 1 hour, 0.1 ml of clear lipoprotein solution was extracted with 5 ml of absolute alcohol; the proteins and the inorganic salts were removed by filtration; and the spectral absorption of the filtrate was determined with the aid of a Cary Recording Spectrophotometer.

For a study on the absorption of the hydroperoxide of methyl linoleate, weanling rats which had been kept on a regular stock diet (24) for eight months were used. A permanent intestinal lymph fistula was made by a modification of the method of Bollman *et al.* (25, 26). Intramedic polyethylene tubing (PE 50), which had been previously wetted with heparin solution, was inserted into the main intestinal lymphatics under anesthesia with Nembutal® (0.5 ml per kg body weight). The rats were kept in restraining cages and allowed access to physiological saline solution and a fat-free, purified diet which consisted of 65% cerelose, 30% casein, 5% Wesson salts, and 100 mg of a water-soluble vitamin mixture (27) per kg of diet. One ml of test sample was administered with the aid of a stomach tube 24 hours after the operation, under slight anesthesia with ether, and the intestinal lymph was collected every 2 hours for a total period of 12 hours. The lymph was defibrinated and its optical density measured at 650 mμ in a silica cell in a light path of 1 mm. The results were expressed as the optical density multiplied by the weight of lymph collected every 2

[1] Nutrisoy 7 B, obtained through the courtesy of Archer-Daniels-Midland, Minneapolis, Minn.

[2] Crystalline bovine plasma albumin, obtained through the courtesy of Armour and Company, Chicago, Ill.

[3] Eastman, Distillation Products, Rochester, N. Y.

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hours. Furthermore, in order to observe whether intestinal absorption of the hydroperoxide of methyl linoleate had taken place, 0.1 ml of lymph was extracted with 5 ml of absolute alcohol, the precipitates were removed, and the absorption spectrum of the alcohol extract was determined in the ultraviolet region with a Cary Recording Spectrophotometer.

In order to observe the *in vivo* effect of the hydro-

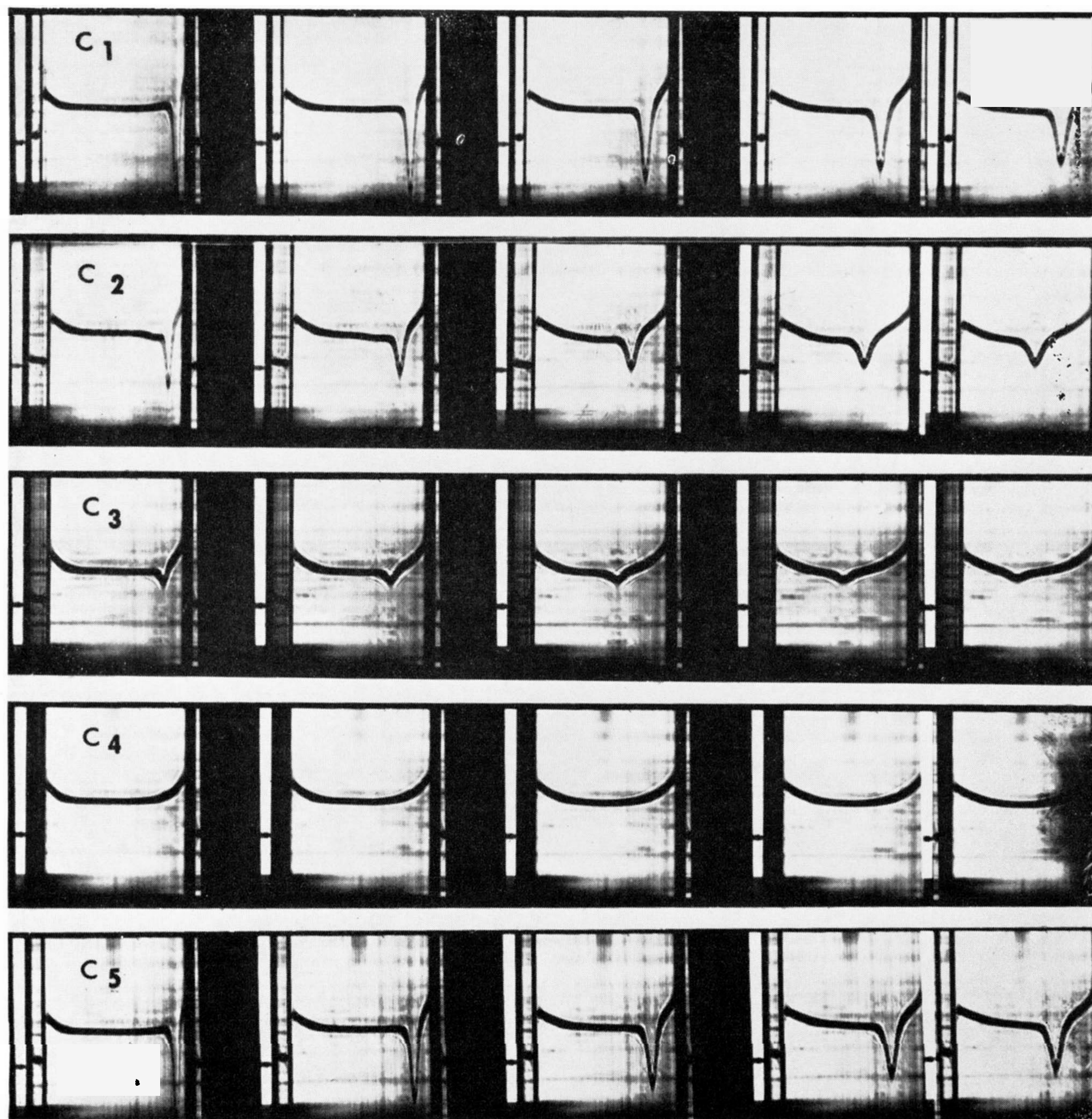


Fig. 1. Progressive changes in the ultracentrifugal flotation pattern of chicken low density lipoproteins after interaction with the hydroperoxide of methyl linoleate. The first series of pictures, labeled $C_1$, represents the flotation patterns of untreated lipoproteins; $C_2$, $C_3$, and $C_4$, the lipoproteins which had been shaken with the hydroperoxide of methyl linoleate for 2, 5, and 10 hours, respectively; and $C_5$, the lipoproteins which had been shaken with methyl linoleate for 10 hours.

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peroxide of methyl linoleate on serum lipoprotein and protein constituents, 1 ml of 10% methyl linoleate, or the hydroperoxide of methyl linoleate in methyl palmitate, was injected intraperitoneally into rats. Approximately 0.2 ml of blood was taken from the tail vein at 0, 6, 12, and 24 hours after the injection, the blood was centrifuged, and 8 or 20 lambda of the serum was applied to the paper for electrophoretic

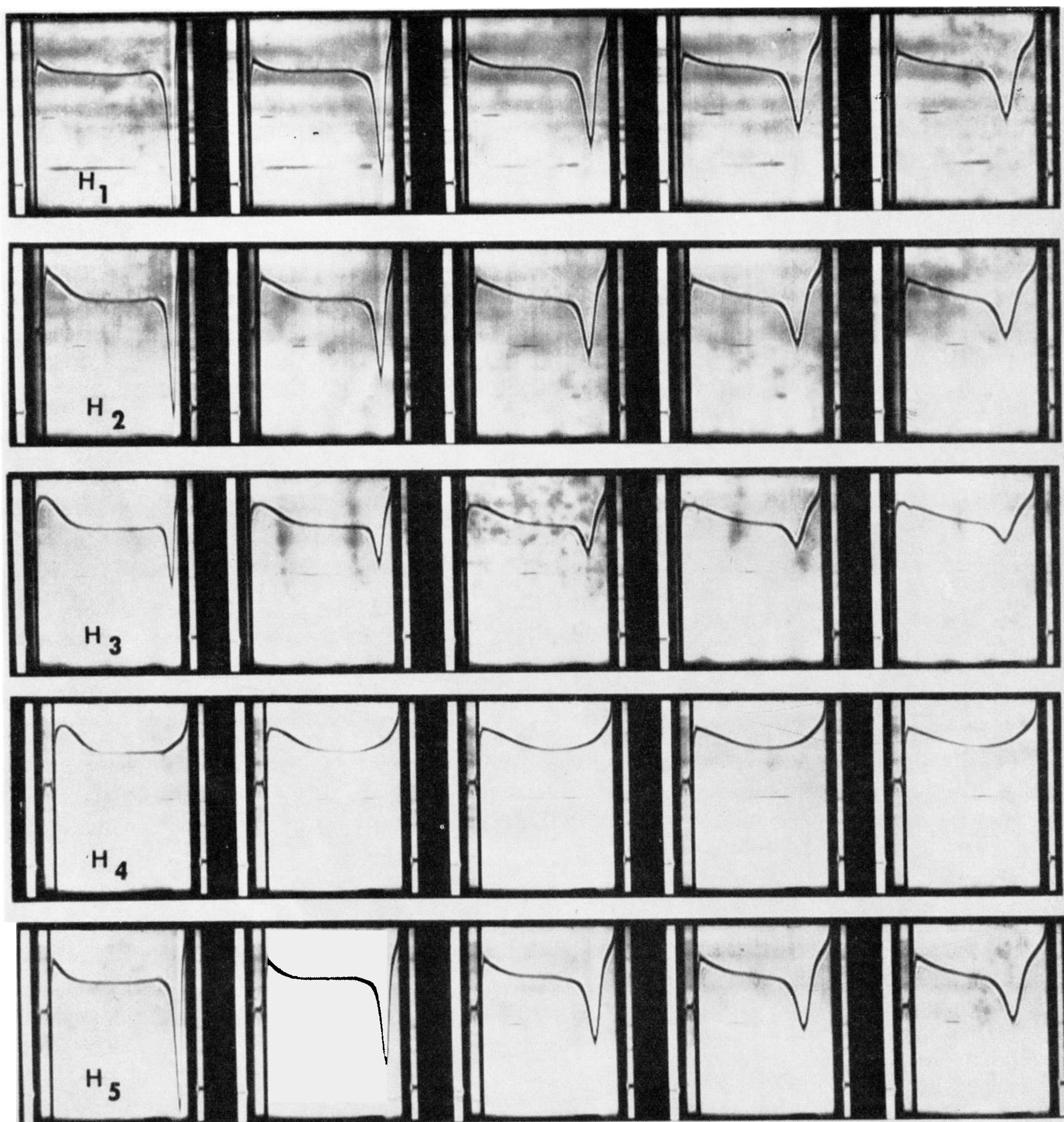


Fig. 2. Progressive changes in the ultracentrifugal flotation patterns of human low density lipoproteins after interaction with the hydroperoxide of methyl linoleate. The first series of pictures, labeled $H_1$, represents the flotation patterns of untreated lipoproteins; $H_2$, $H_3$, and $H_4$, the lipoproteins which had been shaken with the hydroperoxide of methyl linoleate for 2, 5, and 10 hours, respectively; and $H_5$, the lipoproteins which had been shaken with methyl linoleate for 10 hours.

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fractionation of serum proteins or lipoproteins, respectively. The electrophoresis and the staining of the paper strips were performed as described previously.

## RESULTS

The results indicated that the hydroperoxide of methyl linoleate had a marked effect on the stability of low density or β-lipoproteins in chicken or human serum. During the *in vitro* interaction of chicken low density lipoproteins with the hydroperoxide of methyl linoleate, a gradual increase in the flotation rate was noted; and the area under the lipoprotein peak progressively decreased. After 10 hours of exposure to the hydroperoxide of methyl linoleate, the lipoproteins were completely denatured (Fig. 1). However, fresh methyl linoleate did not cause any noticeable change in the flotation pattern of low density lipoproteins. Human low density lipoproteins followed a pattern of denaturation which was similar to chicken lipoproteins, although the increase in the flotation rate in human low density lipoproteins was less pronounced than in chicken low density lipoproteins (Fig. 2).

The results on the *in vitro* interaction of the hydroperoxide of methyl linoleate with human serum indicated that the electrophoretic mobility of β-lipoproteins progressively increased during the interaction (Fig. 3). The concentration of β-lipoproteins which was represented by the area under the peak also decreased, and after 10 hours of interaction approximately 95% of the β-lipoproteins were denatured. However, other lipoproteins (which included lipalbumin, alpha-1, and alpha-2 lipophilic materials) were not subjected to denaturation by the hydroperoxide of methyl linoleate. Although the electrophoretic mobility of the β-lipoproteins in serum which had been shaken with methyl linoleate for 10 hours increased, the degree of increase was less pronounced than for the β-proteins in serum which had been shaken with the hydroperoxide of methyl linoleate; and no significant change in the concentration of β-lipoproteins was observed. Neither the concentration nor the mobility of serum proteins seemed to be affected by the interaction with lipohydroperoxide (Fig. 4). Therefore, these results appeared to indicate that the hydroperoxide of methyl linoleate selectively (or preferentially) denatures only β-lipoproteins.

An initial or primary association of the hydroperoxide of methyl linoleate with low density lipoprotein molecules prior to the denaturation of the lipoproteins was noted. The presence of conjugated diene in the alcohol extract obtained from the lipoprotein fraction which had been shaken with the hydroperoxide of methyl linoleate and freed of denatured lipoproteins and floating materials was shown by absorption in the region of 233 mμ (Fig. 5). The presence of a small amount of carbonyl compounds was also shown by absorption at 265 to 285 mμ. The main absorption at 233 mμ was apparently due to the association of the conjugated dienoic hydroperoxide of methyl linoleate with the low density lipoprotein molecules.

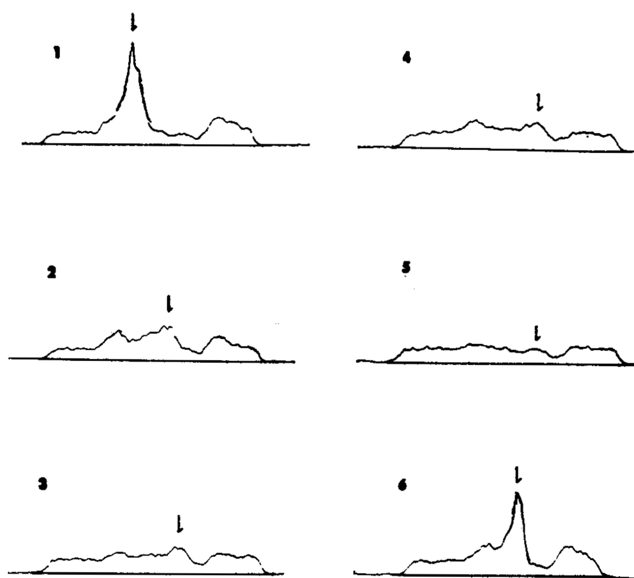


Fig. 3. Progressive changes in the lipid staining pattern as shown on paper electrophoresis of human serum lipoproteins after interaction with the hydroperoxide of methyl linoleate. Picture *1* represents the pattern of normal lipoproteins; pictures *2*, *3*, *4*, and *5*, the lipoprotein patterns after interaction with the hydroperoxide of methyl linoleate for 2, 4, 6, and 10 hours, respectively. Picture *6* represents serum shaken with fresh methyl linoleate for 10 hours.

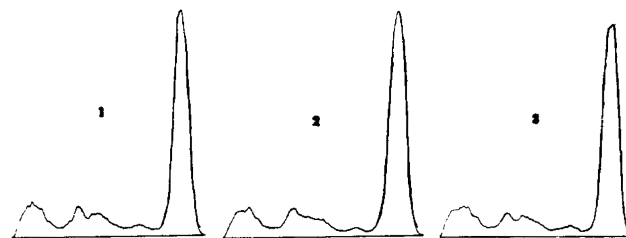


Fig. 4. Paper electrophoretic patterns of human serum proteins before and after interaction with the hydroperoxide of methyl linoleate. Picture *1* represents the protein pattern of normal serum; pictures *2* and *3*, the protein patterns after shaking for 10 hours with the hydroperoxide of methyl linoleate and methyl linoleate, respectively.

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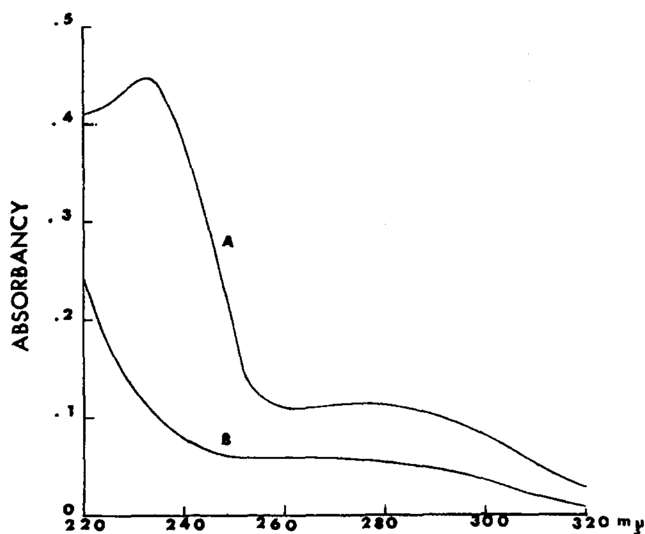


FIG. 5. Spectral absorption. Curve *A* represents the UV absorption spectrum of the alcohol extract of low density lipoproteins shaken with the hydroperoxide of methyl linoleate and freed of denatured lipoproteins and floating material. Curve *B* represents the UV spectrum of the alcohol extract of the original low density lipoprotein solution.

In order to determine the origin of lipohydroperoxide *in vivo* we have conducted a two-phase study: one involved the intestinal absorption of the hydroperoxide of methyl linoleate in lymph-cannulated rats, and the other the intraperitoneal absorption of the hydroperoxide of methyl linoleate in normal rats. The results on the first phase of this study indicated that the hydroperoxide of methyl linoleate inhibited lipid absorption. The lymph obtained from rats 2 to 4 hours after they had been fed methyl linoleate had a value (optical density multiplied by the weight of lymph) of 3.48 (Fig. 6, group *A*), indicating that rapid absorption of methyl linoleate had taken place. In comparison, the lymph from those fed the hydroperoxide of methyl linoleate or methyl linoleate 24 hours after the administration of the hydroperoxide of methyl linoleate had a value of 0.09 and 0.01, respectively (Fig. 6, groups *D* and *E*). These values indicated that less lipid was present in these cases than in the lymph of those given no supplement (Fig. 6, group *C*). When the hydroperoxide of methyl linoleate was diluted with 80% methyl linoleate, the lymph contained more lipid than the lymph from the nonsupplemented group, or values of 0.61 and 0.26, respectively.

The lipid extracted from the lymph obtained from rats given 20% hydroperoxide showed greater absorption at 233 mμ than the lipid from those given the hydroperoxide without any dilution. These results appeared to indicate that hydroperoxides inhibit lipid absorption. However, when diluted with fresh methyl linoleate, lipohydroperoxide seemed to be absorbed.

The intraperitoneal injection of the hydroperoxide of methyl linoleate into rats caused a peritonitis because of its toxic or irritating action and resulted in an accumulation of ascites in the intraperitoneal cavity of the rat. A sufficient quantity of the hydroperoxide to cause a change in the serum lipoprotein pattern did not seem to be absorbed from the intraperitoneal cavity. The marked decrease in the serum albumin fraction of the blood which was taken at 6, 12, and 24 hours after the injection of the hydroperoxide of methyl linoleate as compared to the original serum albumin fraction did not seem to be caused by the direct effect of the hydroperoxide but by the accumulation of ascites in the intraperitoneal cavity of the rat (Fig. 7). These *in vivo* studies indicated that, although some lipohydroperoxides may be absorbed from the intestinal tract, more attention should be paid to the *in vivo* formation of lipohydroperoxides.

## DISCUSSION

The lability of isolated low density lipoproteins has been widely recognized (28). When β-lipoprotein preparations were kept in storage for prolonged periods of time, a series of oxidative changes has been observed (29). The decrease in flotation rate which was caused by prolonged storage was believed to be due to a slight increase in density. Degradative changes in the low density lipoproteins were also noted

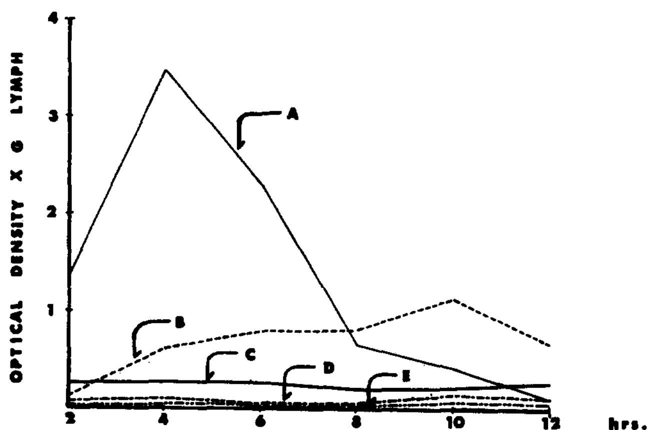


FIG. 6. Effect of administering hydroperoxide of methyl linoleate on intestinal lipid absorption in lymph-cannulated rat. *A*. Methyl linoleate. *B*. 20% hydroperoxide of methyl linoleate in methyl linoleate. *C*. None. *D*. Hydroperoxide of methyl linoleate. *E*. Methyl linoleate 24 hours after the administration of 1 ml hydroperoxide of methyl linoleate.

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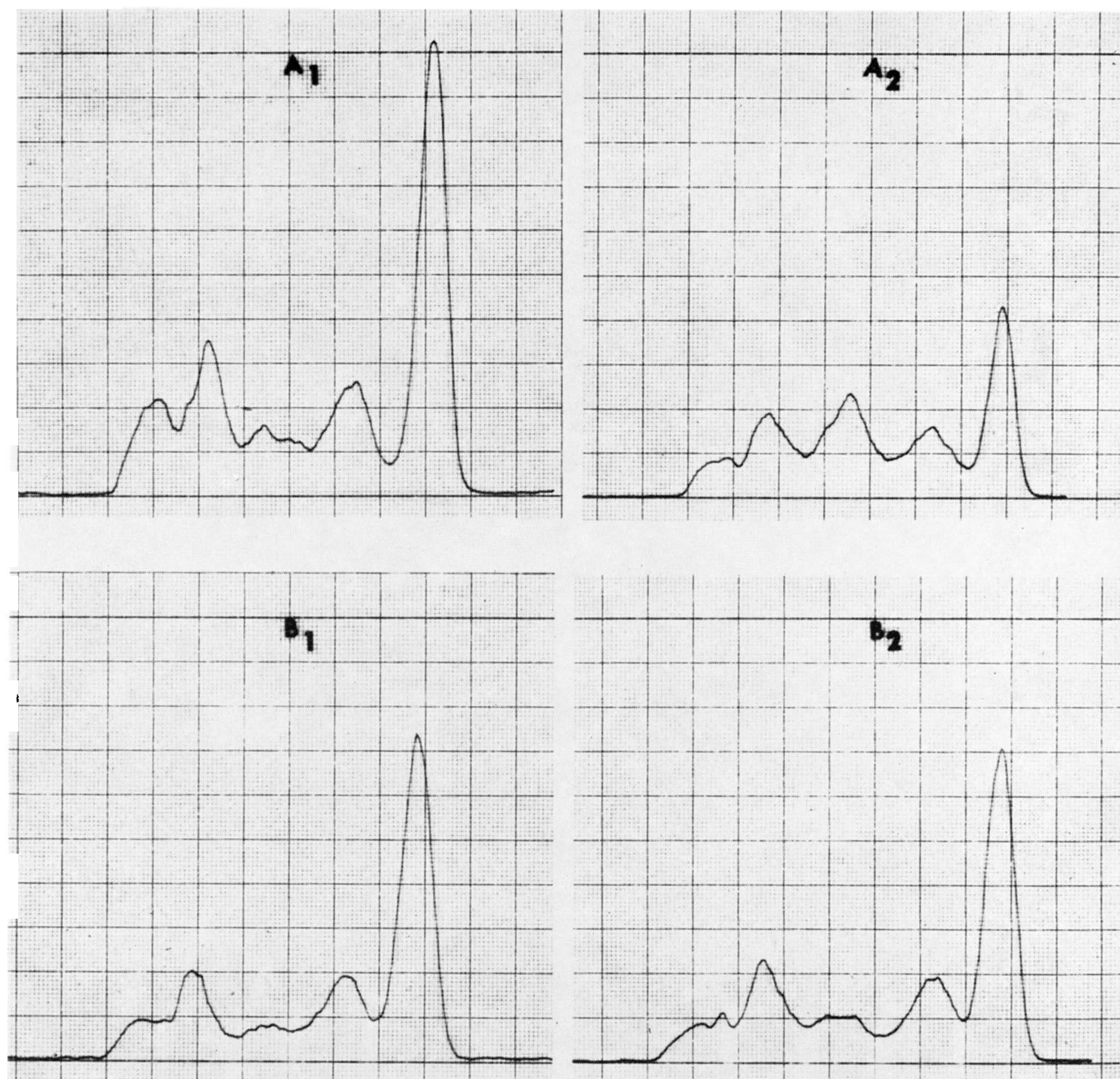


FIG. 7. Paper electrophoretic patterns of rat serum proteins. $A_1$ represents the original serum protein pattern and $A_2$, the protein pattern of blood taken 24 hours after the intraperitoneal injection of 1 ml of 10% hydroperoxide of methyl linoleate in methyl palmitate. $B_1$ and $B_2$ represent the protein patterns before and after the intraperitoneal injection of 1 ml of 10% methyl linoleate in methyl palmitate.

when the lipoproteins of the $S_f$ 5-30 class were dialyzed against buffered saline solutions (30, 31). Degradative changes have been attributed to the oxidation of lipoproteins catalyzed by cupric ion. When all traces of cupric ion were removed from the environment, the lipoproteins were found to be stable upon dialysis.

In the present study it is shown that centrifugally separated low density lipoproteins are stable in the presence of methyl linoleate, but not in the presence of the hydroperoxide of methyl linoleate. As the hydroperoxide of methyl linoleate contained both hydrophilic and lipophilic groups, it may have diffused into the lipoprotein molecules. A gradual oxidative denaturation or degradation of low density lipoprotein molecules by active oxygen in the hydroperoxide may then have occurred. However, it is quite possible that

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after the initial association of the hydroperoxide of methyl linoleate with low density lipoprotein molecules, subsequent decomposition or polymerization of the lipohydroperoxide in the lipohydroperoxide-lipoprotein complex may have then taken place with time. Some of these decomposition products may have facilitated the denaturation of low density lipoproteins or may have combined more firmly with the protein portion of low density lipoproteins than the intact hydroperoxide of methyl linoleate.

The selective or preferential denaturation of β-lipoproteins among serum lipoproteins by interaction with lipohydroperoxide is of interest, as low density or β-lipoproteins are known to be associated with atherosclerosis (32). The marked difference between β-lipoproteins and other lipoproteins in their resistance to the hydroperoxide of methyl linoleate may reflect (*a*) the amount and composition of their lipid components, (*b*) the nature of the bonds between lipids and proteins, or (*c*) their molecular structure. It has been reported that the lipids in low density lipoproteins contain a high concentration of cholesterol esters and triglycerides (33, 34, 35). It may be possible that the active oxygen in the hydroperoxide of methyl linoleate which was incorporated into low density or β-lipoprotein molecules could be transferred into the unsaturated fatty acid portion of lipoproteins by a free-radical chain reaction and could thus facilitate the physical denaturation and chemical degradation of lipoproteins.

The presence of low density or β-lipoproteins in the aorta has been confirmed by various workers (36, 37), and substantial amounts of the $S_f$ 10-100 lipoprotein fraction in the aorta have been reported as evidence of atherosclerosis. Although the present study indicates that the hydroperoxide of methyl linoleate may be absorbed from the intestinal tract when diluted with methyl linoleate, an exogeneous source of lipohydroperoxide may not contribute significantly to the denaturation or degradation of low density lipoproteins *in vivo*. It may be possible that the lipoproteins in the arterial wall undergo oxidative denaturation or degradation by lipohydroperoxide which could be formed *in vivo* by the impairment of normal lipid metabolism, thus initiating plaque formation in the artery. In fact, Glavind *et al.* (9) observed a correlation between the degree of atherosclerosis and the peroxide value of the lipid extracted from the aorta, and it was postulated that the oxidative polymerization of constituents of serum lipoproteins may be the initiating step in atherosclerosis (38). However, in light of the present results, lipohydroperoxide formation, rather than oxidative polymerization, may represent the initiating step in atherosclerosis. It must be emphasized that lipohydroperoxide may not necessarily be formed in quantities which can be detected by chemical means. The formation of very small amounts of lipohydroperoxide in the intra- or intercellular lipids or lipoproteins in endothelial cells may not only cause oxidative denaturation or degradation of lipoproteins but may also inhibit various biological functions in the cells. Oxidative polymerization of lipoproteins in the intima may proceed after the formation of lipohydroperoxides, depending upon the amount of oxygen released in that particular area or the amount of biological catalyst present, such as hematin compounds.

We have previously reported that the substitution of heated oil for fresh oil depressed the serum cholesterol and β-lipoprotein levels, but the incidence of atherosclerosis was at least as high with fresh oil, indicating that the serum cholesterol and β-lipoprotein levels were not necessarily proportional to the degree of atherosclerosis (39). The aggravating effect of heated oil may be partially due to the degradation of lipoproteins by lipoperoxide which might possibly be formed *in vivo* from the oxidation products in heated oil. A number of vitamins, such as A, D, and E, and some water-soluble vitamins, are known to be destroyed by contact with heated oil (40 to 43). Thus the destruction of antioxidants and synergists may cause low density or β-lipoproteins to become less stable toward oxidative degradation or may favor the *in vivo* formation of lipohydroperoxides. A study is in progress in order to clarify the *in vitro* and *in vivo* formation of lipohydroperoxides in relation to atherosclerosis under various conditions.

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