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Changes in Human Serum High-Density Lipoproteins Induced By Disulfide-Exchange Reagents.* (33425)

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In 1962, Shore and Shore (1) found evidence suggesting that lipoproteins of density (D) 1.125–1.20 g/ml contain three identical peptide chains per molecule. In the same year Scanu *et al.* (2) also presented evidence consistent with a similar interpretation for the protein part of the entire alpha$_1$ lipoprotein density range (D 1.063–1.21) (HDL), and later, additional evidence of protein homogeneity was reported by Sandbar and Alaupovic (3). Although an antigenic heterogeneity of HDL present in aged plasma was recently demonstrated by Levy and Fredrickson (4), this was not ascribed to differences in protein composition, but rather to differences either in protein lipidation or polymerization of some basic monomeric unit. Thus, the bulk of the evidence indicates that the HDL protein is homogeneous.

There is less agreement about the cystine content of this protein. Although the presence of cystine had not been found by some (2, 3), more recently using two different methods it has been demonstrated to be present by Shore and Shore (1) and Levy and Fredrickson (4).

In the present report evidence is presented that suggests that the protein component of HDL is heterogeneous in its polypeptide composition and consists of at least two different polypeptides. One variety, called polypeptide C, reacts with a variety of reagent thiols and thiol-disulfide reagent combinations, suggesting it contains cystine-disulfide bonds. The other polypeptide is not reactive with these reagents or their combinations.

Methods. Isolation of HDL. Lipoproteins of density 1.063–1.21 (HDL) were isolated from 80 different sera by ultracentrifugal flotation using a modification of methods described elsewhere (5). Five milliliters of fresh serum were made to D 1.063 by adding 4 ml of a salt (NaCl + KBr) solution of D 1.134; the mixture was spun at 30,000 rpm for 24 hr in a Model L Spinco ultracentrifuge using a 30.2 rotor. The top 3 ml were then removed. The infranatant fluid was then layered over with a salt solution of D 1.063, and the sample was respun for 24 hr. The top 3 ml were removed and the density of the infranatant fluid was increased to D 1.21 by mixing with solid KBr; this mixture then was made to appropriate volume by adding a NaCl-KBr solution of D 1.21. It was spun as before, but only the top 1 ml containing the HDL was removed. It was placed at the bottom of a 9-ml Lusteroid centrifuge tube, carefully layered over with 8 ml of a salt solution of D 1.21, and respun as before. The top 1 ml was removed and dialyzed over-

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night in the cold (4°) versus a Tris-borate-versene buffer (6).

Purity of HDL. Twenty different samples of the isolated HDL were tested for purity immunoelectrophoretically (7), using rabbit antisera to human-serum, -albumin and lipoproteins of density 1.019–1.063 (LDL), and 1.063–1.21 (HDL). When isolated HDL samples were reacted with antisera to HDL a major and a minor precipitin arc were seen in every instance. The latter arc may or may not be the partially delipidated form of HDL, called alpha-LP_b by Levy and Fredrickson (4), which they found developed in all specimens of aged EDTA-plasma. In only 2 of 20 such isolations was a trace of albumin detectable, and in one of these its presence was dubious. No evidence for the presence of beta lipoproteins or other serum proteins was detected.

Preparation of delipidated HDL. The protein content of each HDL isolate was measured (8); it varied from 4 to 9 mg/5 ml of original serum sample. HDL aliquots containing 350 μg protein were then delipidated by mixing each with 100 vol of ethanol:ethyl ether (3:1), and allowing the mixture to stand at 4° for 48 hr or longer. The precipitates were then washed once with ethyl ether.

The adequacy of the delipidation was tested by subjecting the delipidated and nondelipidated material to starch gel electrophoresis in an alkaline Tris-borate-versene buffer. Although the protein in the delipidated and nondelipidated samples stained intensely with amido black, the delipidated protein was unstained by oil red O, under conditions that produced vivid staining of the nondelipidated aliquots of $alpha_1$ LP that served as the control (9). In addition, cholesterol, phospholipid, and triglyceride analyses, performed on the combined alcohol ether extracts by methods described elsewhere (10), also indicated that an essentially complete extraction of the lipids was accomplished. Finally, the delipidated protein was re-extracted with $CHCl_3$:CH_3OH (2:1, v/v); it contained 0.61% phospholipid but no detectable cholesterol or triglyceride. For convenience these partially delipidated high-density lipoproteins are called delipidated HDL.

Electrophoresis of delipidated HDL. The delipidated protein was solubized in a pH 8.6 buffer, containing 0.1 *M* boric acid, 0.025 *M* NaOH, 0.004 *M* Na_2EDTA, and 8 *M* urea. It was then characterized electrophoretically in three different gel systems. The electrophoresis was performed vertically (11) at 120 V for 22 hr at 4° in a pH 3.2 aluminum lactate 8 *M* urea starch gel[1]; a pH 3.2 aluminum lactate starch gel without urea; and a pH 8.6 Tris-borate-versene starch gel (6), also without urea. After electrophoresis each gel was sliced and stained with amido black (12). A total of 80 different serum HDL preparations were studied, including 60 obtained from men and 20 from women. All blood samples were obtained after an overnight fast.

Disulfide exchange reactions. Twenty-four aliquots of the same HDL sample were delipidated and after drying were resolubized in the pH 8.6 borate buffer, containing 8 *M* deionized urea. Three samples were used as controls, one for each of the three gel systems used. The control sample was placed in lane a in each gel. The remaining samples were divided into seven sets of three aliquots per set. Each set of three was reacted with one of seven different reagents or reagent combinations as follows and placed in the lanes of the gel indicated: lane (b) mercaptoethanol (ME), 0.09 *M*; lane (c) diethanoldisulfide (DEDS), 0.1 *M* plus ME, 0.004 *M*; lane (d) DEDS, 0.1 *M*; lane (e), cystamine·2HCl, 0.1 *M* lane (f) cystamine·2HCl, 0.1 *M* plus cysteamine·HCl, 0.004 *M*; lane (g) cysteamine HCl, 0.004 *M*; and lane (h) cysteamine·HCl, 0.09 *M*. Although many other reagent concentrations were tested, those described were found to be the most representative and informative. These reagents were mixed with the appropriate set of aliquots, and these mixtures were allowed to react for 30 min at 37°, before being transferred to the appropriate gel slots, and the electrophoresis and staining were conducted as indicated above.

[1] The authors thank Mr. Michael Sung, University of Wisconsin, for the composition of this buffer.

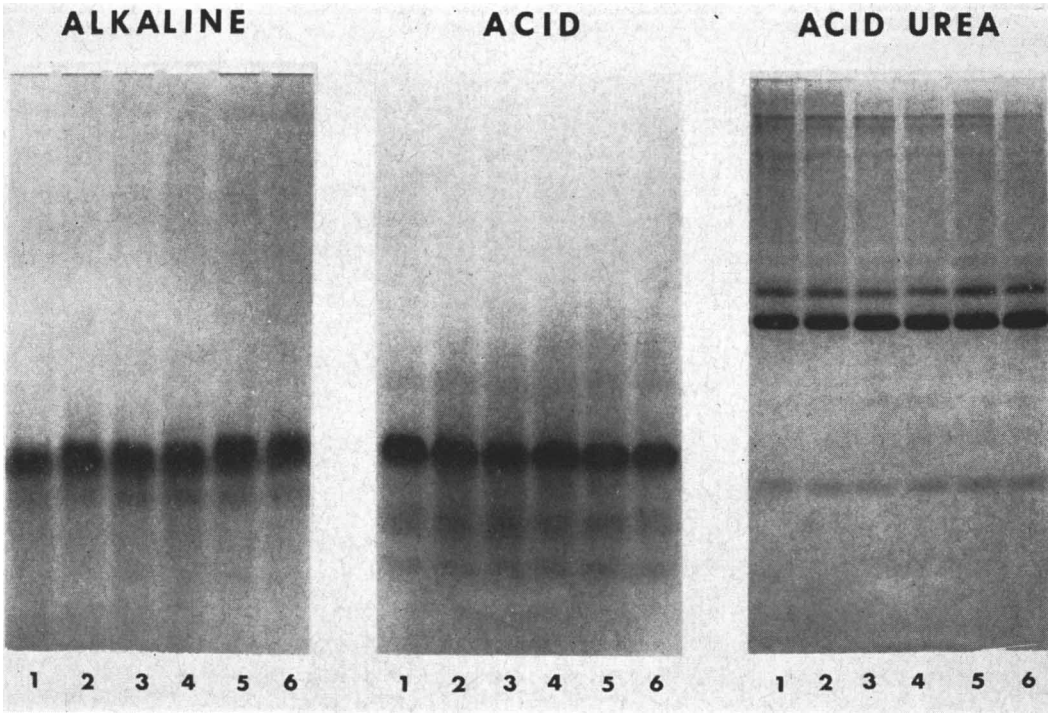

FIG. 1. The appearance of six different delipidated high-density lipoproteins after electrophoresis in three different starch gel systems. The numbers refer to the samples used. Approximately 350 μg was used for each sample. The conditions of the electrophoresis and other details are described in the text.

Results. Eighty different preparations of delipidated HDL were electrophoresed in each of the three gels and stained as described earlier. In a given gel system, all 80 samples had virtually the same appearance. However, each sample had a different pattern in each of the three electrophoretic systems used. Two bands were seen in the alkaline gel; five in the acid gel; and 10–12 in the acid-urea gel (Fig. 1). No sex differences were noted.

The interrelations of these bands to one another were studied. The presence of cystine in HDL (1, 4) suggested that useful data could be obtained by reacting the delipidated HDL with various reagent thiols and disulfides, and their combinations, in the presence of a depolymerizing agent such as urea (14, 15). It was anticipated that these reagents would aid in localizing any component with accessible disulfide or thiol groups. The reagent or reagent combinations used were described in the Methods section. The theoretical reactions of a protein disulfide with these various reagents are summarized in Table I. The effects of these reagents are shown in Fig. 2.

The control sample (lane a) and those treated with the disulfide DEDS, (lane d) and cystamine • 2HCl (lane e) have identical electrophoretic patterns in the alkaline, acid, and acid-urea gels, respectively. This suggests that none of the components in the samples contain accessible thiol groups (15). These observations agree with those of Shore and Shore (1) who by amperometric titration with $AgNO_3$ found no evidence for cysteine in $alpha_1$ LP of D 1.125–1.20.

The quantitatively major component present in all samples was never affected in its electrophoretic behavior by treatment with any of these reagents or their combinations. This unreactive component was consequently assumed to lack any accessible disulfide

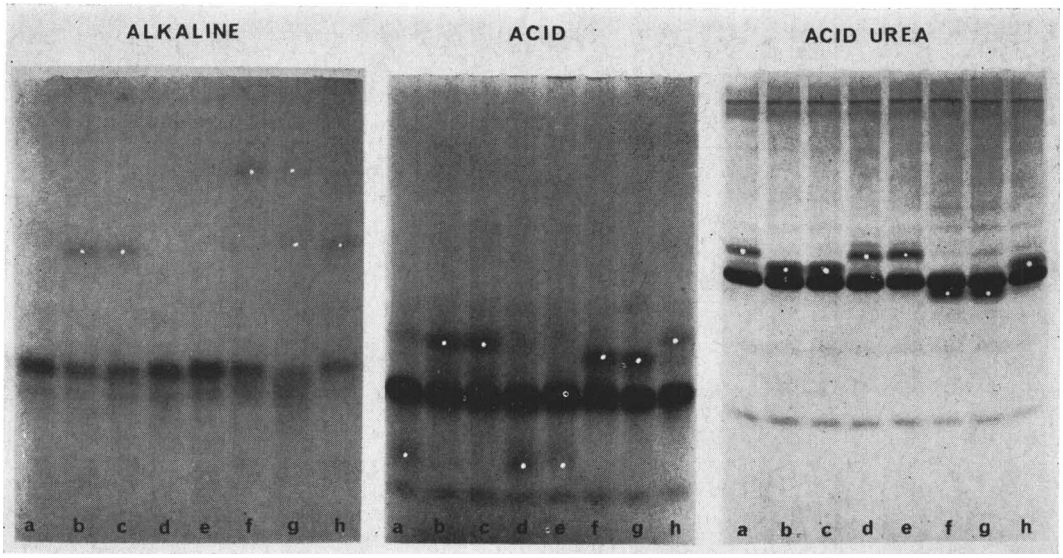

FIG. 2. Starch gel electrophoretic patterns of delipidated high-density lipoproteins (HDL) and alterations induced by reagent thiols and disulfides and their combinations. Each photograph depicts the influence of reagent thiols and disulfides and their combinations on the electrophoretic components of a delipidated HDL in different gels: an alkaline starch gel formed with a pH 8.6 Tris-borate-versene buffer; an acid starch gel formed with a pH 3.2 aluminum lactate buffer; and an acid, 8 *M* urea, starch gel formed with a pH 3.2 aluminum lactate buffer. The letter below each lane refers to the thiol, disulfide, or thiol-disulfide combination, described in the text and in Table I, with which a delipidated HDL was reacted before electrophoresis. The conditions under which the electrophoresis was conducted and the protein was reacted with the disulfide-exchange reagents are also described in the text. The reactive component is indicated by a white dot. It should be noted that an alteration in this component occurs only when the protein sample is reacted with a thiol alone (lanes b, g, and h) or a catalytic amount of thiol in combination with a disulfide reagent (lanes c and f); but not with disulfide reagents alone (lanes d and e). The control specimen is in lane a. In the alkaline gel the concentrations of cysteamine was 0.625 times (lane g) and 0.75 times (lane h) that used in the acid and acid-urea gels.

bonds, and presumably is devoid of cystine and/or cysteine. Several quantitatively minor components were similarly unreactive.

One component, which for convenience is called polypeptide-C, is markedly affected in its migration only by treatment with reagent thiols or thiol-disulfide combinations, and not with disulfide reagents alone. This reactive polypeptide, marked in Fig. 2 with white dots, is consequently assumed to contain a disulfide bond(s), and presumably contains the cystine known to be present in delipidated HDL. The effect of the different reagents on this polypeptide component can be explained as follows:

First, it can be seen that the untreated polypeptide-C (Fig. 2, lane a) migrates in the alkaline gel at the same rate as the major nonreactive component. Whether this is due to complex formation or to a chance similarity in their mobilities is not yet known. In the acid and acid-urea gel systems, the polypeptide-C and the nonreactive components in the control sample do not migrate together; but rather at different rates. This indicates that if complex formation occurs it can be easily dissociated by pH alteration.

Secondly, only the migration of polypeptide-C is changed by treatment with ME alone (Fig. 1, lane b; Table I, reaction b). This suggests that polypeptide-C contains at least one (cystine) disulfide bond that is split by the reagent thiol producing two protein thiols (PS^-). The migration of polypeptide-C is also changed in the same way by treatment with DEDS plus ME (Fig. 1, lane c; Table

TABLE I. Disulfide Exchange Reactions Used in These Studies.[a,b]

(a)[c]	PSSP	→ PSSP
(b)	PSSP + $2R^0S^-$	→ $2PS^- + R^0SSR^0$
(c)	PSSP + R^0SSR^0	$\xrightarrow{R^0S^-}$ $2PSSR^0$
(d)	PSSP + R^0SSR^0	→ No effect
(e)	PSSP + R^+SSR^+	→ No effect
(f)	PSSP + R^+SSR^+	$\xrightarrow{R^+S^-}$ $2PSSR^+$
(g)	PSSP	$\xrightarrow{R^+S^-}$ $2PSSR^+$
(h)	PSSP + $2R^+S^-$	→ $2PS^- + R^+SSR^+$

[a] All reactions were performed at pH 8.6 at 37° and were complete at 30′.

[b] Abbreviations: PSSP (cystine-containing polypeptide); PS^- (cysteine-containing polypeptide); R^0SSR^0 (uncharged reagent disulfide, diethanol-disulfide, DEDS); R^0S^- (uncharged reagent thiol, mercaptoethanol, ME); R^+SSR^+ (positively charged reagent disulfide, cystamine); R^+S^- (positively charged reagent thiol, cysteamine); $PSSR^0$ (mixed disulfide, uncharged); $PSSR^+$ (mixed disulfide, positively charged).

[c] (a) through (h) also correspond to the lanes in the starch gels in which the reaction products were electrophoresed, as seen in Fig. 2.

Thiol symbols above reaction arrow indicate this reagent is present only in catalytic amounts.

I, reaction c). This observation is understandable when it is considered that the free protein thiol, and the mixed disulfide forming between the protein and the uncharged disulfide reagent (DEDS) in the presence of catalytic amounts of thiol, differ only slightly in molecular size and probably not at all in charge. On the other hand, when a charged mixed disulfide ($PSSR^+$) is formed, as occurs when polypeptide-C is treated with cystamine (R^+SSR^+) 0.1 *M* plus cysteamine (R^+S^-) 0.004 *M*, the charged mixed disulfide ($PSSR^+$) migrates differently than PSSR; i.e., slower in the alkaline gel (Fig. 2, lane f) and more rapidly in the acid and acid-urea gels (Fig. 2, lane f). These mobility differences would all be expected after adding a single positive charge to polypeptide-C.

Finally, in the presence of the low cysteamine concentration, 0.004 *M* (Fig. 1, lane g; Table I, reaction g), polypeptide-C appeared to form two products in the alkaline gel, one migrating as does the charged mixed disulfide ($PSSR^+$), and a component migrating more rapidly as does the free protein thiol (PS^-). Presumably, the low concentration (0.004 *M*) of cysteamine gave a mixture of the free protein thiol and the protein-reagent mixed disulfide. If polypeptide-C is treated with a sufficiently high concentration of cysteamine 0.09 *M* (Fig. 1, lane h; Table I, reaction h) or mercaptoethanol, 0.09 *M* (Fig. 1, lane b; Table I, reaction b), then it is completely converted to the free protein thiol (PS^-).

Comment. These observations are consistent with the presence of disulfide bonds (cystine) in the HDL and confirm the earlier findings of Shore and Shore (1). The very interesting and new feature found here is that all of this cystine appears to reside in a single component of the delipidated HDL. This singular localization was evident at two extremely different pH values, and in the presence or absence of urea, and was displayed by taking advantage of newer knowledge of the chemistry of disulfide exchange reactions (15), the availability of several thiol and disulfide reagents, and the electrophoresis of the reaction products in a media that is capable of separating molecules on the basis both of size and charge (13).

This protein component of HDL is thus similar to many other extracellular proteins that characteristically are rich in disulfide bonds (15). Such proteins are also known to be relatively poor in sulfhydryl groups; this also appears to be the case for the HDL.

These data strongly suggest that there are at least two different polypeptides in the HDL; one containing cystine, and one not. This interpretation is supported by the detection of two *N*-terminal amino acids in the HDL apoprotein (1, 4). The major *N*-terminal amino acid, aspartic, may well be associated with the nonreactive (cystine-lacking) polypeptide variety, which is the major polypeptide form seen in the starch gels; the minor *N*-terminal acid, threonine, could be associated with the (cystine-containing) reactive polypeptide-C, which quantitatively represents the minor polypeptide variety. The

physical separation of these two polypeptides from one another, and the delineation of their respective amino acid composition and *N*-terminal acids, should settle this matter, and could assist in a determination of their respective roles in lipid binding. One aspect of the characterization of these polypeptides has already been of preliminary aid in facilitating this separation[2].

The likelihood that the HDL protein consists of two (or more) different polypeptides implies that independently genetically determined variants of each polypeptide would be anticipated. One such inherited variant involving polypeptide-C has already been found, and has been described in preliminary form elsewhere (16).

Summary. High-density lipoproteins, isolated from human serum, were delipidated, and the apoproteins, so obtained, were characterized both electrophoretically, using acid and alkaline buffer systems, and chemically by treatment with a variety of reagent thiols and disulfides and their combinations. The acid system was studied with and without 8 *M* urea. Depending on the electrophoretic system used, two or more components (polypeptides) were seen, presumably reflecting apoprotein aggregational differences under the several conditions of study.

No matter what the complexity of the components, two varieties of component were always demonstrable in all three systems: one variety was reactive in the presence of 8 *M* urea with reagent thiols, or disulfide plus thiol reagent combinations, and one variety was not. Neither was reactive with disulfide reagents alone. These observations indicated that the reactive component contained disulfide bonds, and that the nonreactive component probably did not; and that neither variety of component contained demonstrable thiol groups.

These observations strongly suggest that apo HDL consists of two different polypeptides: one rich in cystine, and one not. If this is so each would be subject to separate genetic control and variants of each polypeptide would be expected to be found in the population.

[2] Lane e shows a decrease in one component (polypeptide-C) when examined in the acid gel–lacking urea. This decrease is due to precipitation of the polypeptide and is being used to develop a method for the separation of the cystine- and noncystine-containing polypeptides.

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