Radioimmunoassay of Beta Lipoprotein–Protein of Rat Serum

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ABSTRACT A double antibody radioimmunoassay for rat serum beta lipoprotein–protein (beta Lp–protein) is described. The protein was purified by ultracentrifugation, selective heparin-manganous precipitation, and gel filtration on Sephadex G-200. Antiserum was prepared in rabbits by biweekly immunization and absorbed with nonbeta lipoprotein containing rat serum. Iodination with ^{125}I and purification by gel filtration provided a radiolabeled protein which was > 98% displaced by purified beta lipoprotein in the immunoassay. The radioimmunoassay was sensitive to beta Lp–protein concentrations from 0.1 to 1.5 μg. Specificity of the immunoassay for beta Lp–protein was established by comparison of the displacement curves obtained with serum very low density lipoprotein (VLDL), low density lipoprotein (LDL), high density lipoprotein (HDL), and density (d) > 1.21 fractions and with the beta and alpha migrating lipoproteins eluted from paper electrophoretograms. Suitability of the assay for measuring beta Lp–protein in serum was established by demonstrating 100% recovery of beta lipoprotein added to whole serum and by the absence of immunoreactive beta Lp–protein in serum of orotic acid–treated rats. Examination of sera from six other vertebrates species revealed partial cross-reactivity. Normal rat serum was found to contain 0.25 ±0.01 mg/ml of beta Lp–protein and hepatic production by an isolated perfused rat liver system was determined as 0.145 mg/hr.

INTRODUCTION

Recent studies characterizing the apoprotein moiety of the rat serum lipoproteins have suggested that, as with human serum lipoproteins, beta apoprotein ("B" protein) is the predominant protein of low density lipoproteins (LDL), and alpha apoprotein ("A" protein) is the major protein constituent of high density lipoproteins (HDL) (1, 2). Both "A" and "B" apoproteins have also been found in rat serum very low density lipoproteins (VLDL), but the "C" apoprotein identified in human VLDL (3) has not, as yet, been reported in any rat lipoprotein fraction. The demonstration of altered rates of hepatic synthesis and (or) release of the apoprotein moiety of serum lipoproteins in nephrosis (4, 5), after alcohol ingestion (6), in carbohydrate-induced hyperlipemia in rats (7, 8) and in the hereditary obese mouse (9), raises the possibility that these changes may represent the primary event underlying the plasma lipid abnormalities seen in these conditions.

The development of a rapid and specific method for the identification, isolation, and measurement of these lipoprotein apoproteins would be of considerable assistance in the study of their metabolism. In the present report, a quantitative radioimmunoassay for rat serum beta lipoprotein–protein (beta Lp–protein) is described. By use of this immunoassay, the basal serum levels of beta Lp–protein, the quantitative contribution of immunoassayable beta Lp–protein to the chylomicron, LDL, VLDL, HDL, and density (d) > 1.21 protein fractions and the rate of hepatic secretion in normal rats have been determined.

METHODS

Preparation of serum beta lipoprotein standard. Blood was aspirated from the aorta of fed rats under ether anesthesia, defibrinated with a wooden stick and the serum isolated by centrifugation at 5°C for 10 min at 2000 rpm. The chylomicron fraction was separated by ultracentrifugation at d = 1.006 for 30 min at 17,000 g in a No. 50 rotor in a Spinco model L ultracentrifuge and was removed by the tube-slicing technique described by Havel, Eder, and Bragdon (10). After this maneuver was repeated on the infranant solution and the chylomicron fraction discarded, the chylomicron-free serum was taken to d = 1.019 and centrifuged for 22 hr at 140,000 g. The top 2.1 cm fraction containing VLDL was removed by tube slicing and discarded, and the infranant

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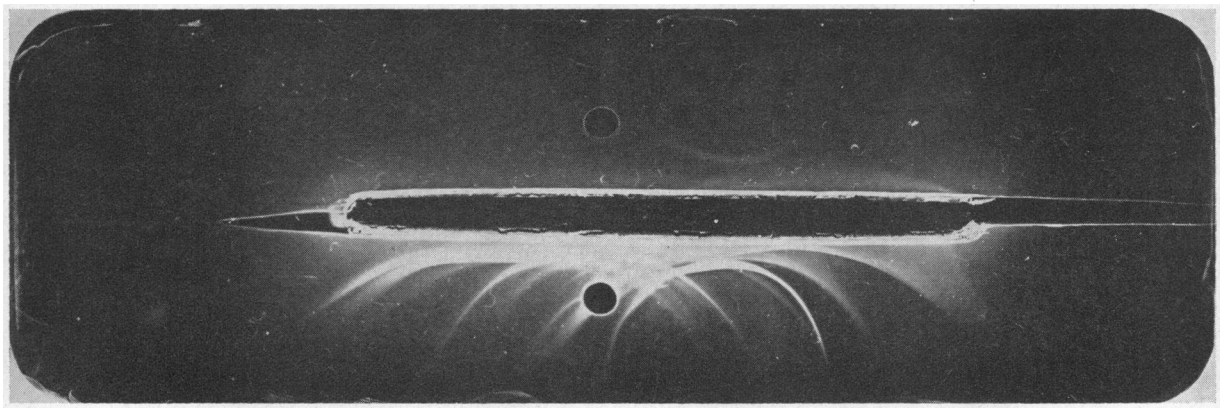

FIGURE 1 Immunoelectrophoretic characterization of low density lipoproteins (LDL) (beta lipoprotein) (upper well) as compared with whole rat serum (lower well). The trough contains goat anti-rat serum obtained from Hyland Laboratories, Los Angeles, Calif. (lot No. 71-229). The stain used is Amidoschwarz.

was suspended in a solution of NaCl and KBr at d = 1.063 (10). After it had been centrifuged for 22 hr at 140,000 g, the top 2.1 cm containing the LDL (beta lipoprotein) was removed by tube slicing, resuspended in the salt solution at d = 1.063, and the LDL fraction isolated after two additional centrifugations. This preparation was then desalted on a 15 cm × 1 cm G-25 coarse Sephadex column previously equilibrated with 0.05 M barbital buffer at pH 8.6. The fractions were monitored by ultraviolet absorption at 280 mμ and by $AgNO_3$ qualitative titration for the bromide ion. The protein "peak" was separated from the bromide "peak" by 4 ml of eluate. The concentration of protein in the isolated fraction was measured by the micro-Lowry procedure (11). This fraction, referred to as "pure" beta lipoprotein standard, was stored undiluted at 5°C with 0.001 M ethylenediaminetetraacetate (EDTA) and used for a maximum of 2 wk as a standard beta lipoprotein preparation for the immunoassay. Immunoelectrophoresis of the LDL (beta lipoprotein) preparation with antiserum directed against whole rat serum demonstrated a single protein precipitin line as shown in Fig. 1, confirming within the limits of this technique the homogeneity of the "pure" beta lipoprotein standard.

Preparation of iodine-125-labeled beta lipoprotein. To prepare ^{125}I-labeled beta lipoprotein, 0.2 mg of fresh standard beta lipoprotein was precipitated by reaction with heparin-Mn^{++} for 15 min according to the method of Burstein and Samaille (12) except that the procedure was carried out at 5°C. 2 volumes of 0.025 M manganous chloride containing 2 mg/ml of heparin was then added to the precipitate with mixing, followed by isolation of the precipitate by centrifugation at 1500 rpm for 10 min at 5°C. After three such washes with 0.025 M $MnCl_2$ containing heparin, the precipitate was washed twice with 2 ml of distilled water. This procedure resulted in a fine white precipitate of beta lipoprotein which was halogen free. 10 μl of 1 M disodium EDTA were added to solubilize the precipitate followed by 100 μl of 0.5 M phosphate buffer, pH 7.6. The solubilized beta lipoprotein was then iodinated by mixing 100 μl of 0.5 M phosphate buffer, 20 μl of lipoprotein solution, and 20 μl of Na-^{125}I (~100 mc/ml) (13). After 1 min, 25 μl of chloramine T (35 mg in 10 ml of 0.05 M phosphate buffer, pH 7.6) was added, and the solution placed on a 15 × 1 cm G50 coarse Sephadex column and eluted with 0.05 M barbital buffer, pH 8.6. The initial radioactive peak appeared in the first 7–10 ml of eluate, while the free iodine-^{125}I was not eluted until after 30–40 ml of buffer had passed through the column. The initial peak was diluted v/v with 0.05 M barbital buffer, pH 8.6, containing 0.001 M EDTA and 6% bovine albumin. This material is referred to as stock beta lipoprotein-^{125}I and was stored at 5°C until further purification by gel filtration before use in the immunoassay. The flotation characteristics of this labeled lipoprotein were examined in two preparations added to a 10% solution of fresh rat serum in saline and were identical with those of the unlabeled LDL (beta lipoprotein). As shown in Table I, approximately 98% of the label could be isolated in the fraction of density 1.019–1.063. The lipid component of this labeled intact lipoprotein, isolated by chloroform: methanol extraction (2:1), contained no radioactivity, a finding indicating exclusive iodination of the protein moiety of the lipoprotein.

Preparation of antiserum to beta lipoprotein-protein. Young female rabbits were immunized by subcutaneous injection with 2 mg of standard beta lipoprotein emulsified with complete Freund's adjuvant and Arlacel oil (1:4 v/v). The rabbits were given booster injections at 2-wk intervals for 6 wk. 2 wk after the last booster injection, blood was obtained by cardiac puncture and the serum assayed for anti-beta lipoprotein activity. Of three rabbits initially immunized, only one produced an antibody of sufficient titer and antigen affinity to be suitable for an immunoassay. This rabbit serum was absorbed with a reconstituted rat serum preparation containing those proteins which sediment at d = 1.21 when centrifuged at 140,000 g.

TABLE I

*Flotation Distribution of Beta Lipoprotein-^{125}I**

Fraction density	Total cpm recovered	
	No. 1	No. 2
d < 1.019	2,040 cpm	1,600 cpm
d = 1.019–1.063	504,000 cpm	340,000 cpm
d > 1.063	5,200 cpm	4,400 cpm

* Ultracentrifugation performed once as described in Methods with carrier serum added to a 10% concentration.

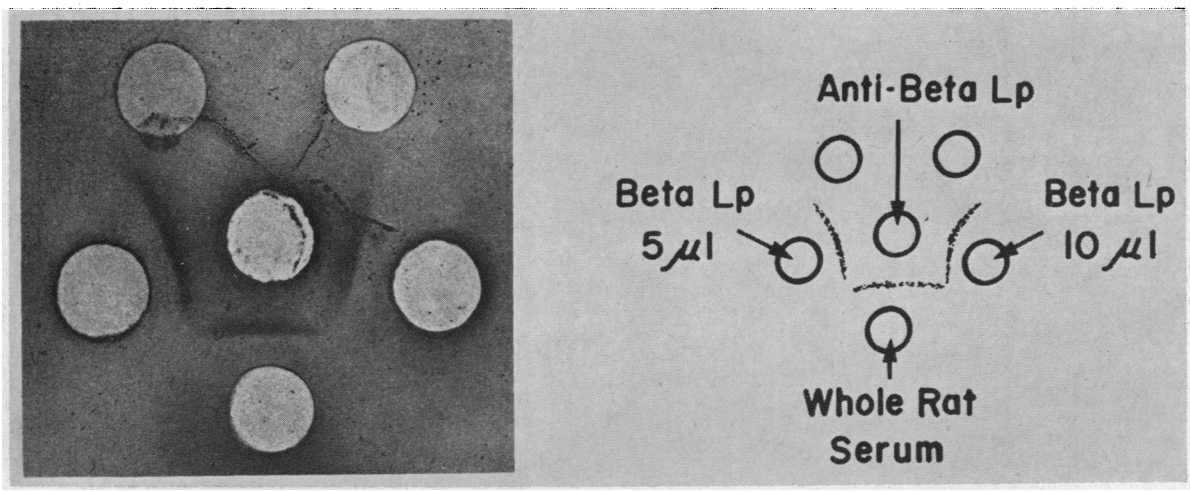

FIGURE 2 Specificity of anti-beta lipoprotein-protein serum by double diffusion. 10 μl of antiserum was placed in the center well; 10 μl of rat serum was placed in the lower well and purified beta lipoprotein (3 μg/μl) in the side wells as indicated. The stain used is Oil-Red-O.

Absorption was carried out six times by incubating 1 ml of rabbit serum with 10 μl of the rat serum preparation for 1 hr at 37°C followed by centrifugation at 3000 rpm for 30 min in a refrigerated centrifuge to remove any resulting antigen-antibody precipitate. A small amount of precipitate was noted in the first of three absorptions, while none could be seen in the final three absorptions. These incubations were followed by absorption three time with the rat serum lipoprotein fraction of d = 1.063–1.21 (HDL or alpha lipoprotein) containing 0.25 mg/ml of protein by Lowry analysis. Finally, the rabbit serum was absorbed three times with alpha lipoprotein eluted from the appropriate migrating band obtained by paper electrophoresis according to the method of Lees and Hatch (14). This preparation contained an undefined concentration of alpha lipoprotein, since the method utilizes a buffer containing albumin which is also eluted with the alpha lipoprotein band. The preparation used was obtained from the electrophoresis of 30 μl of rat serum followed by elution of the appropriate band with 500 μl of saline. No precipitate was visible in any of these absorptions. The specificity of the final antiserum was examined by a double diffusion technique against both whole rat serum and purified beta lipoprotein. As shown in Fig. 2, a single precipitin line containing lipid by Oil-Red-O staining is shared in common by the isolated beta Lp–protein and by whole serum. Thus, within the limits of this technique, the antiserum selectively binds only beta Lp–protein in the presence of all serum proteins. This antiserum was kept frozen at −40°C with 0.1% methiolate added as a preservative. When used in the immunoassay, the absorbed antiserum was appropriately diluted with 0.05 M barbital buffer containing 3% bovine albumin, pH 8.6.

RESULTS

Stability of Lp-^{125}I–protein by gel filtration. Beta lipoprotein–^{125}I was further purified by gel filtration on G200 coarse Sephadex, with a 30 cm × 1.5 cm column prepared in 0.05 M barbital buffer, pH 8.6. Approximately 1 ml of stock beta lipoprotein–^{125}I was placed on the column, and 0.5-ml fractions of the barbital buffer eluate were collected until 100 ml was obtained. Typical elution patterns are shown in Fig. 3, with a flow rate of 1 ml/10 min. Four peaks are seen, referred to as peaks A, B, C, and D. Peak A appeared with the void volume and was the only peak which was immunoreactive with beta lipoprotein–antibody. Neither peak D, corresponding to the elution volume of free iodine, or peaks B or C were bound by the antibody. Fractionation on G200 did not improve the separation found with G150, so the latter column was routinely used for the purification step because of its more rapid flow rate. The stability of the labeled lipoprotein is illustrated in the lower portion of Fig. 3 by the elution pattern of material stored at 5°C for 2½ months. Peak A became progressively smaller throughout the 10 wk of observation, peak B became progressively larger, and peaks C and D were little affected. In spite of these changes in gel filtration pattern, peak A remained reactive with specific antiserum with exactly the same standard curve, while peak B remained unreactive. The identity of peaks B and C have not as yet been established, but studies are in progress to examine the possibility of the presence of heparin-lipoprotein complexes and (or) delipidated-denatured proteins in these fractions. For use in the immunoassay, an aliquot of peak A was assayed for ^{125}I radioactivity in a Packard Autogamma Spectrometer and diluted with 0.05 M barbital buffer, pH 8.6, containing 3% bovine albumin, so that 100 μl of solution contained 10,000 cpm. This purification of labeled lipoprotein was performed on the same day that each immunoassay was begun.

Immunoassay procedure. Incubation conditions were selected to reduce the nonspecific precipitation of lipoprotein-^{125}I observed in this assay system in the absence

TABLE II

Effect of Assay Conditions on the Precipitation of ^{125}I-Labeled Beta Lipoprotein in the Absence of Beta Lipoprotein–Antibody

Buffer	Tube	Albumin concentration	Per cent of total counts precipitated
		g/100 ml	%
0.05 M barbital	Siliclad*	0	8.6
"	"	0.25	3.4
"	"	0.50	3.6
"	"	1.00	2.7
"	"	2.00	3.2
"	"	4.00	3.1
"	"	5.00	3.0
"	Normal	0	3.7
"	"	0.25	3.0
"	"	0.50	4.7
"	"	1.00	11.0
"	"	2.00	18.0
"	"	4.00	21.0
"	"	5.00	19.0
0.05 M phosphate	"	0	40.0
"	"	3.00	20.0
"	Siliclad	0	51.0
"	"	3.00	25.0

* Siliclad, Clay Adams, Inc., New York.

of first antibody (Table II). By use of siliconized tubes[1] and final albumin concentrations ranging from 0.25 to 5%, the precipitable radioactivity ranged from 2.7 to 3.6% of the total radioactivity in the system. This background precipitation rose to 8.6% of the total radioactivity when albumin was omitted. The use of nonsiliconized tubes resulted in a progressive rise in the amount of radioactivity precipitated from 3 to 19% with the same increments in albumin concentration, making this system unsuitable for the immunoassay of serum samples. The substitution of 0.05 M phosphate buffer, pH 7.6, for barbital buffer in all combinations of glass tubes and albumin concentrations resulted in unsuitable nonspecific precipitation of ^{125}I ranging from 20 to 51% of the total radioactivity added. The addition of 0.01 M EDTA had no effect on the background of any of the above systems.

The reaction of antibody and beta lipoprotein–^{125}I was performed with 100 μl of a 1:300 dilution of antiserum, 100 μl of lipoprotein-^{125}I solution containing 10,000 cpm, 100 μl of standard beta lipoprotein or unknown lipoprotein solution, and 200 μl of 0.05 M barbital buffer, pH 8.6, containing 3% bovine albumin. All dilutions were made with barbital buffer. These solutions were placed in 10 × 50 mm siliconized disposable glass tubes, covered, and allowed to react at 5°C for 48 hr. The precipitating antibody (anti-rabbit gamma globulin) was then added in an appropriate dilution so that 100 μl would provide maximum precipitation of the beta lipoprotein–^{125}I antibody complex. After 24 hr of incubation at 5°C, the total radioactivity in each tube was measured in a Packard Autogamma Spectrometer, and the precipitate was isolated by centrifugation at 2000 rpm for 30 min in a refrigerated centrifuge at 5°C. The supernatant solution was aspirated and discarded and the precipitate washed three times with 2 ml of cold 0.05 M barbital buffer. The tube containing the precipitate was placed in a Packard Autogamma Spectrometer, and the radioactivity contained in the washed precipitate was measured with a counting efficiency of 50 ±5%. The results of the assay were expressed in terms of the ratio of radioactivity in the immunoprecipitate (bound) to radioactivity remaining in solution (free), the latter determined as the difference between the total counts per minute originally in each tube and the final counts per minute remaining in the immunoprecipitate.

Standard curve. The curve describing the displacement of beta lipoprotein–^{125}I from antibody by unlabeled standard beta lipoprotein was sigmoid in appearance in a semilog plot, with the steepest portion of the curve occurring with beta protein concentrations of 0.1–1.5 μg of protein, and with the variability of each determination being ±4% (Fig. 4). The terminal portion of the curve changes slope at a bound:free ratio of approximately 0.1, resulting in loss of sensitivity at protein concentrations above 1.5 μg, so that all unknown samples

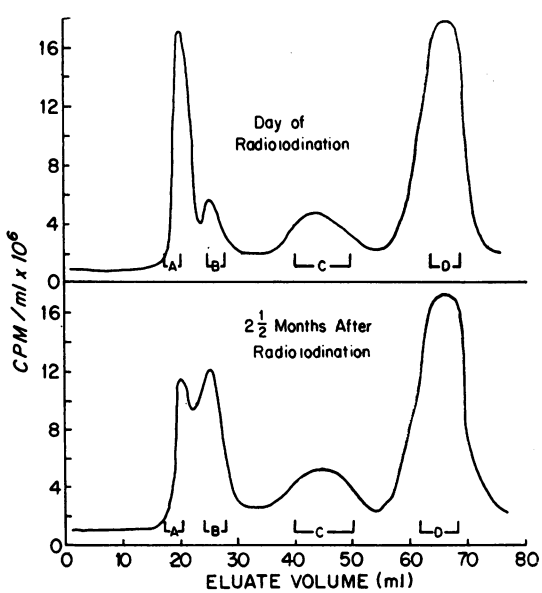

FIGURE 3 Elution pattern of beta lipoprotein–^{125}I on Sephadex G200. Peaks A, B, C, and D were evaluated for immunoreactivity with beta lipoprotein–antibody (see text).

[1] Siliclad, Clay-Adams, Inc., New York.

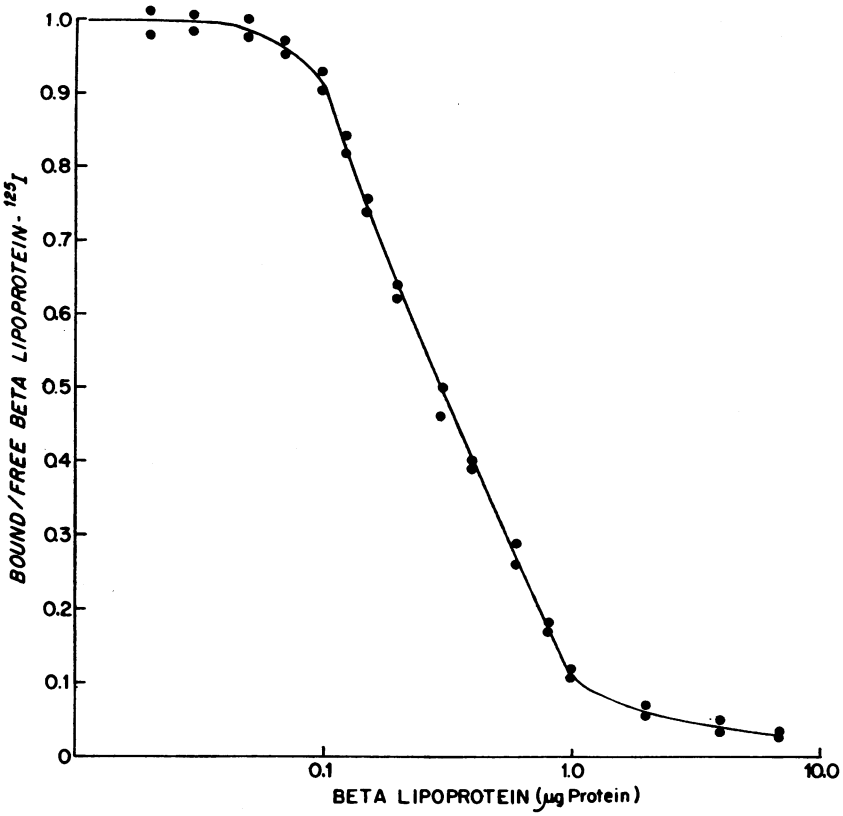

FIGURE 4 The beta lipoprotein immunoassay standard curve. All points were evaluated in duplicate as plotted.

were adjusted to this range of protein concentration. The addition of 7 μg of standard beta Lp–protein displaced > 98% of the label (bound: free = 0.02) with a final antibody dilution of 1:1500. A reproducible standard curve could be obtained with a fresh preparation of standard beta lipoprotein for about 3 wk, after which time the displacement of a given concentration of protein was decreased. The standard could thus be considered stable for only 2 wk, after which time the evaluation of unknown samples would be expected to be spuriously high. Quantitative recovery of standard beta lipoprotein was readily obtained in the presence of a 1:200 dilution of normal rat serum, indicating no inhibition of immunoreactivity in the presence of serum. As shown in Table III a recovery of ~100% was obtained with the addition of both 0.1 μg and 0.4 μg of standard beta Lp–protein to rat serum.

Lipoprotein specificity. The specificity of the immunoassay was determined by comparing the displacement curves of the major lipoprotein fractions isolated by two different methods: paper electrophoresis and flotation ultracentrifugation. By convention, the corresponding fractions are referred to as beta: low density (LD), prebeta: very low density (VLD), and alpha: high density (HD) lipoproteins, respectively. All ultracentrifuged preparations were spun three times at the appropriate density after the original isolation ultracentrifugation and desalted on G25 Sephadex at described under Methods. These preparations, referred to as VLDL, LDL, HDL, and d > 1.21, were assayed for their protein content by the Lowry procedure (11).

TABLE III
*Recovery of Purified Beta Lipoprotein–Protein in the Presence of Rat Serum**

Addition to assay	Beta Lp–protein by immunoassay	Recovery of added beta Lp–protein
	μg/sample	%
Fresh serum, 1/200 dilution	0.11 ±0.01	None added
Fresh serum plus 0.1 μg beta lipoprotein	0.20 ±0.008	90
Fresh serum plus 0.4 μg beta lipoprotein	0.50 ±0.005	98

* Mean ±SEM of six separate determinations each run in triplicate.

Lipoproteins eluted from paper electrophoretic strips were identified by staining parallel strips to identify the rate of flow (R_f) of the respective lipoproteins. Since the electrophoresis was carried out in albumin-containing buffer, the protein present in the extract represents both the derived lipoprotein and albumin. These preparations, referred to as beta, prebeta, and alpha lipoproteins were assayed in serial dilutions and plotted in superimposition with the standard beta lipoprotein curve. The results of these studies are shown in Fig. 5 and Table IV and represent four separate preparations of each lipoprotein fraction assayed in triplicate in a 4-point assay. The displacement curves for LDL isolated by ultracentrifugation over a concentration range of 0.1–1.0 μg and for beta lipoproteins isolated by electrophoresis indicate immunological identity in this system (Fig. 5). The displacement curves exhibited by VLDL isolated by ultracentrifugation (d = 1.006–1.019) in concentrations ranging from 0.8 to 8.0 μg and by the prebeta migrating band obtained with paper electrophoresis are also identical with that observed with beta lipoprotein, identifying the presence of beta apoprotein in this fraction (Fig. 5). However, the quantitative contribution of beta Lp–protein is small, being 9, 12, 20, and 17% on four separate preparations (Table IV). The possibility exists that the "B" protein present in the VLDL protein complex, although exhibiting a reaction of identity, may have fewer sites of beta antigen reactivity exposed per unit of protein than does the "B" protein contained within the beta Lp–protein. Thus our quantitative

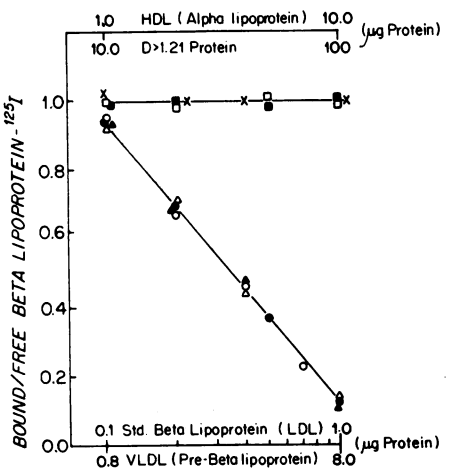

FIGURE 5 Lipoprotein specificity of rat beta lipoprotein–protein immunoassay demonstrated by the competitive displacement curves of standard low density lipoprotein (LDL) (●), beta lipoprotein isolated by paper electrophoresis (○), very low density lipoprotein (VLDL) (△), prebeta lipoprotein isolated by paper electrophoresis (▲), high density lipoprotein (HDL) (■), alpha lipoproteins isolated by paper electrophoresis (□), and proteins of density greater than 1.21 (X).

TABLE IV

Immunoassayable Beta Lipoprotein–Protein in Serum Fractions Isolated by Flotation Ultracentrifugation

Lipoprotein fraction	Protein content by Lowry assay	Beta Lp–protein by immunoassay
density (d)	*μg*	*% total protein*
LDL (d = 1.019–1.063	1.0	100.0
VLDL (d = 1.006–1.019)	1.0	9–20.0
HDL (d = 1.063–1.21)	20.0	0.5
VHDL (d = >1.21)	100.0	Undetectable
Chylomicron (d = <1.006)	0.1	Undetectable

LDL, low density lipoprotein; VLDL, very low density lipoproteins; HDL, high density lipoproteins; VHDL, very high density lipoproteins.

analysis of "immunoreactive beta Lp–protein" may tend to underestimate the "B" protein content of the VLDL protein.[2] However, until quantitative examination of the other constituent proteins, i.e. "A" and "C" proteins has been performed, our data constitute an appropriate initial investigation into the problem.

The cross-reactivity in this assay system of HDL (d = 1.063–1.21) isolated by ultracentrifugation over a concentration range of 1.0–10.0 μg and of alpha lipoproteins isolated by paper electrophoresis is also shown in Fig. 5. At these concentrations, no beta apoprotein was identifiable in these preparations. However, HDL lipoprotein at concentrations of 50.0–100.0 μg did exhibit displacement curves which were identical with those seen with beta lipoprotein at 0.5% of that concentration. With less rigorous purification in the ultracentrifuge, alpha lipoprotein preparations frequently were found to have as much as 25% of the protein immunoreactive as beta Lp–protein with an identical slope of the displacement curve. These observations suggest contamination of purified alpha lipoprotein with beta or prebeta lipoprotein rather than cross-reactivity of similar antigens.

The immunoassay of serum proteins of density greater than 1.21 exhibited no displacement, even though pro-

[2] The demonstration of a reaction of identity (Fig. 5) would suggest that the "B" protein in the VLDL and LDL fractions exhibits equivalent quantitative immunogenicity in our assay system (15). On theoretical grounds, it might be anticipated that blockade of a fraction of the antigenic determinants would result in sufficient change in the antigen to produce both quantitative and qualitative changes in the antigen-antibody reaction. Experimentally, this has proven to be the case. When the immunoreactive behavior of identical subunits, as in the case of limpet hemocyanin (16) and *Escherichia coli* alkaline phosphatase (17), was assayed in the dissociated (monomeric) or associated (polymeric) form, reactions of *partial identity* rather than *identity* have been observed.

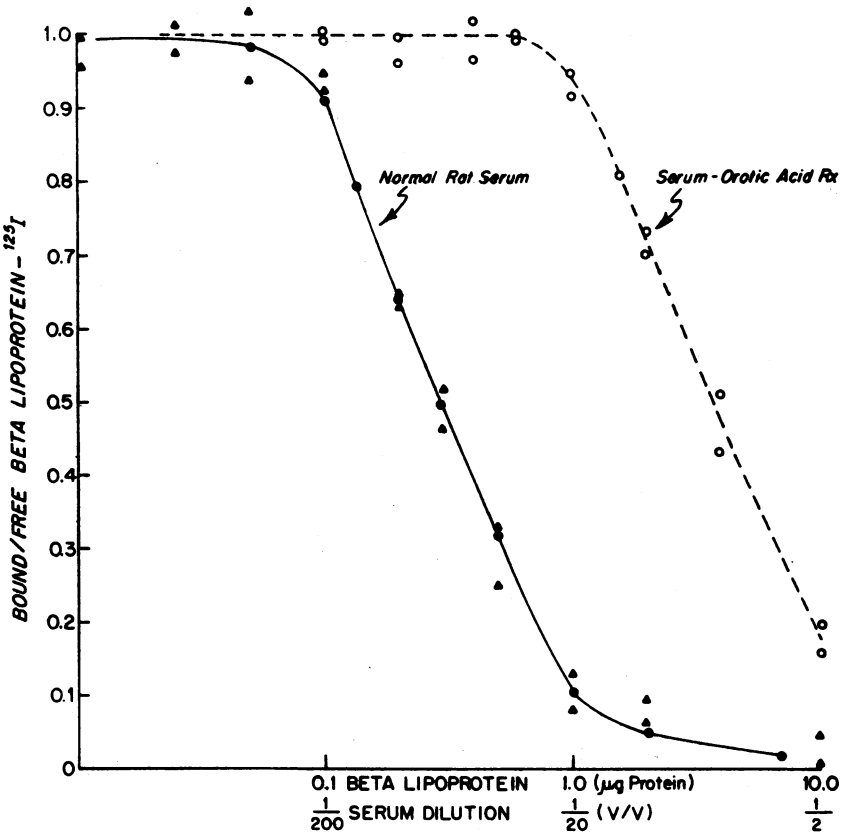

FIGURE 6 Comparison of immunoassay competitive displacement curves of standard beta lipoprotein (●), normal rat serum (▲), and orotic acid-treated rat serum (○). All points are evaluated in duplicate.

tein concentrations from 10.0 to 200.0 μg were examined. With this assay system, therefore, there is no immunoreactive beta Lp–protein in this high density fraction, nor is there indication of partial cross-reactivity with beta lipoprotein.

Assay of beta lipoprotein in whole serum. The competitive displacement curve for whole serum from normal rats was defined using 100 μl volumes of serial dilutions of serum from 1/2 to 1/2000 (v/v). The locus of points is plotted as closed triangles in superimposition with the standard beta lipoprotein curve in Fig. 6 and is identical with it. A duplicate 2-point assay at a 10-fold difference in concentration was performed on 58 separate rat serum samples. The immunoassayable concentration of beta Lp–protein in whole rat serum obtained between 8:00 a.m. and 11:00 a.m. was 0.24 ±0.01 mg/ml. This value represents all the immunoreactive beta Lp–protein in the serum and is a combination of the LDL and that present in the VLDL fraction (e.g. ~12%).

In order to establish the specificity of this assay system for beta Lp–protein in the presence of all other serum proteins, the immunoreactivity of serum obtained from rats fed an adenine-free diet containing 1% orotic acid was determined (18). Windmueller and Levy (1) have shown that these rats are unable to release beta lipoprotein from the liver and thus develop a marked deficiency of circulating beta and prebeta lipoprotein. The competitive displacement curve for this serum prepared in identical serial dilutions with those used for normal rat serum is also shown in Figure 6. The locus of points is again sigmoidal with a slope identical with that seen with standard beta lipoprotein, a finding indicating immunological identity with beta Lp–protein. The entire locus, however, is displaced in the direction of decreased concentration of immunoreactive protein for every comparable serum dilution. In eight such rat sera studied, the immunoreactive beta Lp–protein varied from 0.02 to 0.08 mg/ml, a finding indicating that as much as 90% of the beta Lp–protein was removed from the serum by this diet. This serum provides a uniquely suited control for establishing the specificity of this immunoassay of serum beta lipoprotein.

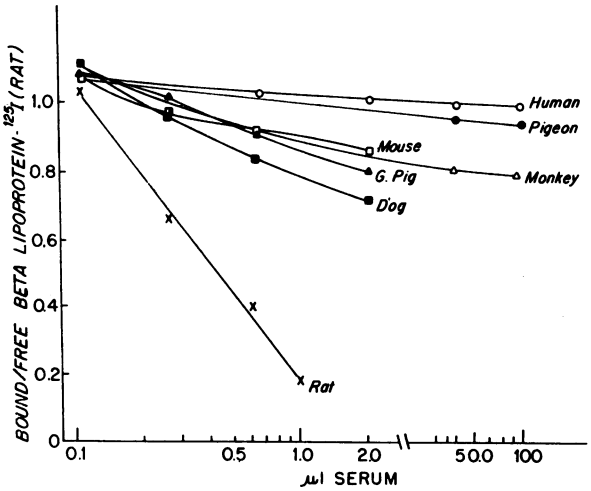

FIGURE 7 Species specificity of rat beta lipoprotein immunoassay demonstrated by the competitive displacement curves of serum from man, pigeon, mouse, monkey, guinea pig, and dog, as compared with the curve obtained with rat serum. All sera were prepared in identical manner and assayed simultaneously in duplicate.

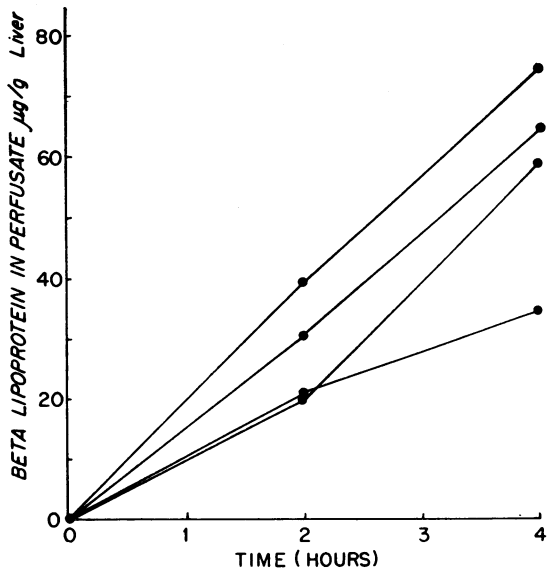

FIGURE 8 Secretion of beta lipoprotein–protein in the perfused liver of normal fed rats. All points were measured in quadruplicate with a variation of ±4% in four preparations.

Species specificity. Serum was obtained from the monkey, dog, guinea pig, mouse, pigeon, and man and immunoassay of beta Lp–protein performed (Fig. 7). The slope of the competitive displacement curve in each case was distinctly different from that observed for rat serum. The slope of the displacement curve for pigeon and human serum remained flat up to concentrations of 100 μl, whereas the slope of the curve for serum from the dog, guinea pig, monkey, and mouse showed a gradual drift with the bound: free ratio falling from 1.0 to 0.7–0.8 when 2.0–20 μl of serum were assayed. These data indicate that proteins with minimal partial cross-reactivity with rat beta Lp–protein may be present in some species.

Hepatic production of beta lipoprotein. The liver has been shown to be the site of synthesis of lipoprotein-protein (1, 2, 5, 7, 19, 20). The rate of release of beta Lp–protein from the liver was measured by an isolated rat liver perfusion technique (reference 21 and footnote 3). Beta Lp–protein in the liver perfusate was established by demonstrating that the slope of the competitive displacement curve obtained with the perfusate was identical with that of a standard beta lipoprotein preparation. As shown in Fig. 8, the rate of release of beta Lp–protein remained linear over a 4 hr period and averaged 14.5 μg/g of liver per hr.

[3] These perfusion studies were performed by Dr. A. Eisenstein, Department of Preventive Medicine, Washington University School of Medicine.

DISCUSSION

Serum lipoproteins exist as a spectrum of lipid-protein species containing varying amounts of triglyceride, cholesterol, and phospholipid and have been classified on the basis of size, charge, and density of the lipid-protein complex. Identification based upon fractionation has required systems of nomenclature relating to the respective physicochemical properties of separation, viz., low density lipoprotein, beta migrating lipoprotein, lipoproteins of $S_f = 0$–20, and heparin-Mn^{++}–precipitable lipoproteins. Gustafson, Alaupovic, Furman, and their colleagues (3, 15, 22–24) have summarized the data indicating that the chemical, physicochemical, structural, and possibly the functional properties of the lipoproteins are largely determined by the presence of specific apoproteins, immunologically defined as A, B, and C proteins (3). These apoproteins provide distinguishing components defining three major classes within the heterogeneous spectrum.

Classification based upon protein constituents has been established by qualitative identification utilizing immunoprecipitin reactions. However, evaluation of normal and abnormal metabolism and biochemistry of lipoproteins requires quantitative methods which depend upon neither isolation techniques requiring normal physicochemical behavior nor assay techniques requiring a normal lipid complement. Radioimmunoassay provides a method of evaluating the protein moiety alone, which utilizes the specificity of antigen-antibody reactivity in place of serum fractionation and combines the quantitative sensitivity of immunological binding with protein

determinants with the accuracy of gamma-counting techniques in the place of colorimetric chemical determinations of lipid constituents. We have confirmed the observation of Alaupovic et al. showing that the protein moiety of the lipid-protein complex can be iodinated with no significant uptake by the lipid (24). Purification of the iodinated lipoprotein by gel filtration provides a rapid method of removing denatured labeled protein from the preparation based on the estimated molecular weight of beta lipoprotein of 2,300,000 (25) and on the experience of previous investigators with gel filtration of serum lipoproteins (26, 27).

The quantitative accuracy of an immunoassay depends upon the purity of the standard reference protein. In the assay reported here, the criterion for purity is primarily separation by repetitive flotation ultracentrifugation. The density ranges used for this preparative fractionation of rat plasma lipoproteins have been shown by Camejo (28) to yield fractions which correspond to the discontinuities in the flotation profiles observed during analagous analytical ultracentrifugation of human lipoproteins (10). He furthermore has reported that the protein : phospholipid : cholesterol : triglyceride composition of the LDL (beta lipoprotein) is comparable to the corresponding fraction in human serum. This type of preparation has been shown by others (1) and confirmed by us to contain a single immunoprecipitin line on immunoelectrophoresis with whole serum antibody (see Fig. 1). These methods do not establish the absence of another protein moiety but only the absence of a protein species which will produce a precipitin line when reacted with the antisera utilized. It is possible that one or more proteins are present in our beta Lp–protein preparation in addition to the "B" apoprotein described by other investigators (1, 3, 15, 22, 24) which remain associated with "B" apoprotein in all the physicochemical maneuvers utilized, but which might be demonstrated with the proper immunologic system. In fact, Aladjem has shown that appropriate alterations in antigen-antibody proportions will result in the appearance of multiple immunoprecipitant lines with human LDL, and he has summarized the evidence for multiple antigens being present in the LDL protein complement (29).

The specificity of our assay for the protein moiety of beta lipoprotein has been established by the competitive displacement of $> 98\%$ of the protein-^{125}I from antibody with standard beta lipoprotein, by the demonstration of a 90% decrease in reactivity in serum from orotic acid–treated rats, and by the total lack of immunoreactivity with the serum fraction of density greater than 1.21 from normal rats. Double diffusion analysis has indicated that the antibody binds only a single protein species in whole serum, and that this protein is identical with beta Lp–protein (Fig. 2). The extremely low concentration of beta Lp–protein in the HDL fraction, less than 0.5%, confirms observations on human sera and rat sera indicating immunologically distinct protein moieties associated with this lipoprotein family. The observed concentration of beta Lp–protein of 9%–20% in the VLDL protein is consistent with the observations of Camejo (28) that approximately 90% of the protein in the VLDL of rat serum may be "A" apoprotein (HDL or alpha Lp–protein). However, studies concerning human VLDL protein by Gustafson et al. (3) have indicated that in man the "B" apoprotein content ranges from 48 to 78% of the total protein. While it is possible that there is a species difference relating to the "B" apoprotein content of the VLDL protein, it is also possible that the "B" protein in the VLDL complex possesses diminished quantitative immunological identity.[a] Such a possibility awaits examination by quantitative immunoassay of both the "A" and "C" apoproteins before the question can be resolved. Other investigators have reported the presence of "B" apoprotein in human chylomicrons, while our studies on rat chylomicrons did not reveal its presence. However, our assay presently is not sensitive to beta Lp–protein concentrations below 0.1 μg, and the largest concentration of total chylomicron protein used was 0.1 μg.

Both Marsh and Whearat (30) and Windmueller and Levy (1) have demonstrated with immunoprecipitin techniques that the perfused rat liver is capable of synthesizing immunologically identifiable beta Lp–protein. In our assay system, the perfusate was found to contain a protein which exhibited a displacement curve of identity with that of standard beta Lp–protein and increased in concentration in a linear fashion over the 4 hr of perfusion. The rate of beta Lp–protein synthesis observed in our perfused rat liver system of 14.5 μg/g per hr compares well with the observations of Marsh et al. (30) of 19 μg/g per hr with nonimmunologic techniques. The fractional turnover rate of this transport protein in the serum can be estimated for these 200 g rats by calculating the intravascular pool as being 1.9 mg and the rate of release from a 10 g liver being 0.145 mg/hr. The plasma fractional turnover rate is thus 7.6%/hr with an intravascular half-life of 9.1 hr. This figure seems inordinately rapid, considering the estimates made for beta protein in human serum in the range of 4–6 days. Estimates for albumin half-life based on liver perfusion data by other investigators have been similarly rapid, and it has been suggested that the high secretory rates obtained with the perfused liver preparation may be due to the loss of feedback control mechanisms seen in the intact animal which would normally reduce the secretion rate (31).

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