Immunochemistry of Human Plasma High Density Lipoproteins. Radioimmunoassay of Apolipoprotein A-II†

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ABSTRACT: Apolipoprotein A-II (ApoA-II) is a major apoprotein of human plasma high density lipoproteins (HDL). The apoprotein has two identical chains of known amino acid sequence; the chains are linked by a single disulfide bond. In the present study, we have developed a specific radioimmunoassay for apoA-II that provides a convenient and reproducible method for measuring 10–100 ng of apoprotein. The assay was based on the competition of apoprotein with isotopically labeled [^{125}I]apoA-II. Dioxane (52%) was used to separate antibody bound [^{125}I]apoA-II from the free apoprotein. The assay has enabled us to begin studies directed toward mapping-out of the antigenic reactive regions of apoA-II. It has also allowed us to determine the interrelationship between the lipid-binding sites and the antigenic sites of the molecule. The antigenic reactivity of apoA-II was approximately the same in HDL and phospholipid–apoA-II complexes as that of the free apoprotein. However, succinylation of apoA-II was associated with a marked decrease in antigenic reactivity.

Human plasma high density lipoproteins (HDL[1]) contain approximately 45% protein and 55% lipid. Cholesteryl ester and phospholipid are the major lipid constituents. HDL have two major proteins which have been designated apoA-I and apoA-II. Morrisett et al. (1975) have recently reviewed the physicochemical properties of these apoproteins. The amino acid sequence of apoA-I has been determined (Baker et al., 1974, 1975; Delahunty et al., 1975). The protein is a single polypeptide chain of 245 amino acid residues. The other major HDL apoprotein, apoA-II, consists of two identical polypeptide chains of 77 amino acid residues that are linked by a single disulfide bond. The amino acid sequence of apoA-II has also been determined (Figure 1) (Brewer et al., 1972; Lux et al., 1972a). ApoA-I and apoA-II have been recombined with phosphatidylcholine and cholesteryl oleate to yield complexes which have compositions and properties similar to native HDL (Lux et al., 1972b). Both apoproteins have increased α-helical structure in the isolated complexes. To determine the role of lipid and conformation on the immunochemical properties of the plasma lipoproteins, we now describe a specific radioimmunoassay for apoA-II. This apoprotein was chosen because previous findings from this laboratory have shown that apoA-II contains specific phospholipid-binding sites (Jackson et al., 1973a, b; Lux et al., 1972c).

Materials and Methods

Preparation of Lipoproteins and Apoproteins. All lipoproteins were isolated by ultracentrifugal flotation in a Beckman/Spinco ultracentrifuge (Model L2-65B) using a 60 Ti rotor. The centrifugations were performed at 8° for 20 hr at 55,000 rpm. Very low density lipoproteins (VLDL) were isolated from plasma of fasting subjects with type IV or V hyperlipoproteinemia (Fredrickson and Levy, 1972) by flotation at plasma density. The VLDL were delipidated and the major apoproteins were fractionated by chromatography of apoVLDL on Sephadex G-150 and DEAE-cellulose (Brown et al., 1969, 1970). Low density lipoproteins (LDL) were obtained from normal fasting subjects by flotation between densities 1.020 and 1.050 g/ml (Gotto et al., 1972). HDL were isolated by ultracentrifugation of plasma between densities 1.063 and 1.210 g/ml. After centrifugation, the HDL were refloated at density 1.210 g/ml and dialyzed overnight against 0.01% EDTA. The HDL were delipidated with diethyl ether–ethanol (3:1) and apoA-I and apoA-II were fractionated on Sephadex G-150 as previously described (Jackson and Gotto, 1972). Homogeneity of all apoproteins used in the study was determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Weber and Osborn, 1969) and urea (Davies, 1964), by amino acid analysis and by immunodiffusion techniques using specific antisera prepared against each apoprotein.

Iodination of ApoA-II. ApoA-II was iodinated with ^{125}I using a modification of the iodine monochloride method of McFarlane (1964). To the apoprotein (1 mg) in 0.1 ml of 0.1 *M* glycine–NaOH buffer (pH 10.0) was added 0.05 ml of 3 mCi of Na^{125}I (Amersham-Searle) in the same glycine buffer. Iodine monochloride (0.05 ml), which was prepared by diluting a stock solution (0.02 *M*) with 2 *N* sodium chloride so as to provide one atom of iodine per molecule of apoA-II (mol wt 17,400), was then added to the apoprotein. After 30 S at 4°, the ^{125}I-labeled apoA-II was diluted to give 0.5 mg/ml and was dialyzed overnight at 4° against 0.05 *M* Tris-HCl (pH 8.0) with several changes of buffer. Chromatography of the labeled apoprotein on Sephadex G-150 yielded a single peak of radioactivity which coincided with the elution profile of unlabeled apoA-II. By immunodiffusion, both [^{125}I]apoA-II and apoA-II gave single precipitin lines of complete identity when tested against anti-apoA-II. The labeled apoprotein was stored at −20° in several vials. A freshly thawed sample of labeled apoprotein was used for each experiment.

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[1] Abbreviations used are: HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins; apoA-I and apoA-II, major apoproteins of HDL; apoC-I, apoC-II, and apoC-III, major apoproteins of VLDL.

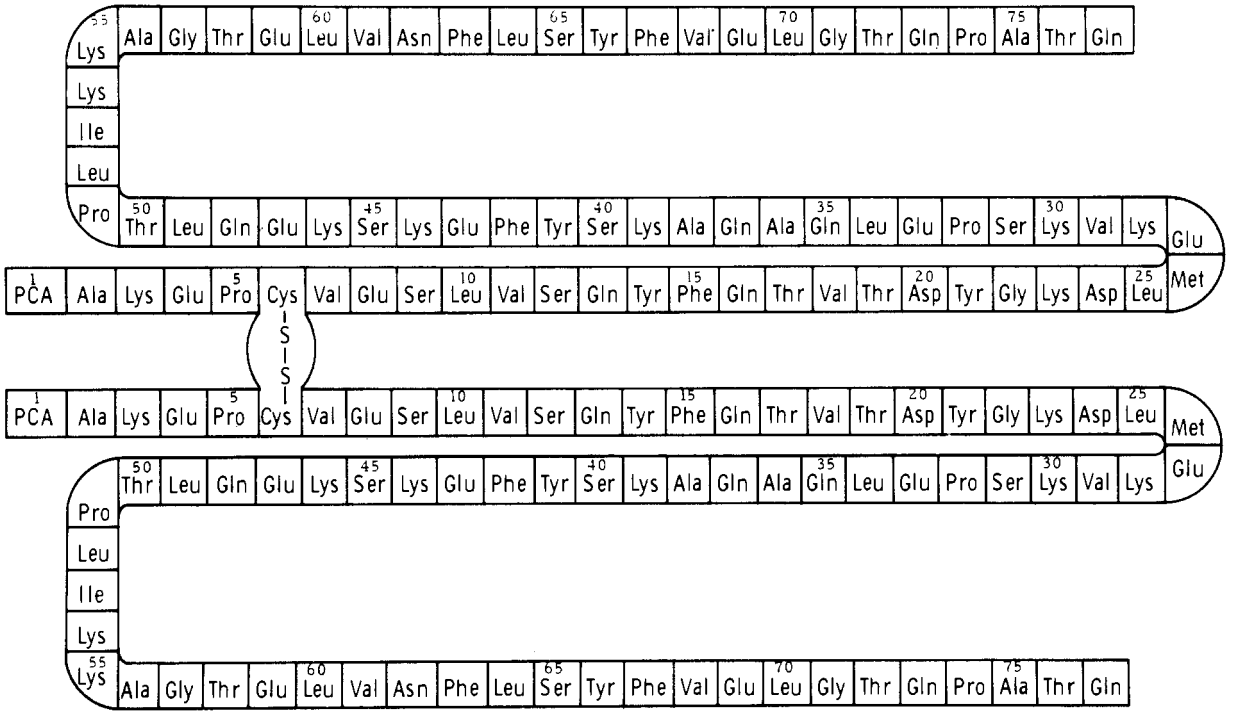

FIGURE 1: The amino sequence of apoA-II (as determined by Brewer et al., 1972; Lux et al., 1972a). Each chain is connected by a disulfide linkage at residue 6. PCA is pyrrolidonecarboxylic acid.

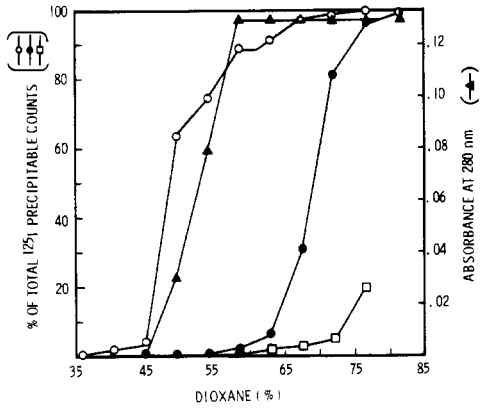

FIGURE 2: Separation of unbound [^{125}I]antigen by 1,4-dioxane. The various antigens were precipitated with different concentrations of 1,4-dioxane. After 5 min at 23°, the precipitates were isolated by centrifugation and counted in an autogamma counter. The antigens used were as follows: rabbit anti-apoA-II-[^{125}I]apoA-II complex (○); nonimmune rabbit γ-globulin (▲); [^{125}I]HDL (●); and [^{125}I]apoA-II (□).

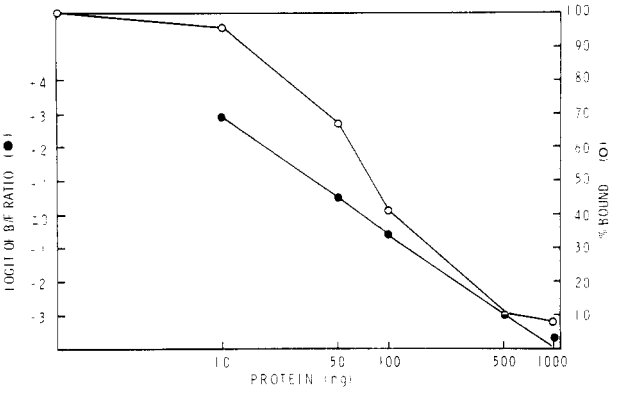

FIGURE 3: Standard curve for the radioimmunoassay of apoA-II. The assay was performed as described in the text. Anti-apoA-II was diluted 1:500. Approximately 2 ng of [^{125}I]apoA-II was displaced with various amounts of apoprotein. The logit of B/F ratio = ln [(cpm of competed antibody bound [^{125}I]apoA-II − cpm in background)/(cpm of total bound [^{125}I]apoA-II − cpm of competed antibody bound [^{125}I]apoA-II)].

Radioimmunoassay Procedures. Rabbit antisera were prepared against apoA-II by previously described methods (Lux et al., 1972d). The antisera were precipitated by adding an equal volume of saturated ammonium sulfate; the precipitate was dissolved in 0.9% NaCl and the precipitation was repeated a second time. The second precipitate was dissolved in 0.9% NaCl and dialyzed overnight. Assays were performed in 12 × 75 mm disposable Falcon polypropylene tubes (Becton, Dickinson and Co., Persippany, N.J.). All dilutions were made in a standard buffer containing 0.05 *M* potassium phosphate, 0.15 *M* NaCl, 0.01% merthiolate, 0.01% EDTA, and 5% nonimmune rabbit γ-globulin (pH 7.2). A typical incubation mixture contained in a final volume of 350 μl the following: 50 μl of [^{125}I]apoA-II (2–10 ng with 5000–15,000 cpm total); 50 μl of anti-apoA-II (1:1000 dilution); 50 μl of unlabeled apoA-II (1:1000 ng); and sufficient buffer to bring the volume to 350 μl. Control tubes which contained no anti-apoA-II were also included in order to evaluate the nonspecific precipitation with dioxane. In routine assays, the tubes were incubated at 23° for 4–6 hr with gentle shaking. Antibody-bound [^{125}I]apoA-II was separated from free apoprotein by 1,4-dioxane according to the procedure of Thomas and Ferin (1968). As shown in Figure 2, an anti-apoA-II–apoA-II complex or nonimmune rabbit globulins were precipitated at a concentration of 52% 1,4-dioxane, whereas radiolabeled HDL or apoA-II required concentrations greater than 65%. Accordingly, 2.0 ml of 60% 1,4-dioxane was added to each assay tube giving a final concentration of 52%; the tubes were incubated for 5 min at 23°. The precipitates were isolated by centrifugation at 6000 rpm for 30 min at 23° and counted at an accuracy of 1.5% in a autogamma counter (Packard Instrument Co.).

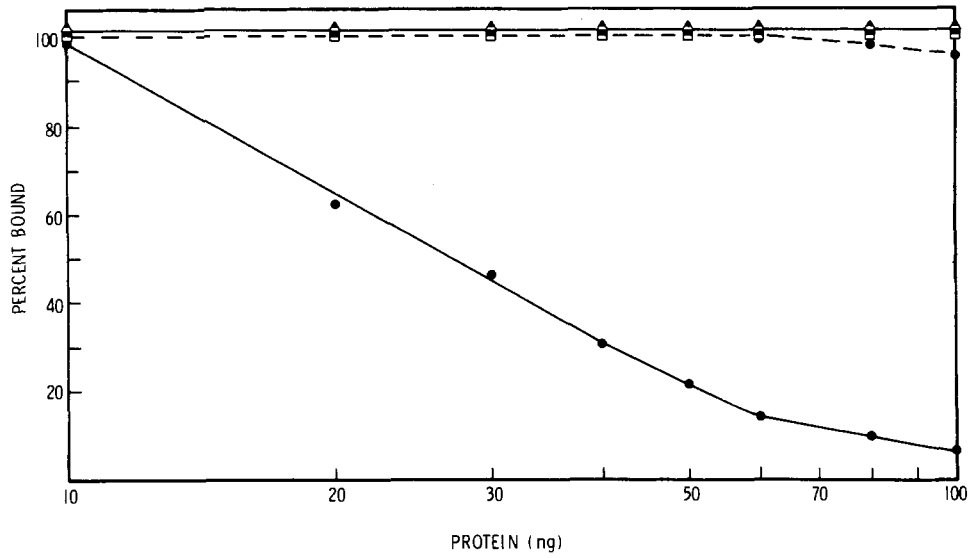

FIGURE 4: Specificity of the apoA-II radioimmunoassay. In these experiments, LDL (▲), apoC-I (△), apoC-II (■), apoC-III (□), apoA-I (● - - ●), and apoA-II (●—●) were assayed as described in the text. The anti-apoA-II was diluted 1:1000 and 10 ng of [^{125}I]apoA-II was used.

The results are expressed as the percent of the total counts bound.[2]

Other Procedures. The two cyanogen bromide fragments for apoA-II were prepared as described previously (Jackson and Gotto, 1972). Protein concentrations were determined by the method of Lowry et al. (1951) or by amino acid analysis.

Results

Quantitation of ApoA-II by Radioimmunoassay. Figure 3 represents a standard curve for the displacement of 2 ng of [^{125}I]apoA-II by unlabeled apoprotein. As increasing amounts of apoA-II were added, a characteristic sigmoidal curve was obtained when the percent [^{125}I]apoA-II bound was plotted vs. apoprotein added; the logit plot of this curve yielded a straight line. Although the assay was linear between 10 and 1000 ng of apoA-II, maximum precision was in the range of 10–100 ng. Within this range, the assay procedure was accurate to within 3% of the concentration as determined by amino acid analysis.

Specificity of Radioimmunoassay. To determine the specificity of the radioimmunoassay, we have measured other human plasma lipoproteins and apolipoproteins. As shown in Figure 4, the apoproteins (apoC-I, C-II, and C-III) isolated from human very low density lipoproteins were all ineffective in displacing [^{125}I]apoA-II. In addition, LDL and the other major HDL apoprotein (apoA-I) were ineffective.

Quantitation of ApoA-II in HDL and ApoA-II Phospholipid Complexes. Human whole plasma HDL and apoHDL gave displacement curves in the radioimmunoassay that were parallel between 80 and 200 ng of protein (Figure 5). In this experiment, 27 ng of apoA-II displaced 50% of the [^{125}I]apoA-II from the antibody. A corresponding degree of displacement was given by 100 ng of HDL-protein and by 140 ng of apoHDL; the latter is equivalent to 20 ng of apoA-II per 100 ng of apoHDL. Thus, the apoA-II concentration in native HDL was 1.4 times greater than that of

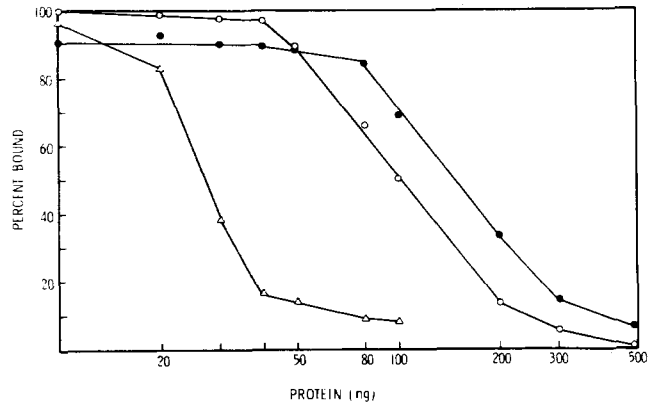

FIGURE 5: Radioimmunoassay of human male plasma HDL and apoHDL. HDL (○), apoHDL (●), and apoA-II (△) were added to 10 ng of [^{125}I]apoA-II and the radioimmunoassay was performed as described in the text. The anti-apoA-II was diluted 1:1000.

apoHDL. ApoA-I and apoA-II account for approximately 90% of HDL protein (Scanu, 1972). Therefore, in 100 ng of apoHDL, there is 18 ng of apoA-II and 72 ng of apoA-I. These values yield a weight ratio of apoA-I (molecular weight 28,331) to apoA-II (molecular weight 17,400) of 4.0:1 or a molar ratio of approximately 3:1. The greater effectiveness of HDL than apoHDL in displacing the [^{125}I]apoA-II was a reproducible finding and somewhat unexpected as discussed below. To determine if this result was related to the association of apoA-II with lipid, we have recombined apoA-II with egg phosphatidylcholine vesicles (Jackson et al., 1973a), isolated the lipid–protein complexes by ultracentrifugation in KBr (Jackson et al., 1974a), and have quantitated apoA-II in the isolated complex. In contrast to the curves for apoHDL and HDL, the curves obtained for both apoA-II and the apoA-II–phospholipid complex were nearly identical (Figure 6).

Antigenicity of Succinylated ApoA-II and of CNBr Fragments. A quantitative estimate of the effects of chemical modification by succinylation on the immunological activity of apoA-II was determined. As compared to apoA-II, succinylated apoA-II was about one-tenth as effective in displacing the [^{125}I]apoA-II (Figure 7).

[2] % bound = [(cpm of competed antibody bound [^{125}I]apoA-II − cpm in background)/(cpm of total antibody bound [^{125}I]apoA-II − cpm in background)].

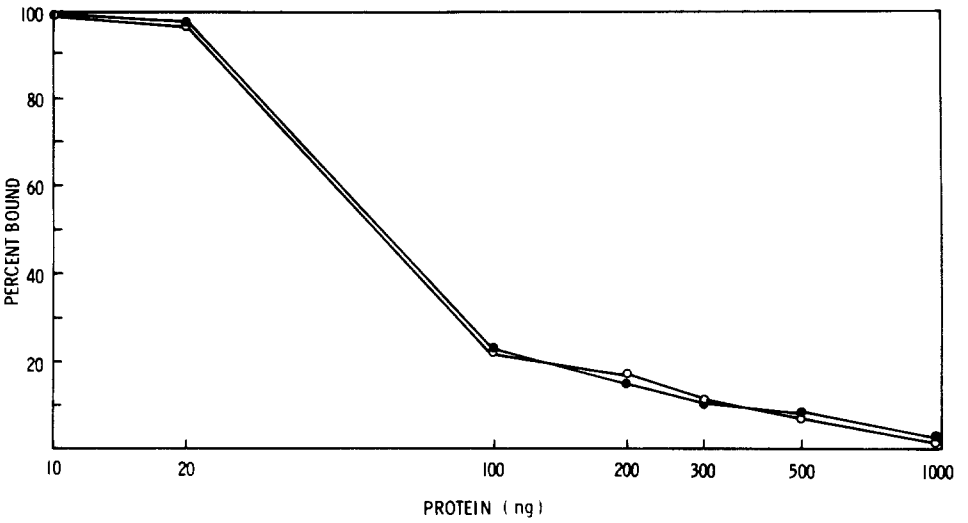

FIGURE 6: Radioimmunoassay of apoA-II and apoA-II recombined with egg phosphatidylcholine vesicles. Phosphatidylcholine vesicles were prepared as described by Jackson et al. (1974a). The phospholipid was added to apoA-II and the complexes were isolated by ultracentrifugation in KBr. The isolated complexes contained 1.2 mg of phospholipid/mg of apoA-II. Various amounts of this complex (○) and apoA-II (●) were added to 2 ng of [^{125}I]apoA-II and the radioimmunoassay was determined as described in the text. The anti-apoA-II was diluted 1:1000.

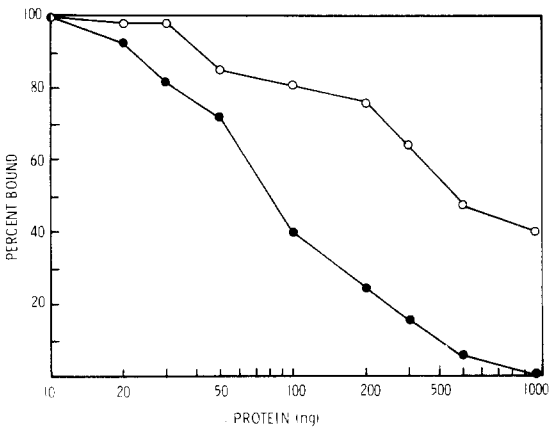

FIGURE 7: Effects of succinylation on the antigenic reactivity of apoA-II. ApoA-II was chemically modified by succinylation with [1,4-^{14}C]succinic anhydride. From the specific activity of the succinic anhydride, it was determined that 8.6 of the 9 lysines in the apoprotein were modified. The radioimmunoassay was performed as described in the text; 2 ng of [^{125}I]apoA-II was used. The anti-apoA-II was diluted 1:1000. ApoA-II (●); succinylated apoA-II (○).

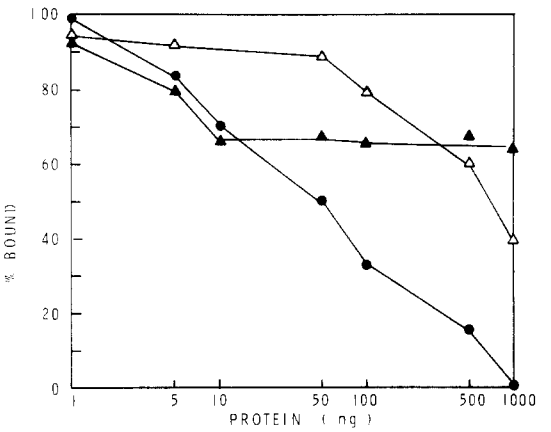

FIGURE 8: Immunogenicity of cyanogen bromide fragments of apoA-II. The two cyanogen bromide fragments of apoA-II were prepared as described by Jackson and Gotto (1972). The fragments were tested in the radioimmunoassay for apoA-II using anti-apoA-II diluted 1:1000. The assay contained 2 ng of [^{125}I]apoA-II and apoA-II (●) or the NH_2-terminal cyanogen bromide fragment, residues 1–26, with the disulfide intact (▲), or the COOH-terminal fragment, residues 27–77 (△).

As an initial approach to mapping the antigenic sites of apoA-II, the two cyanogen bromide fragments of apoA-II (Figure 1) were isolated and tested in the radioimmunoassay (Figure 8). The NH_2-terminal cyanogen bromide fragment (residues 1–26, with the disulfide intact) exhibited displacement up to a peptide concentration of 10 ng. At this concentration, only 30% of the anti-apoA-II antibodies were displaced by this fragment. By contrast, the COOH-terminal fragment (residues 27–77) required at least 50 ng before any displacement of [^{125}I]apoA-II occurred. This fragment gave 50% displacement after an addition of about 600 ng as compared to 55 ng for apoA-II.

Discussion

In the present report, we describe a specific radioimmunoassay for apoA-II. The assay is sensitive between the protein range of 10–100 ng and is specific for apoA-II. Using this assay procedure, it was determined that the amount of apoA-II in HDL was 1.4 times greater than that in apoHDL. The increased reactivity within HDL compared to apoHDL may be the result of denaturation of the protein with delipidation. Greater activity of apoA-II in HDL was, however, unexpected since Schonfeld and Pfleger (1974) found that only 5% of the apoA-I of HDL reacted in their radioimmunoassay. To explain the unavailability of apoA-I, Schonfeld and Pfleger have suggested that apoA-I complexes with lipid so as to make it unavailable to antibody. In a recent report, Fainaru et al. (1975) have detected greater than 90% of apoA-I in HDL by radioimmunoassay. The difference between this finding and that of Schonfeld and Pfleger may be related to differences in antigenic specificity of their respective antibodies or to assay techniques. In any case, the antigenic reactivity of apoA-II as shown in the present study was not affected by lipids as demonstrated by its reactivity in intact HDL (Figure 5) and in phosphatidylcholine vesicles (Figure 6). The fact that all of the apoA-II was detected by the radioimmunoassay suggests that the apoprotein is on the surface of the HDL particle. This as-

sumption is consistent with a number of physical and chemical studies (Jackson et al., 1975). Small angle X-ray scattering measurements of HDL_3 (Laggner et al., 1973) have suggested that the protein in HDL is in an outershell of 12 Å. A surface location is also consistent with the fact that over 90% of the lysine residues of the HDL apoproteins can be succinylated with no apparent effect on the structure of HDL (Scanu et al., 1968). However, succinylation of apoA-II does cause a tenfold decrease in the antigenic reactivity of the apoprotein which is similar to a previous report from this laboratory on the effects of maleylation on lipid binding and antigenic reactivity of apoA-II (Jackson et al., 1974). Whether this decreased antigenic reactivity is due to the negative charge introduced into the molecule or gross conformational changes as a result of the chemical modification remains to be answered.

Finally, to map-out the antigenic sites within the apoprotein, we have isolated the cyanogen bromide fragments from apoA-II and have shown that there are high affinity antibodies to the NH_2-terminal half of apoA-II but that the majority of the antibodies are directed toward the COOH-terminal half. Using the radioimmunoassay described in the present report, experiments are now in progress to determine the antigenic reactive regions within the COOH-terminal portion of the apoprotein.

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