Modification of human high density lipoprotein (HDL_3) with tetranitromethane and the effect on its binding to isolated rat liver plasma membranes

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Abstract Apolipoprotein E-free high density lipoproteins (HDL) bind to various cells and cell membrane preparations, with properties typical of ligand-receptor interactions. In order to further characterize the binding sites and to investigate the functional role of binding, a chemically modified HDL without the specific binding properties would be highly desirable. We have reacted human HDL_3 with tetranitromethane, a relatively specific nitrating reagent for tyrosine residues, in 50 mM Tris HCL buffer, pH 8.0, and at a reagent concentration 10 times the molar excess of tyrosine residues. The resulting nitrated HDL_3 completely lost its ability to bind to high affinity saturable binding sites of rat liver plasma membranes, as determined by competitive binding with ^{125}I-labeled HDL_3, and also by direct binding assays using ^{125}I-labeled nitrated HDL_3. Although nitrated HDL_3 did not bind to the high affinity saturable binding sites, it bound to the membranes, but the binding was not saturable, and was not competed for by unlabeled nitrated HDL_3. On agarose gel electrophoresis, pH 8.6, the nitrated HDL_3 moved ahead of the control HDL_3, indicating an increase in negative charges in the molecule. No difference in size was noted in the nitrated HDL_3 when analyzed either by negative stain electron microscopy or by gel filtration chromatography. Spectroscopic analysis of the nitrated HDL_3 at pH 8.0 revealed a prominent absorption with maximum at around 360 nm, but none in the region expected for nitrotyrosine residues. At pH 10.0, however, the nitrated HDL_3 showed an absorption band with a maximum at around 440 nm, possibly related to nitrotyrosine residues. Nitrotyrosine was detected in the nitrated HDL_3 on amino acid analysis. Comparison of the amino acid analysis of the nitrated HDL_3 and control HDL_3 showed no difference in composition of any of the amino acids except tyrosine; tyrosine content was reduced more than 90% in the nitrated HDL_3. SDS-polyacrylamide gel electrophoresis analysis of apoproteins of nitrated HDL_3 revealed changes in apolipoprotein profile. Bands corresponding to the apolipoproteins of the starting HDL_3 almost disappeared and a series of new bands appeared at the high molecular weight region of the gel, indicating extensive cross-linking of apolipoproteins during the reaction. In addition, a substantial amount of phospholipids and cholesteryl esters, but not unesterified cholesterol, was found covalently linked, possibly through the unsaturated centers of the fatty acid chains, to apolipoproteins. The loss of the binding activity of HDL_3 by the nitration reaction appears to be related to covalent cross-linking of lipids to apolipoproteins and of apolipoproteins themselves. The availability of a modified HDL_3 preparation with no binding ability to the high affinity saturable binding sites may be useful for studies related to the functional aspects of HDL binding sites. —**Chacko, G. K.** Modification of human high density lipoprotein (HDL_3) with tetranitromethane and the effect on its binding to isolated rat liver plasma membranes. *J. Lipid Res.* 1985. **26:** 745–754.

Supplementary key words HDL binding sites • nitration of tyrosine • cross-linking of apolipoproteins and lipids

Although high-affinity saturable binding sites for high density lipoproteins (HDL) have been detected in a variety of cells (1–17) and membrane preparations (18–23), very little information is available on the nature of the binding sites or their physiological function. They have been implicated in the efflux of unesterified cholesterol from cells (24, 25). In addition, the HDL binding sites detected in rat steroidogenic tissues appear to be involved in the delivery of cholesterol to these tissues for the synthesis of steroid hormones (4–6, 13, 20–22). In order to investigate the possible role of the HDL binding in the above proposed functions, a chemically modified HDL preparation without the specific HDL binding properties would be highly desirable. Recent attempts to inhibit the binding of human HDL_3 to rat liver and rat testes plasma membrane preparations by chemical modification of the lysine or the arginine residues of HDL_3 were not successful (26). In the present studies, we have reacted human HDL_3 with tetranitromethane, a presumably specific nitrating reagent for tyrosine residues. The resulting nitrated HDL_3 completely lost its ability to bind to the HDL binding sites of rat liver plasma membranes. The results of these studies plus those on the characterization of the nitrated HDL_3 are given in this report.

Abbreviations: HDL, high density lipoproteins; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

MATERIALS AND METHODS

Materials

Human HDL_3 ($1.125 < d < 1.21$ g/ml) was isolated by differential ultracentrifugation as described (27). It was further processed by heparin-Sepharose affinity chromatography (28) to remove any traces of apolipoprotein E, if present. Apolipoprotein A-I constituted about 90% of human HDL_3 apolipoproteins as determined by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Coomassie blue staining and densitometric scanning (29); the other components were apolipoprotein A-II and C apolipoproteins. Occasionally, traces of albumin were detected. HDL_3 was labeled with ^{125}I using the iodine monochloride procedure (30). The specific activities ranged from 70 to 100 cpm/ng protein. An averge of about 2% of the label was associated with lipids. Fatty acid-free bovine serum albumin (BSA) and 3-nitrotyrosine were from Sigma Chemical Co. (St. Louis, MO). Tetranitromethane was purchased from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were of reagent grade.

Methods

Isolation of membrane fractions. Fischer 344 strain male rats weighing 100–150 g were used. Liver plasma membranes were isolated according to the procedure of Ray (31), as described previously (23). The membranes were suspended in 10 mM Tris-HCl buffer, pH 7.4 (containing 0.15 M NaCl and 0.5 mM $CaCl_2$) at a protein concentration of 5 mg/ml, and used for the binding studies.

Binding of ^{125}I-labeled HDL_3 and nitrated ^{125}I-labeled HDL_3 to isolated membranes. The binding of ^{125}I-labeled HDL_3 to isolated membranes was determined according to the procedure described previously (26). Briefly, aliquots of membranes (200 μg of protein) were incubated with ^{125}I-labeled HDL_3 at room temperature for 1 hr in a total volume of 0.2 ml, containing 10 mM Tris-HCl, pH 7.4, 0.5 mM $CaCl_2$, and 1% BSA. After incubation, 0.175-ml aliquots of the incubation mixture were centrifuged in a Beckman 42.2 Ti rotor at 30,000 rpm for 15 min to recover the membranes. The membrane pellets were washed once with 0.175 ml of incubation medium and the tubes containing the membranes were assayed for radioactivity in a Beckman Model 300 gamma scintillation spectrometer with a ^{125}I counting efficiency of 66%. Nonspecific binding of ^{125}I-labeled HDL_3 to the membranes was determined in samples run in parallel that also contained a 100-fold excess of unlabeled HDL_3. The difference between the ^{125}I-labeled HDL_3 bound to the membranes in the absence and in the presence of excess unlabeled HDL_3 was taken as the amount of specific binding. The effect of nitration of HDL_3 on the binding of HDL_3 to the membranes was investigated by studying its ability to compete for the binding of ^{125}I-labeled HDL_3 to the membranes. Similar procedures were used to determine the binding characteristics of nitrated ^{125}I-labeled HDL_3 to the membranes.

Preparation of nitrated HDL_3 and nitrated ^{125}I-labeled HDL_3. Unless otherwise described, the following procedure employing a 10-fold molar excess of reagent—based on seven tyrosine residues per molecule of apolipoprotein A-I—was used for the preparation of nitrated HDL_3. In a typical experiment, 75 μl of 0.84 M tetranitromethane in 95% ethanol was added to 25 mg of HDL_3 (protein) in 2.5 ml of 0.05 M Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl, and immediately mixed. After incubation for 1 hr at room temperature, during which the solution would turn yellow, the reaction mixture was applied to a Biogel P-6 DG desalting column (1 × 30 cm) and eluted with 0.05 M Tris-HCl, pH 8.0 buffer, containing 0.1 M NaCl. The nitrated HDL_3, eluting as the first yellow component from the column was recovered and analyzed. The yield was 24.5 mg of protein. Nitrated ^{125}I-labeled HDL_3 was prepared from ^{125}I-labeled HDL_3 in the same manner.

Chemical and physical analyses. Protein was determined by the method of Lowry et al. (32); protein in nitrated HDL_3 was determined according to the Biuret procedure (33). BSA was used as the standard. Polyacrylamide gel electrophoresis of apolipoproteins was performed on 10% gels containing 0.1% SDS (29).

Total lipids were extracted from lipoprotein preparations using the procedure of Bligh and Dyer (34). Lipid phosphorus was measured according to the procedure of Rouser, Siakotos, and Fleischer (35). For the determination of phosphorus linked to apolipoproteins, the lipids were extracted from the lipoprotein samples by the methanol–chloroform–ethyl ether extraction procedure of Lux, John, and Brewer (36) and the apolipoprotein residue was analyzed for phosphorus by the procedure of Rouser et al. (35). Cholesterol was determined by gas–liquid chromatography using coprostanol as an internal standard. Unesterified and total cholesterol represented, respectively, cholesterol determined before and after saponification (37). Esterified cholesterol was obtained by difference. For the determination of esterified cholesterol covalently linked to apolipoproteins, the lipoprotein samples were first delipidated, according to the procedure of Lux et al. (36), the apolipoprotein residue was saponified (37), and the extract was analyzed for cholesterol by gas–liquid chromatography.

Agarose gel electrophoresis was carried out in barbital buffer, pH 8.6, in the apparatus and according to the direction of Bio-Rad Laboratories (Richmond, CA). After electrophoresis, the lipoproteins were visualized by staining with Sudan Black B. For electron microscopy, the lipoprotein samples were negatively stained with 2% sodium phosphotungstrate, pH 7.0, and examined in a

Zeiss 10 transmission electron microscope operating at 80 KV and at 50,000 × magnification. Gel filtration of HDL_3 and nitrated HDL_3 was done in a 85 × 2 cm Sephacryl S-200 column (Pharmacia Fine Chemicals, Upsala, Sweden); the column wsa eluted with 0.15 M NaCl containing 0.02% EDTA, pH 7.4, and 0.02% sodium azide, at a flow rate of 20 ml/hr. Fractions (3.5 ml) were collected and analyzed for absorption at 280 nm and 360 nm. The control and the nitrated HDL_3 were hydrolyzed in 6 N HCl under vacuum for 24 hr at 110°C and the amino acid composition was determined on a Beckman 6300 amino acid analyzer.

RESULTS

Reaction of tetranitromethane with HDL_3

The reaction conditions described by Sokolovsky, Riordan, and Vallee (38) for the nitration of the tyrosine residues of protein were applied for the nitration of HDL_3. **Fig. 1** shows the course of nitration of HDL_3 with a 10 molar excess of tetranitromethane at pH 8.0, as followed by measuring the increase in absorption at 350 nm as a function of time. The absorption at 350 nm is due to nitroformate, a byproduct formed in the reaction (38). The reaction was rapid and complete in 60 min. Gel filtration of the reaction mixture served to remove the excess reagent and the nitroformate from the nitrated HDL_3. The recovery of the nitrated HDL_3 was almost quantitative. The nitrated HDL_3 was quantitated by the Biuret method (33); the nitration of HDL_3 affected the protein estimation by the procedure of Lowry et al. (32).

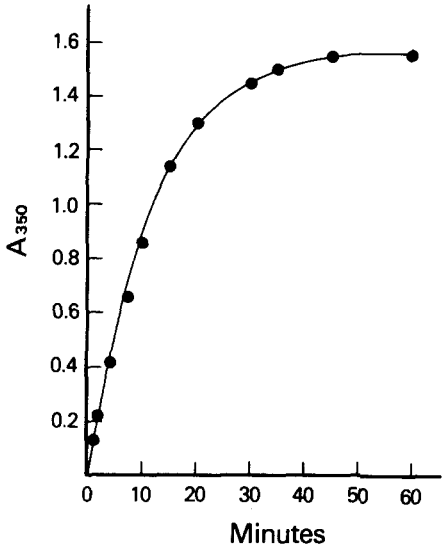

Fig. 1. Increase in absorbance at 350 nm on nitration of HDL_3; 1.0 mg of HDL_3 protein in 3.5 ml of 0.05 M Tris-HCl, pH 8.0, containing 0.1 M NaCl, and 3 μl (0.84 M in 95% ethanol) of tetranitromethane (10 molar excess). The absorbance at each time point was corrected for absorbances due to HDL_3 and due to the reagent.

In order to detect nitrotyrosine residues in the nitrated HDL_3, the absorption spectrum of the sample between 310 and 500 nm was determined and is given in **Fig. 2A**, along with that of standard 3-nitrotyrosine (Fig. 2B). At pH 8.0 nitrotyrosine had an absorption maximum at approx. 420 nm and it shifted to approx. 360 nm at pH 6.0. The nitrated HDL_3 at pH 8.0 had a strong absorption band with a maximum of approx. 370 nm but no absorption was seen in the region near 420 nm, expected for nitrotyrosine residues at this pH. At a higher pH of 10, however, the nitrated HDL_3 showed a new absorption band with a maximum at approx. 440 nm; the 360 nm band shifted to a maximum of approx. 370 nm. The spectrum of nitrated HDL_3 in 0.1 N NaOH was similar to that at pH 10.0. Control HDL_3 did not have any significant absorption in the range of 310 to 550 nm at pH 6.0, 8.0, or 10.0.

On agarose gel the nitrated HDL_3 migrated as a single band and it moved farther toward the anode than did the control HDL_3 (**Fig. 3**) indicating the generation of negatively charged groups during the nitration of HDL_3. Control and nitrated HDL_3 were well separated from one another when a mixture was electrophoresed. The amino acid compositions of the control and the nitrated HDL_3 were similar except for tyrosine; only traces of tyrosine (retention time 24.3 min relative to 25.83 min for phenylalanine) were detected in the acid hydrolysate of nitrated HDL_3 (**Fig. 4**). A new peak with a retention time of 26.81 min was detected in the nitrated HDL_3. The retention time of this new component was the same as that of standard 3-nitrotyrosine (Fig. 4).

Effect of the nitration of HDL_3 on the binding

For the determination of the effect of nitration on the binding of HDL_3 to the membranes, the ability of nitrated HDL_3 to compete with ^{125}I-labeled HDL_3 for the binding sites was studied. As is shown in **Fig. 5**, the nitrated HDL_3 did not significantly affect the binding of ^{125}I-labeled HDL_3 to rat liver plasma membranes, whereas control HDL_3 reduced the binding more than 80%. Nitration of HDL_3, therefore, resulted in particles with no ability to compete with ^{125}I-labeled HDL_3 for the binding sites. In order to relate the loss of the ability of HDL_3 to compete for the binding sites to the nitration reaction, HDL_3 was nitrated with varying molar concentrations of tetranitromethane, and the resulting nitrated HDL_3 was analyzed for its ability to compete with ^{125}I-labeled HDL_3 for the binding sites (**Fig. 6**). The HDL_3 preparation nitrated with 10 molar excess of tetranitromethane completely lost its ability to compete for the binding sites, whereas that nitrated with 1 or 2 molar excess of reagent showed reduced ability to compete for the binding sites.

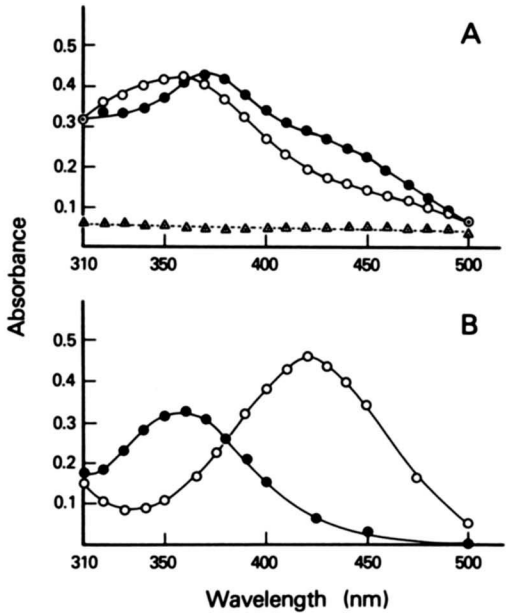

Fig. 2. Effect of pH on the absorption spectra of nitrated HDL_3 and 3-nitrotyrosine. A, Nitrated HDL_3 (○) 0.5 mg/ml 0.05 M Tris-HCl, pH 8.0, 0.1 M NaCl; nitrated HDL_3 (●), 0.5 mg/ml 0.05 M Tris-HCl, pH 10.0, 0.1 M NaCl; HDL_3, (△), 1.0 mg/ml 0.05 M Tris-HCl, pH 8.0, 0.1 M NaCl and pH 10.0, 0.1 M NaCl. B, 3-Nitrotyrosine (○), 0.1 mM, 0.05 M Tris-HCl, pH 8.0, 0.1 M NaCl; (●), 0.1 mM, 0.15 M NaCl, 2 mM EDTA, pH 6.0.

The loss of ability of HDL_3 nitrated with 2 molar excess of reagent to compete for the binding sites was greater than the sample treated with a 1 molar excess of reagent.

As further characterization of the loss of the ability of nitrated HDL_3 to compete for the binding sites, the binding characteristics of nitrated ^{125}I-labeled HDL_3, prepared by nitration of ^{125}I-labeled HDL_3 with a 10 molar excess of tetranitromethane, were studied. **Fig. 7A** shows the concentration-dependent binding of nitrated ^{125}I-labeled HDL_3 to rat liver plasma membranes. The binding of nitrated ^{125}I-labeled HDL_3 increased linearly with increasing concentration; the binding in the presence of a 50-fold excess of unlabeled nitrated HDL_3 was also linear. The presence of an excess of unlabeled nitrated HDL_3 did not significantly reduce the binding of nitrated ^{125}I-labeled HDL_3 to the membranes. Thus, although the nitrated ^{125}I-labeled HDL_3 bound to the membranes, the binding did not appear to be displaceable or saturable. Competitive binding experiments showed that neither HDL_3 nor nitrated HDL_3 reduced significantly the binding of nitrated ^{125}I-labeled HDL_3 (Fig. 7B), suggesting that the nitrated ^{125}I-labeled HDL_3 was not binding to the HDL binding sites and that it was binding nonspecifically to the membranes.

Characterization of nitrated HDL_3

In **Fig. 8** is shown a comparison of the apolipoprotein patterns of nitrated HDL_3 with those of control HDL_3

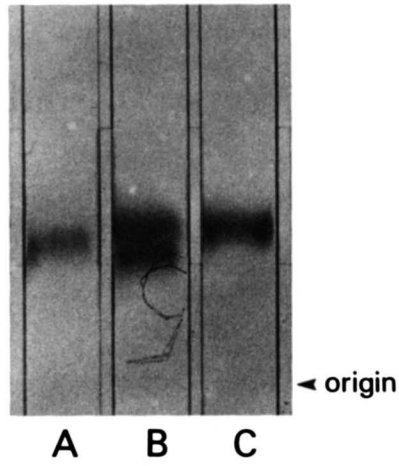

Fig. 3. Agarose gel electrophoresis of control HDL_3 and nitrated HDL_3. A, Control HDL_3; B, mixture of control and nitrated HDL_3; C, nitrated HDL_3.

determined by SDS-PAGE. A marked reduction in the apolipoproteins of HDL_3 was seen in the nitrated HDL_3. A series of Coomassie blue-stained bands appeared at the high molecular weight region of the gel, suggesting covalent cross-linking of apolipoproteins to high molecular weight polymers during the nitration of HDL_3 with tetranitromethane. The small amount of albumin that was present as a contaminant in the preparation of HDL_3 appeared to remain unchanged during the nitration reaction. There was no indication of cross-linking between HDL particles when the nitrated HDL_3 was analyzed by

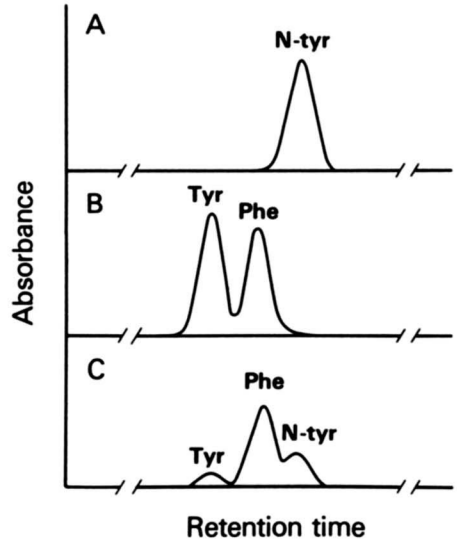

Fig. 4. Chromatographic analyses of the amino acids of control HDL_3 (B), nitrated HDL_3 (C), and standard 3-nitrotyrosine (A). Each sample was chromatographed separately. To facilitate presentation, that portion of the chromatograms containing tyrosine, phenylalanine, and 3-nitrotyrosine peaks is shown; the rest of the chromatogram was identical for control HDL_3 and nitrated HDL_3.

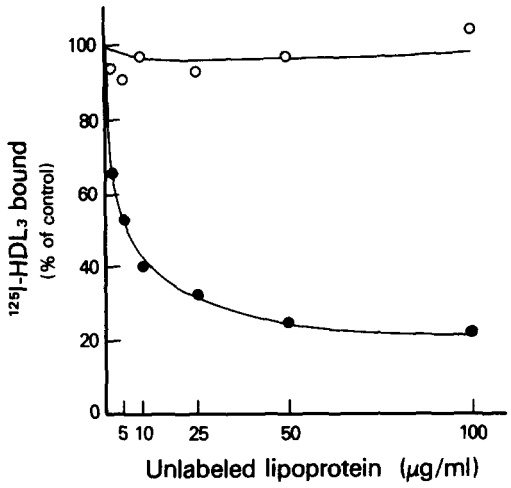

Fig. 5. Effect of HDL_3 and nitrated HDL_3 on the binding of ^{125}I-labeled HDL_3 to rat liver membranes. Each incubation mixture contained 200 μg of membrane protein, 10 μg ^{125}I-labeled HDL_3 protein/ml of incubation medium (0.15 M NaCl, 0.5 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.4, 10 mg/ml of bovine serum albumin) and indicated concentration of either HDL_3 (●) or nitrated HDL_3 (○). After incubation at 22°C for 1 hr the amount of ^{125}I-labeled HDL_3 bound to the membranes was determined as described in Materials and Methods.

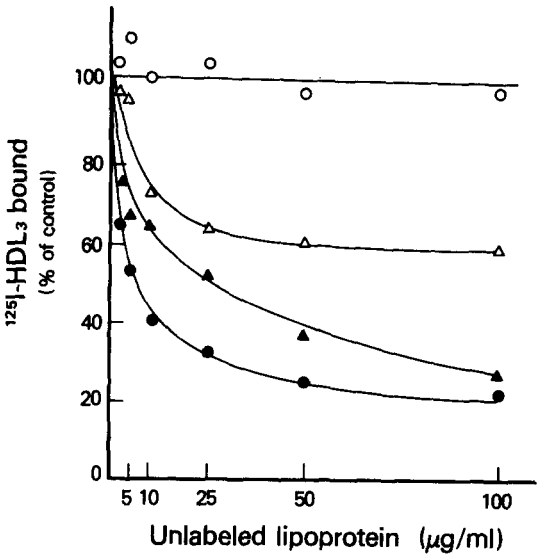

Fig. 6. The relationship between the extent of nitration of HDL_3 and its ability to compete with ^{125}I-labeled HDL_3 for the HDL binding sites in rat liver plasma membranes. Aliquots of HDL_3 were nitrated with 1 (▲), 2 (△), and 10 (○) molar excess of tetranitromethane as described in Materials and Methods. The amount of phospholipid phosphorus found in the apolipoprotein residues were 1.1, 1.6, and 2.5 μg/mg protein, respectively. The nitrated HDL_3 samples, along with control HDL_3 (●) were studied for their ability to compete with ^{125}I-labeled HDL_3 for the binding sites. The experimental protocols were the same as given in the legend of Fig. 5.

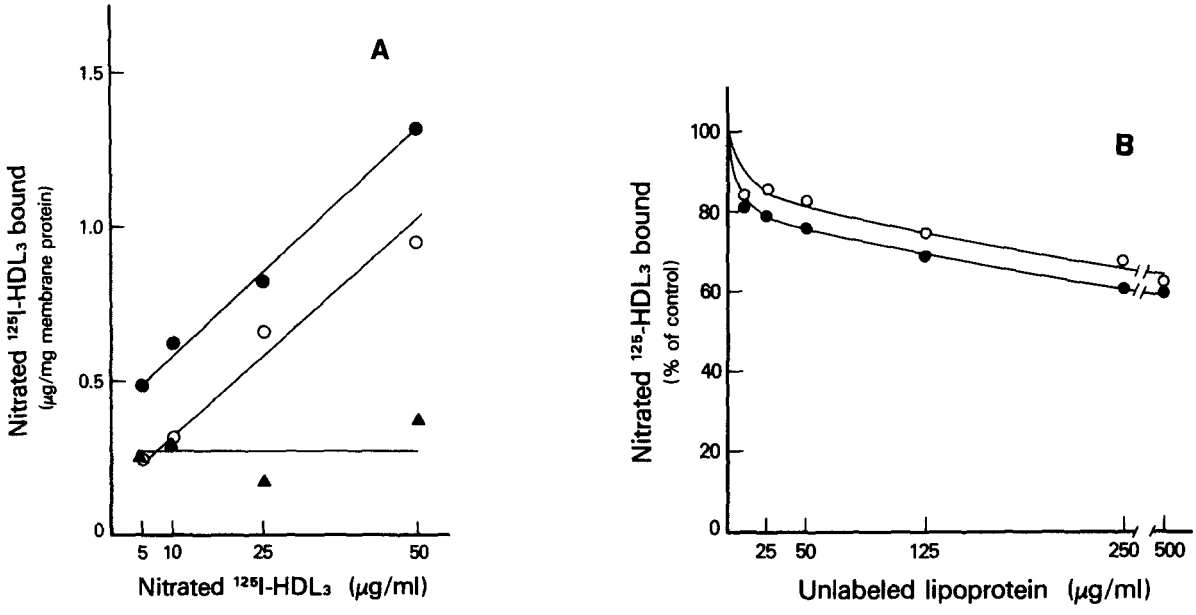

Fig. 7. A, Binding of nitrated ^{125}I-labeled HDL_3 to rat liver membranes as a function of its concentration. Aliquots of rat liver membranes (200 μg of protein) were incubated with indicated concentrations of nitrated ^{125}I-labeled HDL_3 in 0.2 ml of incubation medium containing 10 mg/ml bovine serum albumin for 1 hr at 22°C in the absence (●) and in the presence (○) of 50-times excess of unlabeled nitrated HDL_3. The bottom line (▲) represents the difference between the binding of nitrated ^{125}I-labeled HDL_3 to the membranes in the absence and in the presence of excess of unlabeled nitrated HDL_3. The data are the average of three determinations using three different nitrated ^{125}I-labeled HDL_3 preparations. B, Effect of control and nitrated HDL_3 on the binding of nitrated ^{125}I-labeled HDL_3 to rat liver membranes. Each incubation mixture contained 200 μg of membrane protein, 10 μg of nitrated ^{125}I-labeled HDL_3 protein/ml in a total volume of 0.2 ml incubation medium, and indicated concentrations of either control (○) or nitrated (●) HDL_3. The experimental protocols were the same as given in the legend of Fig. 5. The data are the average of two different experiments.

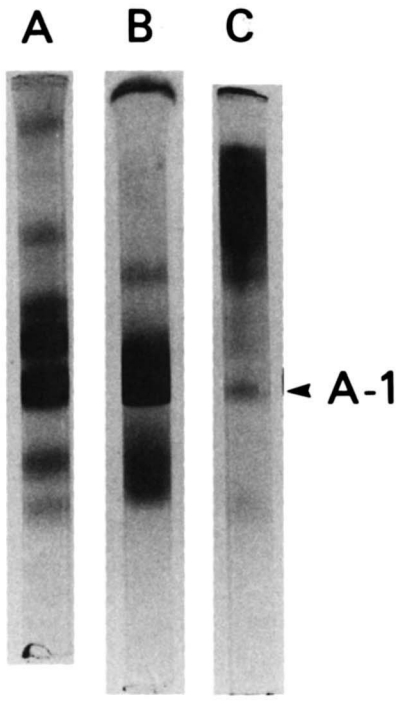

Fig. 8. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of control (B) and nitrated (C) HDL_3 apolipoproteins. On the left (A) is shown the pattern of rat HDL apoproteins, used as a standard. Fifty μg of protein/gel was employed; A-I refers to the apolipoprotein A-I, the major apolipoprotein of human HDL_3. It is almost completely lost on nitration, appearing as a series of Coomassie blue-stained bands at the high molecular weight region of the gel.

negative stain electron microscopy (**Fig. 9**). No aggregate particles were detected in the preparation. Both the control and the nitrated HDL_3 were of similar size. Control HDL_3 had a mean diameter of 96.3 Å and nitrated HDL_3 had a mean diameter of 97.7 Å; 200 particles of each were measured. Gel filtration chromatography of a mixture of control and nitrated HDL_3 on a Sephacryl S-200 column also showed that the modified HDL_3 had the same size as the control HDL_3 (**Fig. 10**). When fractions from the column were analyzed at 280 nm for protein and at 360 nm for nitrated HDL_3, overlapping peaks were obtained.

In **Table 1** is shown the lipid composition of the control and the nitrated HDL_3. Although there was no difference in the phosphorus content between the control and the nitrated HDL_3, the lipid extract from the nitrated HDL_3 gave a lower phosphorus value than the lipid extract from the control HDL_3, suggesting that part of the phospholipid in the nitrated HDL_3 was linked covalently to the apolipoprotein residue. This was indeed found to be the case when the apolipoprotein residue was analyzed for bound phosphorus. While only negligible phosphorus was found in the apolipoprotein residue of control HDL_3, an amount of phosphorus equal to the difference between the phosphorus in the lipid extract of control HDL_3 and the phosphorus in the lipid extract of the nitrated HDL_3 was found in the apolipoprotein residue of the nitrated HDL_3. A part of esterified cholesterol also was covalently linked to apolipoproteins during the nitration reaction. Thus, the esterified cholesterol content in the lipid extract of the nitrated HDL_3 was less than that in the lipid extract of the control HDL_3 (Table 1). Saponification of the apolipoprotein residue of the nitrated HDL_3 yielded cholesterol in amounts comparable to the amount found diminished in the content of esterified cholesterol in the lipid extracts of nitrated HDL_3 (**Table 2**). These results suggested that, during nitration of HDL_3 by tetranitromethane, a part of phospholipids and of esterified cholesterol, but not unesterified cholesterol, was covalently linked to apolipopro-

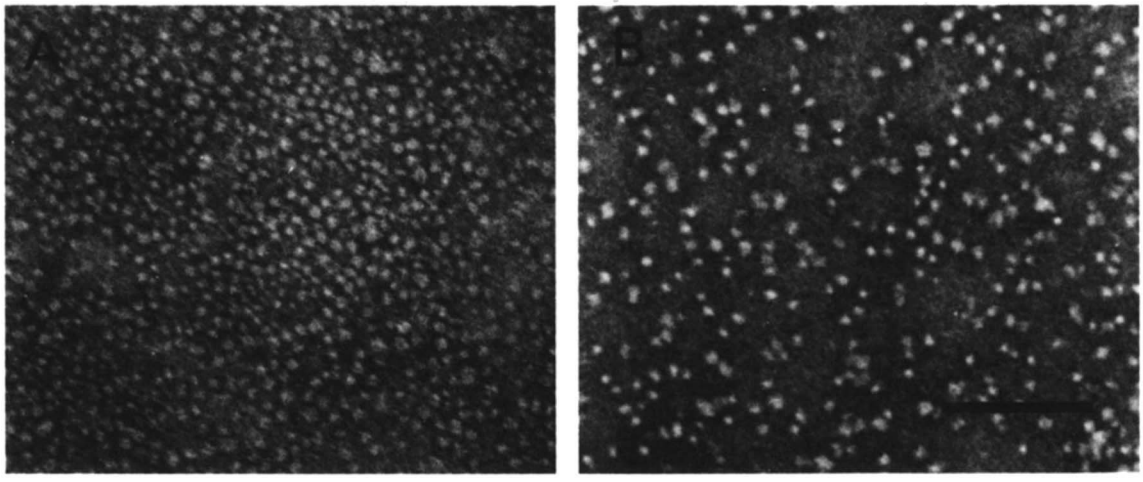

Fig. 9. Electron micrographs of negatively stained control (A) and nitrated (B) HDL_3 × 50,000.

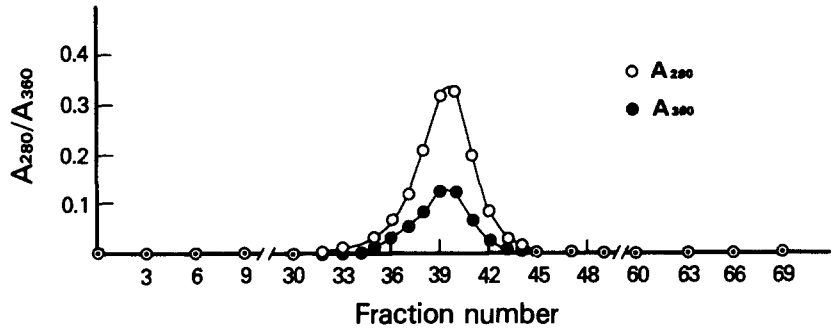

Fig. 10. Elution profile obtained upon gel filtration chromatography of a mixture of control HDl_3 (1.3 mg of protein) and nitrated HDl_3 (2.4 mg of protein) on a Sephacryl S-200 column (2 × 85 cm) in 0.15 M NaCl containing 0.02% EDTA, pH 7.4, and 0.02% sodium azide. Flow rate, 20 ml/hr. Fractions (3.5 ml) were analyzed for absorption at 280 nm (protein) (○) and at 360 nm (nitrated HDL_3) (●).

teins. This might occur through the unsaturated centers of the fatty acid chains.

DISCUSSION

The present study has shown that chemical modification of HDL_3 with tetranitromethane abolished its ability to interact with the high-affinity saturable HDL binding sites of rat liver plasma membranes. Thus, HDL_3 on treatment with 10 molar excess of tetranitromethane at pH 8.0 for 1 hr completely lost its ability to compete with ^{125}I-labeled HDL_3 for the HDL binding sites. In addition, ^{125}I-labeled HDL_3 after nitration did not bind to membranes with high affinity or with saturable kinetics. Furthermore, a dose-dependent inactivation of HDL_3 by tetranitromethane suggested that the loss of the ability of HDL_3 to interact with the HDL binding sites of the membranes was related to the reaction of tetranitromethane to HDL_3. Although nitrated HDL_3 did not bind to the high-affinity saturable binding sites, it exhibited binding ability to the membranes, but the binding was nonspecific, i.e., the binding was not saturable and was not competed for by unlabeled nitrated HDL_3.

Tetranitromethane is used widely as a specific and mild reagent for the modification of tyrosine residues of proteins (39, 40). Although it is reasonably specific as a nitrating agent for tyrosine residues, oxidation of sulfhydryl groups as well as reaction with histidine, methionine, and tryptophan residues (39, 40) have been reported. The reaction with tyrosine proceeds optimally at alkaline conditions; a pH of 8.0 (0.05 M Tris-HCl) is usually used (38), which was the condition selected for the nitration of HDL_3 in the present study. That the nitration of tyrosine residues of HDL_3 did occur during the treatment with tetranitromethane was indicated by the following results: *a*), the formation of nitroformate (a byproduct formed in the reaction of tetranitromethane with tyrosine residues) as measured by its absorptions at 350 nm (Fig. 1); *b*), an increase in the anodic electrophoretic mobility of modified HDL_3 in the agarose gel, indicating the possible generation of negatively charged nitrotyrosine groups (Fig. 3); *c*), an apparent reduction in the protein content of modified HDL_3 when determined according to the Lowry procedure (32); *d*), the appearance in the modified HDL_3 of a new pH-dependent absorption band with a maximum of around 440 nm at alkaline pH, presumably due to nitrotyrosine residues (Fig. 2); and finally *e*), the

TABLE 1. Lipid composition of control and nitrated HDL_3

	Experiment 1		Experiment 2	
	Control HDL_3	Nitrated HDL_3	Control HDL_3	Nitrated HDL_3
Phosphorus, μg/mg protein	10.1	9.8	12.3	11.6
Phosphorus (Bligh-Dyer extract), μgP/mg protein	10.2	7.7	12.6	6.8
Phosphorus in apolipoprotein residue, μg/mg protein	0.1	2.4	0.2	3.8
Total cholesterol, μg/mg protein	168.7	149.3	203.8	131.6
Unesterified cholesterol, μg/mg protein	18.5	18.8	26.2	21.3
Esterified cholesterol, μg/mg protein	150.2	130.5	177.6	110.3

TABLE 2. Cholesterol content of control and nitrated HDL_3[a]

	Control HDL_3	Nitrated HDL_3
Unesterified cholesterol, μg/mg protein[b]	20.8	18.1
Esterified cholesterol, μg/mg protein[b]	138.1	105.8
Esterified cholesterol bound to apolipoprotein, μg/mg protein[c]	0	24.2

[a] Average of two determinations.
[b] Determined in the Bligh-Dyer extract.
[c] Determined after saponification of the apolipoprotein residue (37). Apolipoprotein residue was prepared by the methanol-chloroform-ethyl ether extraction procedure of Lux, John, and Brewer (36).

disappearance of tyrosine residues with a concomitant appearance of nitrotyrosine residues, but with no change in the composition of any other amino acids, in the amino acid analysis of modified HDL_3 (Fig. 4).

As compared to reports on other proteins (38, 39), the reaction of tetranitromethane with HDL_3 proteins appears to be rapid and requires lower concentrations of reagent. More than 90% of the tyrosine residues was modified when HDL_3 was reacted with a relatively low concentration of 10 molar excess of tetranitromethane. Attempts to estimate the extent of nitration of tyrosine residues by spectrophotometric means were not successful, in that a value higher than the expected maximum of tyrosine residues for apolipoprotein A-I was obtained. Spectrophotometric properties of modified HDL_3 require further comments. At pH 8.0, nitrated HDL_3 did not show any prominent absorption at 420 nm as is seen in standard nitrotyrosine (Fig. 2B); however, it had a strong absorption band with a maximum at 360 nm. When the sample was taken in pH 10, a new absorption band appeared, as a shoulder, with a maximum at approx. 440 nm. In addition, there was a reduction in intensity of the 360 nm band as well as a shift to a higher wavelength of 370 nm maximum. A simple explanation for these observations is that only a part of the 360 nm band is due to nitrotyrosine residues, that these residues exist in a hydrophobic environment, and this absorption is shifted to 440 nm in pH 10. The remaining absorption in the 360 nm band is due to byproducts, such as nitroformate, dissolved in the core of the lipoprotein particles.

Extensive cross-linking of apolipoprotein and lipid components was detected in the modified HDL_3. As is seen in the SDS-polyacrylamide gel pattern (Fig. 8), very little of the apolipoprotein of original HDL_3 was seen in the nitrated HDL_3, obviously converted to high molecular weight polymers. Cross-linking was not limited to apolipoproteins. Evidence for covalent linkage of lipids to apolipoproteins was obtained when analysis of the chloroform-methanol extract of modified HDL_3 was made. A reduction in the content of phospholipid and cholesteryl ester was seen. They were detected, as phosphorus in the case of phospholipids and as unesterified cholesterol after saponification in the case of cholesterol ester, in the apolipoprotein residue. The extent of polymerization of apolipoproteins and the covalent association of phospholipid are related to the concentration of reagent used for the modification of HDL_3.

Since the reaction involves free radical intermediates (40), inter- and intra-molecular cross-linking can be expected during the nitration of HDL_3 with tetranitromethane. Cross-linking and aggregation have been reported during the nitration of several proteins (39). The extent of cross-linking is dependent on variables such as the concentration of the reagent used, the protein concentration, the nature of the protein being studied, and the solvent conditions (e.g., pH). The chemical nature of the cross-linking has not been unambiguously established. Linkage between tyrosine side chains is likely, although other types of linkage are possible in the case of HDL_3, including that involving unsaturated centers of the lipids of lipoproteins. It is likely that the phospholipids and cholesteryl esters found linked to apolipoproteins are attached through the double bonds. The extensive cross-linking of apolipoproteins and lipids at a relatively low reagent concentration during the nitration of HDL_3 may be related to the increased solubility of tetranitromethane in the apolar core of the lipoprotein particles and the presence of reactive unsaturated centers in the lipids. The location of tyrosine residues in the hydrophobic region of the amphipathic helix would favor their cross-linking to fatty acid chains. The linkage of cholesteryl esters to apolipoprotein is compatible with the idea that tetranitromethane partitions in the lipid core of HDL_3 and reacts with components in, and in association with, the lipid core.

No definite conclusion can be drawn from this study on the mechanism by which tetranitromethane inactivates HDL_3 so that it cannot interact with its binding sites. It could be argued that tyrosine-containing peptide segments of apolipoprotein(s) are involved in recognition of the HDL binding sites and that the nitration of tyrosine interfered in their interaction. Such a specific role of tyrosine does not seem compatible with the recent finding of the relatively nonspecific properties of HDL binding sites (26). It is more likely, however, that the inactivation may be related to cross-linking of the apolipoproteins to themselves and to lipid components during the nitration reaction. Whatever the mechanism of inactivation, the availability of a modified HDL_3 preparation with no binding ability to the high-affinity saturable binding sites will be useful for studies related to the functional aspects of HDL binding sites.

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