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Immunochemistry of Human Very Low Density Lipoproteins: Apolipoprotein C-III†

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ABSTRACT: Apolipoprotein C-III (apoC-III) is a major protein constituent of human plasma very low density lipoproteins (VLDL) and a minor constituent of high density lipoproteins (HDL). The apoprotein is a single polypeptide chain of 79 amino acids and occurs in several forms differing only in their content of sialic acid. In the present report a quantitative radioimmunoassay (RIA) has been developed in order to study the immunochemical properties of apoC-III. Two individual rabbit antisera were used. It was observed that the COOH-terminal half (residues 41–79) of apoC-III containing 1 mol of sialic acid (apoC-III_1) contains the antigenic reactive region(s), whereas the NH_2-terminal half of the molecule was unable to react with anti-apoC-III antibodies. When the positively charged lysines of apoC-III_1 were chemically modified by acetic anhydride, the immunoreactivity was decreased by ~40% as measured by RIA in one rabbit antiserum. However, there was no decrease of immunoreactivity in another rabbit antiserum, suggesting that with the former antibody several populations exist directed at different regions of the apoprotein molecule. In addition, the immunoreactivity of apoC-III_1–phospholipid complexes was investigated and was found to be indistinguishable from that of apoC-III_1. Since the conformation of the apoprotein has drastically changed upon the addition of dimyristoylphosphatidylcholine (DMPC), this finding indicates that the gross conformational change of apoC-III_1 does not affect the immunochemical properties and that the antigenic reactive sites are probably located at the surface in apoC-III_1–DMPC complexes. The immunoreactivity of apoC-III was also found to be approximately the same in HDL or VLDL as that of the delipidated apoHDL or apoVLDL. Thus, the antigenic sites of apoC-III must be fully exposed on the surface of the lipoproteins.

ApoC-III[1] is the most abundant of the C-proteins in human plasma very low density lipoproteins (VLDL). The protein exists in at least three forms which differ only in their content of sialic acid. The apoprotein contains 79 amino acids and has no cysteine, cystine, or isoleucine; the amino acid sequence (Figure 1) has been determined (Shulman et al., 1974; Brewer et al., 1974). The physicochemical and lipid-binding properties of the protein have been reviewed in great detail (Jackson et al., 1976; Morrisett et al., 1977; Bradley & Gotto, 1978; Smith et al., 1978). A previous report from this laboratory (Sparrow et al., 1977) has shown that thrombin digestion of apoC-III_1 yielded two fragments (residues 1–40 and 41–79) of the protein by cleavage at Arg_{40}-Gly_{41}. Only the COOH-terminal half (residues 41–79) was able to interact with phospholipid. The immunochemical properties of apoC-III_1 and the thrombin cleavage fragments have not been established. In the present study we report the immunoreactivity of the thrombin fragments and the role of lysine toward the immunochemical properties of apoC-III_1. Modification of the lysine residues was chosen because in the known antigenic structures of sperm whale myoglobin (Atassi, 1975) and of egg white lysozyme (Atassi & Lee, 1978) positively charged lysines were found in all of the antigenic reactive regions. Since the apoprotein displays a helical structure in isolated protein–phospholipid complexes (Morrisett et al., 1973), the role of conformation

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[1] Abbreviations used: HDL, high density lipoproteins; apoA-I and apoA-II, apoprotein constituents of HDL; VLDL, very low density lipoproteins; apoC-III, an apolipoprotein constituent of VLDL; apoC-III_1, apoC-III containing 1 mol of sialic acid; DMPC, dimyristoylphosphatidylcholine; CD, circular dichroism; RIA, radioimmunoassay.

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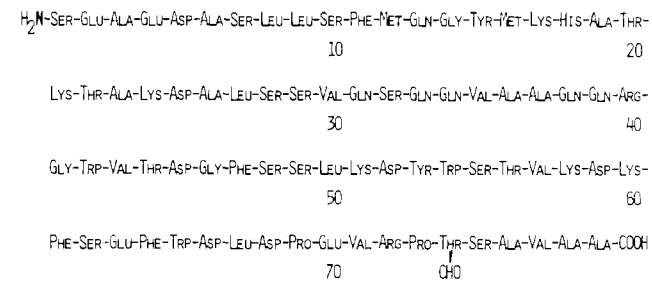

FIGURE 1: Amino acid sequence of apoC-III as determined by Brewer et al. (1974).

in the presence of phospholipid on the immunochemical properties of apoC-III is also discussed.

Materials and Methods

Preparation of ApoC-III$_1$ and Isolation of the Thrombin Fragments. The VLDL proteins were fractionated by a modification of the procedure of Morrisett et al. (1973). A column (2.5 × 200 cm) of Sephadex G-100 was prepared and equilibrated with 0.001 M sodium decyl sulfate and 0.1 M Tris-HCl, pH 8.0. Lyophilized, lipid-free apoVLDL proteins were dissolved in a minimal volume of the same buffer and applied to the column at room temperature. The fractions containing the C-proteins were pooled and desalted. ApoC-III$_1$ was isolated by DEAE-Sephadex chromatography at 4 °C according to the method described by Morrisett et al. (1973). Homogeneity of the apoC-III$_1$ was determined by polyacrylamide gel electrophoresis in the presence of 8 M urea, pH 8.2 (Davies, 1964), by amino acid analysis, and by immunodiffusion techniques using specific antisera against each apoprotein. The 1–40 and 41–79 fragments of apoC-III$_1$ were obtained from thrombin cleavage as described by Sparrow et al. (1977). The purity of each fragment was judged by its migration as a single band on polyacrylamide gel electrophoresis in 8 M urea, pH 8.2. Amino acid compositions were in good agreement with the sequence.

Acetylation of ApoC-III$_1$. Ten milligrams of apoC-III$_1$ was acetylated with acetic anhydride according to the method of Bethune et al. (1964). To the apoprotein in 2 mL of 50% saturated sodium acetate, pH 7.5, containing 3 M guanidine hydrochloride was added a 10-fold excess of acetic anhydride in 0.1 mL of redistilled benzene. The pH was maintained at 7.5 by the addition of 0.1 M NaOH. After 30 min at 24 °C, the modified protein was treated with 2 mL of 2 M NH_2OH, pH 8.0, for 1 h at 4 °C to remove any acetyl groups from tyrosine. The protein was desalted on Bio-Gel P-2 and lyophilized. The homogeneity of the acetylated apoC-III$_1$ was determined by polyacrylamide gel electrophoresis at pH 8.2 in the presence of 8 M urea. The ultraviolet spectrum showed that the acetylated apoC-III$_1$ had a similar spectrum to apoC-III$_1$. For determination of the extent of the acetylation, guanidination of the ϵ-amino groups of the protein was attempted according to the procedure of Klee & Richards (1957).

Preparation of Antisera. New England white rabbits were immunized as described by Jackson et al. (1977). Each rabbit first received 200 μg of apoC-III$_1$ in 0.5 mL of saline (0.9%) vortexed with 0.5-mL of complete Freund's adjuvant. The emulsion was then injected intradermally into 50 sites on the back of the animals. After 30 days the rabbits were boosted intramuscularly with a dose of 1 mg of apoC-III$_1$ in 0.5 mL of complete Freund's adjuvant and then boosted every week until reasonable titers were achieved. The titer of each antiserum was observed by the double-immunodiffusion technique and by radioimmunoassay using ^{125}I-labeled apoC-III$_1$ (Figure 5). The antiserum was specific against all forms of apoC-III and did not cross-react with other apoproteins.

Iodination of ApoC-III$_1$. ApoC-III$_1$ was iodinated with ^{125}I by using a modification of McFarlane's method (McFarlane, 1964). Detailed procedures have been described elsewhere (Mao et al., 1975). By immunodiffusion, both [^{125}I]apoC-III$_1$ and apoC-III$_1$ gave single precipitin lines of complete identity when tested against anti-apoC-III$_1$. The labeled apoprotein was stored at −20 °C in several vials. A freshly thawed sample of labeled apoprotein was used for each experiment.

Radioimmunoassay Procedures. Assays were performed in 12 × 75 mm disposable Falcon polypropylene tubes (Becton, Dickinson and Co., Persippany, NJ). All dilutions were made in a buffer containing 0.05 M potassium phosphate, 0.15 M NaCl, 0.01% NaN_3, 0.01% EDTA, and 5% nonimmune rabbit γ-globulin (pH 7.2). A typical incubation mixture contained the following in a final volume of 350 μL: 50 μL of [^{125}I]-apoC-III$_1$ (10–20 ng); 50 μL of unlabeled apoC-III$_1$ (30–4000 ng); anti-apoC-III$_1$ (1:10–1:20 dilution); sufficient buffer to bring the volume to 350 μL. Control tubes which contained nonimmune rabbit serum were also included in order to evaluate the nonspecific binding. Less than 2% of the total [^{125}I]apoC-III$_1$ was found in nonspecific binding. In routine assays, the tubes were incubated at 37 °C for 1 h with gentle shaking. The tubes were then incubated at room temperature for 8–12 h. Antibody-bound [^{125}I]apoC-III$_1$ was separated from free apoprotein by adding 2 mL of 1,4-dioxane (75%); the final concentration was 65% 1,4-dioxane. The tubes were incubated at room temperature for 15 min. The precipitates were centrifuged at 5000 rpm for 30 min at 24 °C, the supernatant was removed, and the tubes were counted in an Autogamma counter (Packard Instrument Co.). The results are expressed as follows:

$$\% \text{ bound} = B/B_0 = \frac{[(\text{cpm of bound } [^{125}\text{I}]\text{apoC-III}_1 \text{ in the presence of apoprotein}) - (\text{cpm in *NSB})]}{[(\text{cpm of bound } [^{125}\text{I}]\text{apoC-III}_1) - (\text{cpm in *NSB})]} \quad (1)$$

*NSB = nonspecific binding of [^{125}I]apoC-III$_1$ to nonimmune rabbit serum; in all the assays less than 2% of the total [^{125}I]apoC-III$_1$ was bound nonspecifically.

Isolation of Phospholipid–Protein Complexes. Details of the isolation procedure of the lipid–protein complexes are described elsewhere (S. J. T. Mao, J. T. Sparrow, and A. M. Gotto, unpublished experiments). The apoprotein (0.5 mg) was initially mixed with DMPC in a w/w ratio of 1:1, 4:1, and 16:1 to obtain complexes with different ratios of lipid to protein. The mixtures were incubated at 24.5 °C for 48 h, pH 7.4, and were then subjected to density gradient ultracentrifugation in KBr (Mao et al., 1977) to isolate the DMPC–apoC-III$_1$ complexes. The isolated complexes had a lipid/protein ratio of 1.9:1, 4.2:1, and 16.1:1, respectively.

Other Methods. Circular dichroism (CD) was measured at 25 °C on a Cary 61 spectropolarimeter using cells of 0.5-mm path length. The percent α helix = $100(\theta_{222} + 3000)/(36\,000 + 3000)$ (Morrisett et al., 1973). Peptide concentrations were determined by amino acid analysis. Phosphorus was determined by the method of Bartlett (1959).

Results

Acetylation of ApoC-III$_1$. ApoC-III$_1$ was chemically modified with acetic anhydride to study the role of lysine in the antigenic reactivity of apoC-III$_1$. The homogeneity of the derivative was determined by polyacrylamide gel electropho-

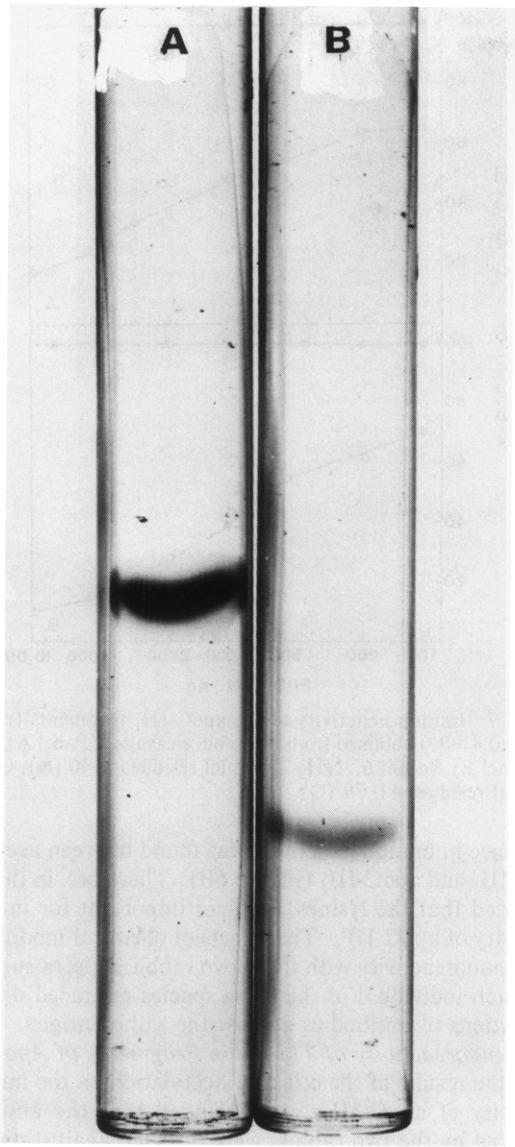

FIGURE 2: Polyacrylamide gel electrophoresis of apoC-III_1 and acetyl-apoC-III_1. The gels were run on 7.5% acrylamide in a 0.03 M Tris–glycine buffer containing 8 M urea, pH 8.2, for 3 h at 3 mA/gel. Each gel was loaded with 40 μg of the protein; the gels were stained with Coomassie blue. (Panel A) ApoC-III_1. (Panel B) Acetyl-apoC-III_1.

resis in the presence of 8 M urea, pH 8.2. As shown in Figure 2, the acetyl-apoC-III_1 was electrophoretically homogeneous and the electromobility was greater than that of apoC-III_1. The extent of modification was further confirmed by reaction with 1 M *O*-methylisourea. After reaction, acid hydrolysates analyzed by amino acid analysis revealed no detectable homoarginine, confirming that the acetylation was complete. Under identical conditions, 98% of the lysines in native apoC-III_1 were converted to homoarginine.

Circular Dichroism of Acetyl-apoC-III_1. A previous report from this laboratory (Morrisett et al., 1973) has shown that apoC-III_1 exhibited a disordered structure containing ~16% helical content as determined by CD measurements. In the present study it was found that chemical modification of apoC-III_1 by acetic anhydride did not significantly alter the conformation of the apoprotein (Figure 3A).

Separation of Bound and Unbound [^{125}I]ApoC-III_1 from Anti-apoC-III_1. As shown in Figure 4, at a concentration of 65% 1,4-dioxane, anti-apoC-III_1 complexes or nonimmune rabbit γ-globulins were precipitated, whereas more than 95%

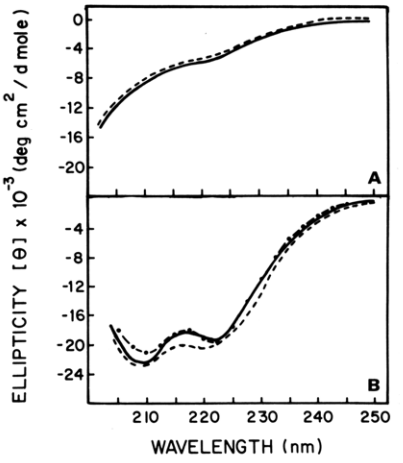

FIGURE 3: Circular dichroic spectra of apoC-III_1, acetyl-apoC-III_1, and apoC-III_1–phospholipid complexes. The spectra were recorded at 25 °C at a protein concentration of 0.66 mg/mL. (Panel A) ApoC-III_1 (—) and acetyl-apoC-III_1 (- - -) in the absence of lipid. (Panel B) The complexes were obtained from apoC-III_1–phospholipid mixtures by ultracentrifugation in KBr density gradients between a density of 1.15 and 1.04 g/mL by using a Beckman SW 50.1 rotor at 45 000 rpm (248000*g*) for 96 h at 25 °C. Initial ratios of protein to phospholipid (w/w) were 1:1 (–·–), 4:1 (—), and 16:1 (- - -).

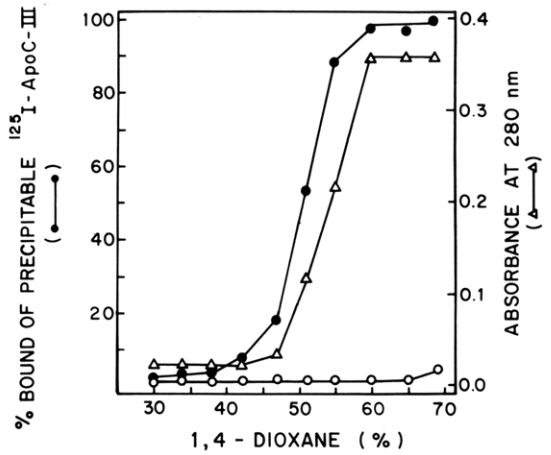

FIGURE 4: Separation of unbound [^{125}I]apoC-III_1 by 1,4-dioxane. The [^{125}I]apoC-III_1–antibody complex was precipitated with different concentrations of 1,4-dioxane. After incubation for 5 min at 24 °C, the precipitates were centrifuged at 5000 rpm for 30 min and were counted in an Autogamma counter. [^{125}I]ApoC-III (○), [^{125}I]-apoC-III–anti-apoC-III_1 complex (●), and anti-apoC-III_1 (△). In the case of anti-apoC-III_1, the precipitation was estimated by light scattering at 280 nm.

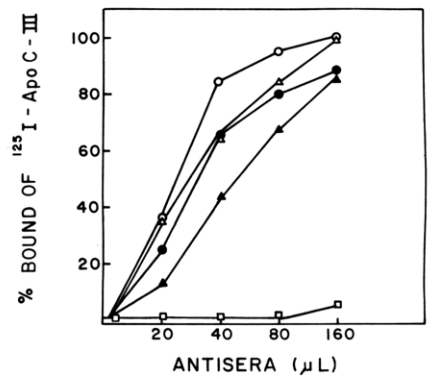

FIGURE 5: Binding curves of [^{125}I]apoC-III_1 to anti-apoC-III_1 antiserum. [^{125}I]ApoC-III_1 (10 ng) was used to determine the titers of rabbit antiserum 3-1 (▲) and 3-2 (△) and antiserum 6-1 (●) and 6-2 (○). Nonimmune rabbit serum (□) was used as a control.

of radiolabeled apoC-III_1 remained in solution. These results were similar to the previous report on an apoA-II radioim-

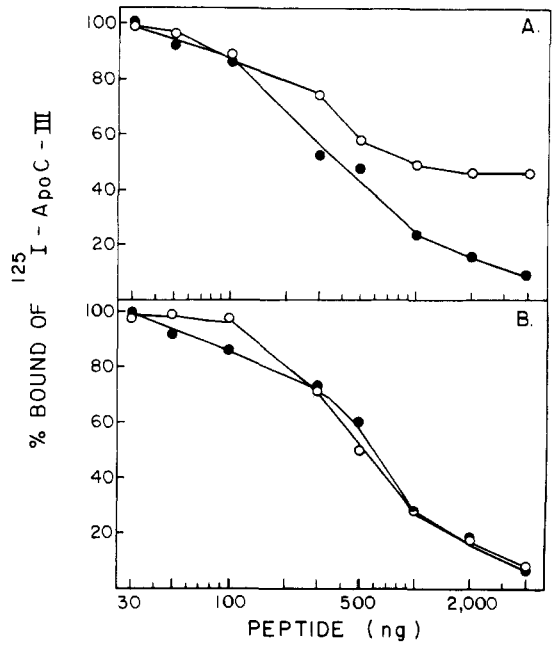

FIGURE 6: Effects of acetylation on the immunoreactivity of apoC-III$_1$. (Panel A) Rabbit 3. (Panel B) Rabbit 6. The antiserum was the pool of rabbit antiserum 3-1 and 3-2 and of rabbit antiserum 6-1 and 6-2, respectively. The antiserum was diluted 1:10 for rabbit 3 and 1:12 for rabbit 6; 10 ng of [^{125}I]apoC-III$_1$ was used. ApoC-III$_1$ (●); acetyl-apoC-III$_1$ (○).

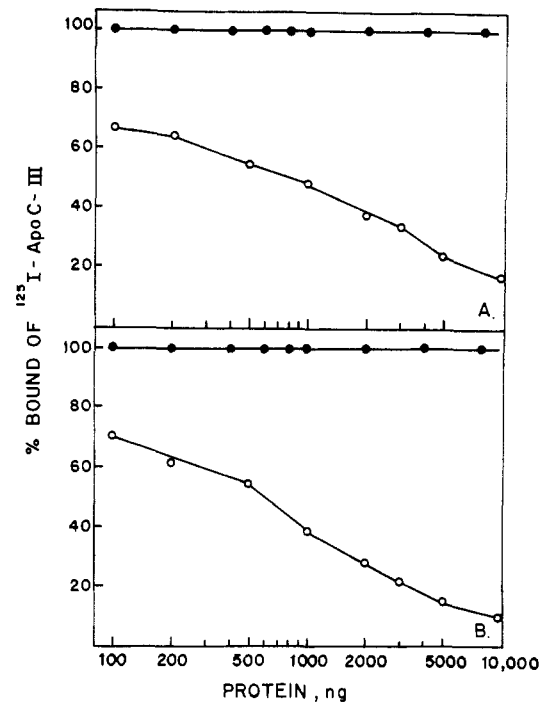

FIGURE 7: Immunoreactivity of the apoC-III$_1$ fragments (residues 1–40 and 41–79) obtained from thrombin cleavage. (Panel A) Rabbit 3. (Panel B) Rabbit 6. NH_2-Terminal residues 1–40 (●); COOH-terminal residues 41–79 (○).

munoassay (Mao et al., 1975).

Radioimmunoassay. The titer of each antiserum was examined, and the results are expressed as percent bound of radiolabeled apoC-III$_1$ to anti-apoC-III$_1$ (Figure 5). Two antisera from rabbit 3 and rabbit 6 were employed separately for the assays. Antiserum 3-1 and 3-2 or antiserum 6-1 and 6-2 represented two bleeding periods after six and seven injections, respectively. The addition of 120 μL of antiserum 3-2 or 6-2 (1:10 dilution) to [^{125}I]apoC-III$_1$ (10 ng) resulted in maximum binding. Rabbit antiserum 6 was shown to have a better binding titer to [^{125}I]apoC-III$_1$ than antiserum 3. Nonimmune rabbit serum did not bind to [^{125}I]apoC-III$_1$. Two antisera (3-1 and 3-2 or 6-1 and 6-2) from each rabbit were then pooled for the studies of the immunochemical properties of apoC-III$_1$. The standard curves from each rabbit antiserum are given in Figure 6. In panels A and B (Figure 6), both antisera 3 and 6 showed a sensitivity in the range of 100–2000 ng of unlabeled apoprotein for displacing 10 ng of [^{125}I]-apoC-III$_1$. For inhibition of 50% binding of [^{125}I]apoC-III$_1$ to anti-apoC-III$_1$, 300–500 ng of unlabeled apoC-III was required.

Immunochemistry of Acetylated ApoC-III$_1$. A quantitative estimate of the effects of chemical modification by acetylation on the immunochemical activity of apoC-III$_1$ was determined by using the above antiserum. The result of the RIA using antiserum from rabbit 3 showed that the acetylated apoC-III$_1$ was about threefold less effective in displacing 50% of [^{125}I]apoC-III$_1$ from its antibodies as compared to apoC-III$_1$ (Figure 6A). The maximal displacement was achieved when 1000 ng of acetyl-apoC-III$_1$ was employed. Thus, the immunoreactivity was decreased ~40% as shown in the RIA curve. The observed decrease in immunoreactivity of acetylated apoC-III$_1$ was probably not due to a conformational change after chemical modification since both native and acetylated apoC-III$_1$ exhibited nearly identical CD spectra (Figure 3A). These findings suggested that the lysines were important in the immunoreactivity of apoC-III$_1$ with this antibody. However, in rabbit antiserum 6 no significant difference in immunoreactivity was found between acetylated apoC-III$_1$ and apoC-III$_1$ (Figure 6B). Therefore, in this case, it seemed that the lysines were not important for immunoreactivity of apoC-III$_1$. The effects of chemical modification on immunoreactivity with these two rabbit antisera suggested that each individual of the same species produced different populations of antibodies against the same antigen.

Immunoreactivity of Thrombin Fragments of ApoC-III$_1$. From the results of the effect of acetylation on the immunoreactivity of apoC-III$_1$, we concluded that the antibodies produced by the two rabbits were directed against different regions of apoC-III$_1$. It was, therefore, of interest to investigate the location of the major antigenic determinants in these two different antisera by utilizing the two thrombin fragments of apoC-III$_1$ reported by Sparrow et al. (1977). We found (Figure 7) that regardless of the antiserum, the NH_2-terminal half of the apoC-III$_1$ molecule (residues 1–40) did not displace the [^{125}I]apoC-III$_1$ from the antibodies even when 10000 ng of the fragments was used. By contrast, at a concentration of 10000 ng of the COOH-terminal half (residues 41–79), more than 80% of the [^{125}I]apoC-III$_1$ could be displaced from both antisera. Obviously, most of the antibody populations were directed toward the COOH-terminal half of the protein. For inhibition of 50% binding of [^{125}I]apoC-III$_1$ to the antibodies (Figure 7), ~600–1000 ng of unlabeled 41–79 fragment was required. Compared to the displacement by whole apoC-III$_1$ (Figure 6), the 41–79 fragment has only about one-half of the immunoreactivity of the apoC-III$_1$, thus suggesting that the 41–79 fragment has less affinity for the antibodies than apoC-III$_1$.

Immunoreactivity of ApoC-III$_1$ in Isolated ApoC-III$_1$–Phospholipid Complexes. Morrisett et al. (1973) have reported that apoC-III was able to recombine with egg phosphatidylcholine and DMPC vesicles and have isolated the lipid–protein complexes by ultracentrifugation in KBr. In this report, we have isolated the complexes with different lipid/protein ratios in order to test the effects of the presence of

Table I: Immunoreactivity[a] (Percent) of ApoC-III$_1$ in the Presence of Phospholipid As Determined by RIA

lipid/protein ratio (w/w)	rabbit antiserum 3			rabbit antiserum 6		
	amount of protein in the complex			amount of protein in the complex		
	300 ng	500 ng	1000 ng	300 ng	500 ng	1000 ng
protein alone	63.0	48.1	33.3	45.0	37.4	26.6
1.9	63.5	48.3	35.6	46.3	38.5	29.2
4.2	62.5	49.3	36.0	44.5	36.2	26.4
16.1	63.0	48.8	31.4	37.7	33.8	26.6
mean ± SD	63.0 ± 0.40	48.6 ± 0.54	34.1 ± 2.14	43.4 ± 3.86	36.5 ± 2.02	27.2 ± 1.33

[a] Immunoreactivity was determined by the percent bound of [^{125}I]apoC-III$_1$ to anti-apoC-III$_1$ antibodies.

phospholipid on the immunoreactivity of apoC-III$_1$. The initial mixtures (w/w) of phospholipid (DMPC) to protein of 1:1, 4:1, and 16:1 were subjected to density gradient ultracentrifugation in KBr. The isolated complexes had lipid/protein ratios (w/w) of 1.9:1, 4.2:1, and 16.1:1, respectively, as given in Table I. [Experimental details and discussions are discribed elsewhere. (S. J. T. Mao, J. T. Sparrow, and A. M. Gotto, unpublished experiments.)] The content of α helix in apoC-III$_1$ was drastically increased in the presence of phospholipid to ~75% as determined by CD measurements (Figure 3B) of the ellipiticity at 222 or 208 nm of the protein–DMPC complexes.

When the immunoreactivities of the isolated complexes were compared to those of the apoprotein (Table I), similar immunoreactivity was found in each rabbit antiserum. At 300, 500, and 1000 ng of apoprotein or recombined apoC-III$_1$, we found the percent bound [^{125}I]apoC-III$_1$ to be 63.0 ± 0.40, 48.6 ± 0.54, and 34.1 ± 2.1%, respectively, in rabbit 3 and an average value of 43.4 ± 3.9, 36.5 ± 2.0, and 27.2 ± 1.3% in rabbit 6. Therefore, we conclude that increased amounts of phospholipid bound to the apoprotein did not affect the immunoreactivity of apoC-III$_1$.

Immunoreactivity of ApoC-III in HDL and VLDL. Since the phospholipid (DMPC) did not affect the immunoreactivity of apoC-III$_1$ in recombined phospholipid–protein complexes, it was of interest to determine whether the presence of other proteins and neutral lipids such as triglycerides or cholesterol would affect the immunoreactivity of apoC-III$_1$. In Figure 8A, we show that the immunoreactivity of apoC-III$_1$ in apoHDL and HDL was indistinguishable. The displacement curves were parallel to apoC-III$_1$ standards. For displacement of 50% of [^{125}I]apoC-III$_1$ from antibodies, ~70 ng of apoC-III$_1$ was equivalent to 3400 ng of HDL. Human VLDL and apoVLDL gave displacement curves in the RIA that were parallel between 125 and 1000 ng of protein (Figure 8B). A slightly higher immunoreactivity of apoC-III was found in VLDL than in apoVLDL. At 50% displacement of [^{125}I]-apoC-III$_1$ from antibodies, ~65 ng of apoC-III was equivalent to 500 ng of VLDL. Thus, approximately 2% (w/w) of apoC-III is present in HDL and 15% in VLDL, resulting in an apoC-III concentration in VLDL 7 times greater than that in HDL at equivalent protein weight.

Discussion

Using the radioimmunoassay techinque, we have shown that although different rabbits produced different antibodies against apoC-III$_1$, the majority of the antibodies were directed toward the COOH-terminal of the apoprotein. Acetylation of apoC-III$_1$ affected the immunoreactivity with one antiserum, but the protein was still able to inhibit 50% of the [^{125}I]apoC-III$_1$ binding to anti-apoC-III$_1$ antibodies (Figure 6A), thus suggesting that there was probably more than one antigenic site in apoC-III$_1$. Since the NH_2-terminal half was immunoinactive, we suggest that one or more of the three lysines (Figure 1, residues 51, 58, and 60) in the sequence 41–79 are involved in the antigenic sites. Since all the lysines were modified simultaneously, we cannot determine which lysine falls within the reactive region.

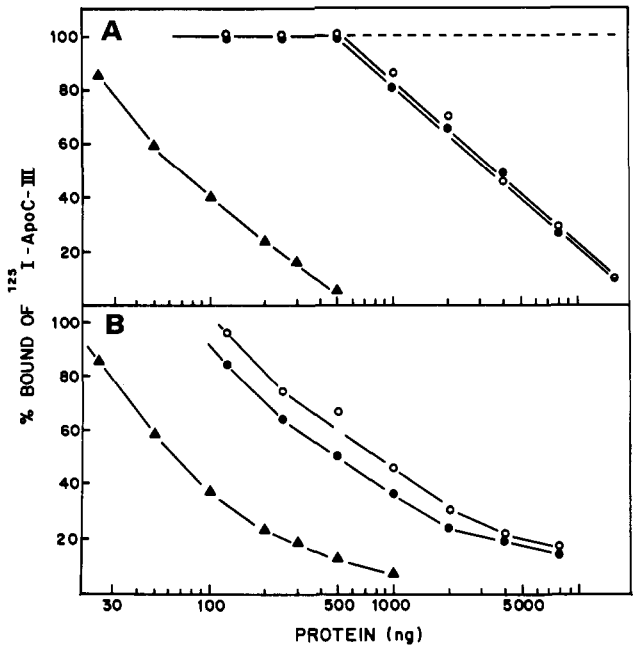

FIGURE 8: Radioimmunoassay of apoC-III in HDL and in VLDL. Rabbit antisera 3 and 6 were pooled. The antiserum was then diluted 1:120; 1 ng of [^{125}I]apoC-III$_1$ was used. (Panel A) HDL (●); apoHDL (○); apoC-III (▲); apoA-I, -A-II, -C-I, and -C-II (---). (Panel B) VLDL (●); apoVLDL (○); apoC-III (▲).

When apoA-II was recombined with lipid in vitro (Mao et al., 1975; Schonfeld et al., 1977a), the apoA-II was fully quantitated by RIA. However, only 10% of the expected apoA-I could be detected in HDL (Schonfeld & Pfleger, 1974). From these results, Mao et al. (1975) and Schonfeld et al. (1977b) have thus concluded that apoA-II was more exposed on the surface of HDL than apoA-I. Therefore, it was of interest to investigate the effects on immunoreactivity of binding of phospholipid (DMPC) with apoC-III$_1$.

When isolated apoC-III$_1$ was recombined with phospholipid, there was no decrease of immunoreactivity regardless of the lipid to protein stoichiometry (Table I), indicating that the antigenic reactive regions of apoC-III$_1$ reside on the surface of the lipid–protein complexes. Likewise, nearly identical immunoreactivity was observed in apoHDL and HDL or in apoVLDL and VLDL, respectively, (Figure 8), suggesting that the apoprotein is on the surface of the HDL and VLDL particles. Therefore, neutral lipids such as triglycerides, cholesteryl esters, and cholesterol did not affect the immunoreactivity of the apoprotein. The slightly decreased reactivity within apoVLDL compared to VLDL may be the result of

aggregation of the proteins during the delipidation procedure. These results indicate that the antigenic structures of apoC-III between HDL and VLDL are similar. Since more than 85% of the immunoreactivity of apoC-III is located in the COOH-terminal half (residues 41-79), we suggest that the orientation of this part of the molecule in HDL and VLDL is probably identical.

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Immunochemical Study of the Structure of Poly(adenylic acid)†

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ABSTRACT: Antibodies specific for poly(adenylic acid) (M_r ~700 000) [poly(A)] were generated by immunization of rabbits with poly(A) covalently linked to bovine serum albumin via the 3′-terminal residue of the polynucleotide. The antibodies precipitated with poly(A) as well as with nucleotide–protein conjugates, regardless of the purine or pyrimidine base, but not with conjugates of nucleosides. Precipitation was also seen with DNA and RNA and with polyribose phosphate. Agglutination of *Hemophilus influenzae* type b, which has a capsule of polyribose phosphate, could also be demonstrated. In a competitive binding assay, the antibodies bound poly(A) with an affinity at least 4 orders of magnitude greater than that of oligo(adenylic acids) as large as A_{10}. Cross-reaction was seen with poly(I), poly(C), and poly(G) and with polyribose phosphate. Unlike antibodies generated by poly(A)–MBSA, the specificity of these antibodies was directed at ribose phosphate residues, suggesting a conformation of poly(A) in solution in which the purine residues are stacked "inside" a single-stranded helix with the ribose phosphate residues extending outward. This conformation is apparently disrupted by reaction with MBSA. Earlier physicochemical studies are in agreement with the type of structure derived from our data. Poly(A) can, therefore, be envisaged as a dense cloud of negative charges arranged in a helical array. There is evidence that the conformation of poly(A) in RNA is the same; therefore, any theory for the role of the poly(A) "tail" in mRNA should take into account the contribution of a highly negatively charged segment at the 3′ end. These studies support the efficacy of using immunochemical methods to study nucleotide conformation if care is taken to ensure that the conformation of the nucleotide in the antigen is in the native state.

The ability of antibodies to recognize conformation in polypeptides and proteins is well established (Gerwing & Thompson, 1968; Young & Leung, 1970; Brown, 1962; Lehrer & Van Vunakis, 1965; Arnon, 1973). Similar recognition of polynucleotide conformation comes from immunological studies on ribosomal RNA (Souleil & Panijel, 1968) and on double- and triple-helical oligonucleotides (Stollar, 1975), as well as from experiments with antibodies to specific oligonucleotide sequences (D'Alisa & Erlanger, 1974, 1976). With respect to the latter, it was shown that antibodies specific for AAA cross-reacted only slightly with A and poly(A) and not at all with denatured DNA, despite the fact that AAA is the triplet coden for lysine. Thus, the conformation of AAA must be unlike that of the triplet in DNA or in poly(A) and it is

† From the Department of Microbiology, Cancer Center/Institute of Cancer Research, Columbia University, New York, New York 10032. *Received April 30, 1979; revised manuscript received October 29, 1979.* This research was supported by grants from the National Institutes of Health (AI-06860 and GM 22966) and the National Science Foundation (NSF-PCM-77-19280). It is part of a dissertation submitted by Z.E.K. in partial fulfillment of the Ph.D. degree in the Department of Microbiology, Columbia University.

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