Cross-linking of apoproteins in high density lipoprotein by dimethylsuberimidate inhibits specific lipoprotein binding to membranes

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Abstract Apoprotein E-free high density lipoproteins (HDL) bind to various cells and cell membrane preparations with properties typical of ligand-receptor interactions. This specific binding can be inhibited by treatment of HDL with tetranitromethane (TNM). During treatment of HDL with TNM, in addition to the expected nitration of tyrosine residues, cross-linking of lipids to apoproteins and of apoproteins to each other occurs. We have recently shown that cross-linking of phospholipids to apoproteins is not responsible for the inhibition of binding (1987. Chacko, G. K., et al. *J. Lipid Res.* **28:** 332–337). To determine the role of cross-linking of apoproteins to each other in the inhibition, we used the bifunctional reagent dimethylsuberimidate (DMS) to cross-link the apoproteins in HDL_3. Over 80% of apoproteins in DMS-HDL_3 were cross-linked, as analyzed by SDS-polyacrylamide gel electrophoresis. DMS-HDL_3 was similar to control HDL_3 in its lipid composition. Gel filtration chromatography did not reveal any significant difference in size between DMS-HDL_3 and control HDL_3. As determined by competitive binding with ^{125}I-labeled HDL_3, DMS-HDL_3 was almost completely unable to bind specifically to rat liver plasma membranes and human skin fibroblasts. It is concluded from these results that TNM inhibits the specific binding of HDL_3 to membranes by a mechanism that involves cross-linking of apoproteins to each other in HDL_3 particles. This observation implies that the specific binding of HDL_3 to cells may depend on the native quaternary structure of apoproteins in the HDL particle. Because of its reduced ability to bind to the specific binding sites, DMS-HDL_3 may be useful for studies related to the functional aspects of HDL binding sites.—**Chacko, G. K., F. H. Mahlberg, and W. J. Johnson.** Cross-linking of apoproteins in high density lipoprotein by dimethylsuberimidate inhibits specific lipoprotein binding to membranes. *J. Lipid Res.* 1988. **29:** 319–324.

Supplementary key words apoA-I • tetranitromethane

Because of the putative role of high density lipoprotein (HDL) as a protective lipoprotein against arteriosclerosis (1), interaction of HDL with various cells and membranes has been under intensive study (2). High affinity saturable binding sites for apoE-free HDL have been detected in a variety of tissues and cells of different species (2). These binding sites have properties different from those of the LDL (apoB/E) receptor (3) or the apoE receptor (4); they are insensitive to proteolytic digestion (5–8), to chemical modification of the lysine or arginine residues of the lipoprotein (4, 9, 10), and to calcium concentration (11, 12). Recently, investigators in several laboratories (13, 14) including ours (15) have shown that the treatment of HDL with tetranitromethane (TNM) inhibits specific HDL binding. The mechanism of this inhibition is not known. During treatment of HDL with TNM, in addition to the expected nitration of tyrosine residues of apoproteins, cross-linking of lipids to apoproteins and of apoproteins to each other occurs (15). Previously we have shown that cross-linking of phospholipids to apoproteins is not responsible for the inhibition (16). In the present study, we have investigated the role of cross-linking of apoproteins to apoproteins as a possible mechanism of inhibition of HDL binding during the treatment of HDL with TNM. The bifunctional cross-linking reagent, dimethylsuberimidate (DMS), was used to cross-link apoproteins in HDL_3. Upon apoprotein cross-linking, HDL_3 lost its ability to bind to specific HDL binding sites of isolated rat liver membranes and of GM3468 human skin fibroblasts.

MATERIALS AND METHODS

Materials

Human HDL_3 ($1.125 < d < 1.21$ g/ml) was isolated by differential ultracentrifugation (17) from fresh human plasma that had been treated with 5 mM N-ethylmaleimide

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

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to inhibit lecithin:cholesterol acyltransferase (18). It was further processed by heparin-Sepharose affinity chromatography (19) to remove traces of apoprotein E. Apoprotein A-I constituted about 70% of the human HDL_3 apoproteins as determined by SDS-polyacrylamide gel electrophoresis (PAGE), followed by Coomassie blue staining and densitometric scanning (20); the other apoprotein components were apoprotein A-II and the C apoproteins. HDL_3 was labeled with ^{125}I using the iodine monochloride procedure (21). The specific activities ranged from 70 to 100 cpm/ng protein. No more than 2% of the label was associated with lipids. Fatty acid-free bovine serum albumin (BSA) and dimethylsuberimidate were from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade.

Methods

Isolation of rat liver plasma membranes. Sprague-Dawley male rats weighing 100–150 g were used. Liver plasma membranes were isolated according to the procedure of Ray (22), as described previously (10). 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 of protein/ml and used for the binding studies.

Binding of ^{125}I-labeled human HDL_3 to plasma membranes. The binding of ^{125}I-labeled human HDL_3 to isolated membranes was determined according to the procedure described previously (10). Briefly, aliquots of rat liver plasma membranes (200 μg of protein) were incubated with ^{125}I-labeled human HDL_3 at room temperature for 1 hr in a total volume of 0.2 ml, containing 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.5 mM $CaCl_2$, and 1% BSA (incubation medium). 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 the incubation medium, followed by centrifugation and aspiration of the supernatant. The tubes containing the membranes were assayed for radioactivity in an NE 1600 gamma counter (Nuclear Enterprises Ltd, Edinburgh, Scotland) with a ^{125}I counting efficiency of 75%. The effect of apoprotein cross-linking on the binding of HDL to the membranes was investigated by studying the ability of cross-linked HDL to compete for the binding of ^{125}I-labeled HDL_3 to membranes; DMS-HDL_3 and ^{125}I-labeled HDL_3 were added simultaneously.

Binding of ^{125}I-labeled human HDL_3 to cells. The binding assay was as described by Oram, Brinton, and Bierman (23). Briefly, cell monolayers were rinsed three times with 2.0 ml of PBS at 37°C. Then 0.5 ml of serum-free culture medium containing 2 mg/ml BSA and ^{125}I-labeled HDL_3 with or without unlabeled lipoproteins (prewarmed for 1 hr at 37°C) was added to cells, and the plates were covered and incubated at 37°C for 1 hr. To end incubations, the culture plates were chilled on ice, and the medium was removed. The cells were rinsed five times with PBS-BSA (2 mg of BSA/ml) (4°C) and two times with PBS (4°C). After the final rinse, the cells were digested with 1.0 ml of Markwell Lowry A solution (24) (37°C overnight in humidified air) and aliquots were taken for the determination of ^{125}I radioactivity and protein (24).

Preparation of DMS-HDL_3. After a series of trials, the following reaction conditions, patterned after the published procedures of Davies and Stark (25) and Swaney (26), were used for the preparation of highly cross-linked HDL_3: HDL_3 concentration, 5 mg of protein/ml; DMS concentration, 5 mg/ml; incubation medium, 0.09 M triethanolamine –HCl buffer, pH 9.5; and duration of reaction, 2 hr at room temperature. Freshly made DMS solution was used. After incubation, the reaction mixture was applied to a Bio-Gel P-6 DG column (1 × 30 cm) and components were eluted with saline–EDTA (2 mM) buffer, pH 7.4. Fractions were collected and analyzed. Fractions containing protein were combined. DMS-HDL_3 was recovered in almost quantitative yield.

Chemical and physical analyses. Protein was determined by the method of Lowry et al. (27). BSA was used as the standard. Polyacrylamide gel electrophoresis of apoproteins was routinely performed on 10% gels containing 0.1% SDS (20). Apoproteins in DMS-HDL were also analyzed by gradient (3–27%) polyacrylamide slab gel (28).

Total lipids were extracted from lipoprotein preparations using the procedure of Bligh and Dyer (29). Lipid phosphorus was measured according to the procedure of Rouser, Siakotos, and Fleischer (30). For the determination of phosphorus linked to apoproteins, the lipids were extracted from the lipoprotein samples by the methanol–chloroform–ethyl ether extraction procedure of Lux, John, and Brewer (31) and the apoprotein residues were analyzed for phosphorus by the procedure of Rouser et al. (30). Free and total cholesterol were determined by gas–liquid chromatography using coprostanol as an internal standard (32). Esterified cholesterol was the difference between total and free cholesterol. Agarose gel electrophoresis of lipoprotein samples was carried out in barbital buffer, pH 8.6, in a Bio-Rad apparatus and according to the direction of Bio-Rad Laboratories (Richmond, CA). After electrophoresis, the lipoproteins were visualized by staining with Fat Red B. Gel filtration of HDL_3 and DMS-HDL_3 was done in an 85 × 2 cm Sepharose 4B column (Pharmacia Fine Chemicals, Upsala, Sweden); the column was 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 (2.1 ml) were collected and analyzed for absorbance at 280 nm.

RESULTS

Preparation of apoprotein-cross-linked DMS-HDL_3

The present procedure for preparing apoprotein-cross-linked DMS-HDL_3 differs from that of Swaney (26) in two respects: *1)* use of high concentrations of HDL_3 (5 mg of

protein/ml) and dimethylsuberimidate (5 mg/ml), and *2)* the use of a desalting column to isolate the modified HDL_3 from the excess reagent at the end of the reaction. In **Fig. 1** is shown a comparison of the apoprotein pattern of control and DMS-HDL_3, as determined by SDS-slab gel electrophoresis. The predominant apoprotein in control HDL_3 was apoA-I; the minor band corresponded to a mixture of apoA-II and the C apoproteins (A). Extensive cross-linking of apoproteins was seen in DMS-HDL_3 (B); a marked reduction in the concentration of both apoA-I and apoA-II/apoCs occurred. A diffuse high molecular weight band with a mean molecular weight of 95,000 was seen in DMS-HDL_3. Densitometric scanning of the SDS-PAGE revealed that over 80% of the apoproteins were cross-linked. A prolonged incubation period or addition of more reagent did not result in complete cross-linking. In order to detect particle to particle cross-linking during preparation of DMS-HDL_3, the DMS-HDL_3 was chromatographed on a column of Sepharose 4B and the elution profile was compared to that of control HDL_3. As is shown in **Fig. 2**, the elution profiles of control and DMS-HDL_3 were similar, indicating no appreciable cross-linking between particles in DMS-HDL_3. Except for the cross-linking of apoproteins in HDL_3 particles, the composition of DMS-HDL_3 was similar to that of control HDL_3 (**Table 1**). On agarose gel electrophoresis, the DMS-HDL_3 moved as a single band (**Fig. 3**) and it moved less toward the anode than did the control HDL_3. The reason for this reduced anodic mobility is not known.

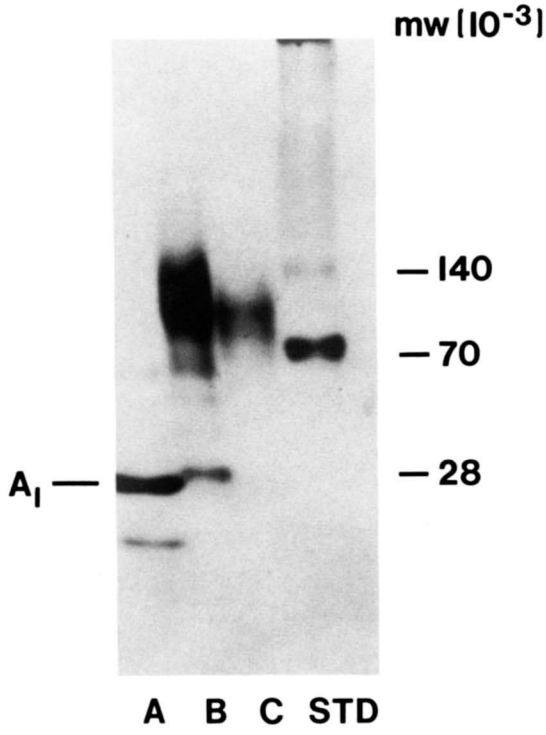

Fig. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of HDL_3 and DMS-HDL_3 on a 3–27% gradient slab. Lanes: A, ca. 100 μg of HDL_3 protein; B, ca. 100 μg of DMS-HDL_3 protein; C, ca. 10 μg of DMS-HDL_3 protein; STD, cross-linked hemocyanins as molecular weight markers. The gel was stained with Coomassie blue.

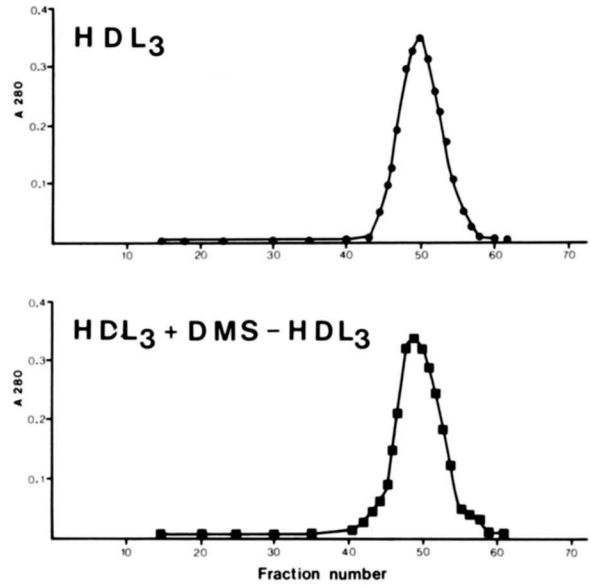

Fig. 2. Comparison of the elution profiles obtained upon gel filtration chromatography of control HDL_3 (5 mg of protein) (A) and mixture of control HDL_3 (2.5 mg of protein) and DMS-HDL_3 (2.5 mg of protein) (B) on a Sepharose 4B column (2 × 85 cm) in 0.15 M NaCl containing 0.02% EDTA, pH 7.4, and 0.02% azide. Fractions (2.1 ml) were analyzed for absorbance at 280 nm.

Effect of apoprotein cross-linking on the binding

In order to determine the effect of apoprotein cross-linking on the binding of HDL_3, the ability of DMS-HDL_3 to compete with ^{125}I-labeled HDL_3 for the binding sites of rat liver plasma membranes and of human skin GM3468 fibroblasts was studied. The results obtained with rat liver plasma membranes are shown in **Fig. 4.** At 25-fold excess concentration of lipoprotein, DMS-HDL_3 reduced the binding of ^{125}I-labeled HDL_3 to the membranes by only about 25%, whereas control unlabeled HDL_3 reduced the binding more than 80%. Thus, cross-linking of apoproteins in HDL_3 resulted in particles with reduced ability to compete with ^{125}I-labeled HDL_3 for the binding sites. The extent of cross-linking of HDL_3 apoproteins could be varied by changing either the concentration of DMS or the length of exposure to DMS. In this case, the ability of the unlabeled HDL_3 to inhibit ^{125}I-labeled-HDL_3 binding correlated with the amount of non-cross-linked apoA-I remaining in the treated HDL (**Table 2**). Essentially similar results were obtained when the effect of apoprotein cross-linking on the binding of HDL_3 to human skin fibroblasts was studied (**Table 3**). As compared to control HDL_3, the DMS-HDL_3 was much less effective in inhibiting the binding of ^{125}I-labeled HDL to GM3468 human skin fibroblasts. Thus whereas five-fold excess of unlabeled control HDL_3 reduced

TABLE 1. Lipid composition of control HDL_3 and DMS-HDL_3[a]

Composition	Control HDL_3	DMS-HDL_3
Phosphorus, μg/mg protein	18.82 ± 0.35	18.79 ± 3.1
Phosphorus in apoprotein residue, μg/mg protein[b]	0.42 ± 0.03	0.75 ± 0.05
Total cholesterol, μg/mg protein[c]	216.8 ± 2.4	219.7 ± 3.9
Unesterified cholesterol, μg/mg protein[c]	35.5 ± 0.37	33.5 ± 0.1
Esterified cholesterol, μg/mg protein	181.3 ± 1.9	186.2 ± 6.3

[a]Average of three determinations (mean ± SD).
[b]Determined after saponification of the apoprotein residue (32); the apoprotein residue was prepared by the methanol–chloroform–ethyl ether extraction procedure of Lux, John, and Brewer (31).
[c]Determined in the Bligh-Dyer extract.

the binding of ^{125}I-labeled HDL_3 to fibroblasts by 66%, DMS-HDL_3 at similar concentration reduced the binding by only 10%.

DISCUSSION

Although saturable and specific binding of apoE-free HDL to a variety of cells and isolated membranes has been identified, no physiological role for this binding has been identified. One of the difficulties in identifying a physiological role for HDL binding has been the inability to inhibit the binding by specific chemical or enzymatic modification of either HDL or the binding sites. However, it was found recently that the treatment of HDL with tetranitromethane, a nitrating reagent of tyrosine residues, inhibits the specific binding of HDL to cells and isolated membranes. Using TNM-treated HDL, Brinton et al. (13) performed experiments which suggest that the HDL binding sites may facilitate the HDL-dependent removal of cholesterol from cultured skin fibroblasts. By the use of a similar preparation , Nestler, Chacko, and Strauss (33) have concluded that HDL specific binding sites are not required for the delivery of cholesterol from HDL to rat ovarian cells for steroidogenesis.

During the treatment of HDL_3 with TNM, it has been found (15) that, in addition to the expected nitration of tyrosine residues in apoproteins, two major side reactions occur, namely, the cross-linking of lipids (phospholipids and cholesteryl esters) to apoproteins and of apoproteins to each other. Recently, we have shown that cross-linking of phospholipids to apoproteins is not responsible for the inhibition of binding. The purpose of the present study was to examine the role of cross-linking of apoproteins to each other as a possible mechanism of inhibition.

Since dimethylsuberimidate (DMS) has been shown previously to readily cross-link apoproteins in HDL (26), we selected this cross-linking reagent for our studies. DMS is a diimidoester reagent, specific for primary amino groups and has a relatively long spacer alkyl chain capable of cross-linking all apoproteins on an HDL particle. We utilized the procedure of Swaney (26) except that higher concentrations of HDL_3 and DMS were used. Analysis of the treated HDL_3 revealed extensive cross-linking of apoproteins without appreciable cross-linking of lipids to apoproteins or of particle to particle. As shown by SDS-PAGE analysis (Fig. 1), very little native apoprotein is seen in DMS-HDL_3; more than 80% of Coomassie blue-stained material appeared at a region of the gel corresponding to an average molecular weight of 95,000. This molecular weight of the cross-linked apoprotein is in agreement with the reported value for human HDL (26). Except for apoprotein cross-linking, DMS-HDL_3 was similar to control HDL_3 in its composition (Table 1). There was very little detectable cross-linking of apoproteins to phospholipids in DMS-HDL_3. This result is reasonable, since the ethanolamine and serine phospholipids that can participate in cross-linking constitute only a minor fraction of the HDL phospholipids (34). A small increase in the amount of bound phosphorus in the apoprotein residue of DMS-HDL_3 may represent these minor phospholipids cross-linked to apoproteins via the primary amino group. That the cross-linking in apoproteins occurred via lysine residues was checked by amino acid analysis of control and DMS-HDL_3; as compared to control HDL_3, the content of lysine residues only was reduced in DMS-HDL_3 (data not shown). An unexpected finding

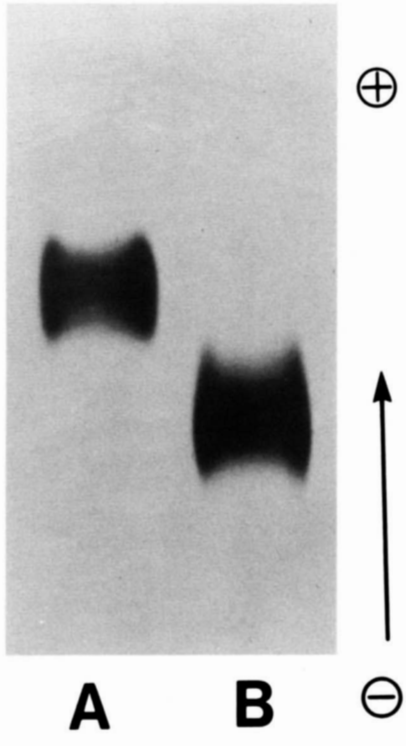

Fig. 3. Agarose gel electrophoresis of control HDL_3 and DMS-HDL_3. A, Control HDL_3; B, DMS-HDL_3. Lipoprotein bands were visualized by staining with Fat Red B.

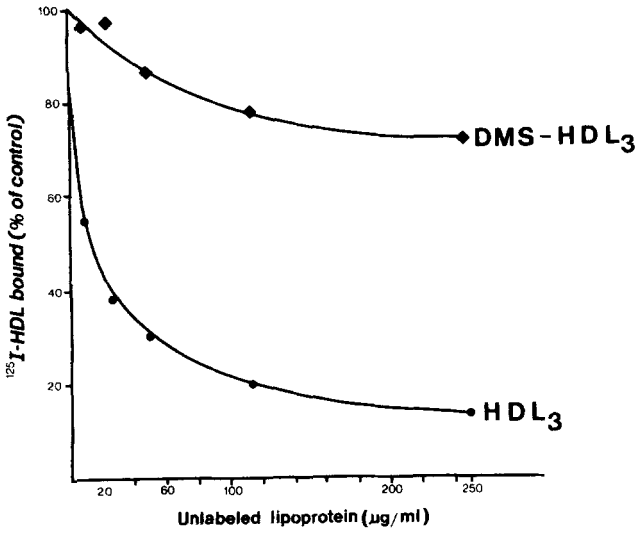

Fig. 4. Effect of control HDL_3 and DMS-HDL_3 on the binding of ^{125}I-labeled HDL_3 to rat liver membranes. Each incubation mixture contained 200 μg of membrane protein, 10 μg of ^{125}I-labeled HDL_3 protein/ml of incubation medium (0.15 M NaCl, 0.5 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.4), 10 mg/ml of BSA, and the indicated concentration of either control HDL_3 (●) or DMS-HDL_3 (♦). After 1 hr incubation at 22°C, the amount of ^{125}I-labeled HDL_3 bound to the membranes was determined. Data in this figure are from one experiment, and are representative of five separate experiments.

in the properties of DMS-HDL_3 was its reduced anodic electrophoretic mobility on agarose gel. No difference in electrophoretic mobility was expected, as the amidine group that results from the reaction of imidoester group to primary amine group was thought to have charge characteristics similar to the original primary amine. The reason for this reduced mobility is not clear.

TABLE 2. Competitive inhibition of ^{125}I-labeled HDL_3 binding by DMS-HDL_3: effect of varying degrees of cross-linking

Unlabeled Competitor		Binding of ^{125}I-labeled HDL_3 (% of uncompeted binding)
% of Cross-Linking[a]	Concentration	
	μg/ml	
0	0	100
	50	30
	250	13
35	0	100
	50	66
	250	53
55	0	100
	50	86
	250	71
80	0	100
	50	100
	250	89

The binding of ^{125}I-labeled HDL_3 (10μg of protein/ml) to the membranes was determined as described in the legend to Fig. 4.

[a]The extent of cross-linking was determined by spectrophotometric scanning of Coomassie blue-stained gels.

TABLE 3. Effect of control HDL_3 and DMS-HDL_3 on the binding of ^{125}I-labeled HDL_3 to GM3468 human fibroblasts

Unlabeled Lipoprotein	Concentration	^{125}I-Labeled HDL_3 Bound
	μg of protein/ml	*ng HDL protein/mg cell protein*
Control HDL_3	0	81.6 ± 1.0 (100%)
	25	27.9 ± 1.8 (34%)
	100	18.4 ± 2.3 (22%)
DMS-HDL_3	0	77.2 ± 2.4 (100%)
	25	69.4 ± 4.0 (90%)
	100	59.1 ± 6.6 (76%)

Cells were grown in MEM-Bicarb, containing 10% fetal bovine serum and incubated with 5 μg/ml of ^{125}I-labeled HDL_3 with the indicated amounts of unlabeled control HDL_3 or DMS-HDL_3. ^{125}I-Labeled HDL_3 bound was determined as described in Materials and Methods (mean ± SD, n = 3).

The treatment of HDL_3 with DMS produced an inhibition of HDL binding which is similar to the inhibition previously obtained with TNM treatment (15). This similarity suggests that TNM treatment inhibits binding by a mechanism involving the cross-linking of apoproteins and not by nitration of tyrosine residues. This observation also suggests that the specific binding of HDL_3 to cells depends on the native quaternary structure of apoproteins in the HDL_3 particle.

In summary, dimethylsuberimidate can be used to extensively cross-link apoproteins in HDL_3 without significant changes in composition and without causing HDL_3 particles to aggregate with each other. As determined by competitive binding assays, the DMS-HDL_3 binds poorly to HDL binding sites of rat liver plasma membranes and human fibroblasts. Because of its reduced ability to bind to the high affinity specific binding sites, and because of the absence of lipid cross-linking, DMS-HDL_3 may be useful for studies related to the functional aspects of HDL binding sites.

We gratefully acknowledge the excellent technical assistance of Beth Bailey and Bernard Bury. We are indebted to Dr. John Swaney for the gradient SDS-PAGE slab gel analysis and to Joan Letizia for the gel filtration chromatography. This research was supported by NIH grants HL 37550 and HL 22633. FHM was supported by a grant from the Comité des Aides à la Recherche Fournier-Dijon-France.

Manuscript received 10 July 1987, in revised form 26 August 1987, and in re-revised form 12 October 1987.

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