Translation in vivo and in vitro of proteins resembling apoproteins of rat plasma very low density lipoprotein

Rick Hay and Godfrey S. Getz[1]

Departments of Pathology and Biochemistry, The University of Chicago and The Pritzker School of Medicine, Chicago, IL 60637

Abstract Antibodies raised against rat plasma apoVLDL and a purified fraction of arginine-rich peptides (ARP) were labeled with $Na^{125}I$ and were shown to bind to polyribosomes isolated from rat liver. Antibody fractions enriched by selective affinity chromatography exhibited increased levels of binding to polysomes. Anti-apoVLDL immunoreactivity was further resolved into anti-ARP and anti-apoB components, each reactive with a distinct polysome population. Binding was specific for rat polysomes, and was directed toward nascent polypeptide chains. About 2% of normal rat liver polysomes were recovered by indirect immunoprecipitation with anti-apoVLDL. Ribonucleic acid (RNA) extracted from this immunoprecipitate contained species with polyadenylate (poly[A]) sequences characteristic of eukaryotic messenger RNA (mRNA). These species, purified by affinity chromatography on poly(U)–Sepharose, stimulated the in vitro synthesis of immunoprecipitable apoVLDL-like proteins by about 17-fold when compared to unfractionated rat liver mRNA. Most of the in vitro translation products precipitated by purified anti-ARP migrated identically on polyacrylamide gel electrophoresis with unlabeled purified ARP. Some implications of these findings with respect to plasma VLDL biosynthesis are discussed.—**Hay, R., and G. S. Getz.** Translation in vivo and in vitro of proteins resembling apoproteins of rat plasma very low density lipoprotein. *J. Lipid Res.* 1979. **20:** 334–348.

Supplementary key words affinity chromatography · anti-apolipoprotein antibodies · apolipoprotein B · arginine-rich peptides · cell-free systems · immunoprecipitation · lipoprotein biosynthesis · messenger ribonucleic acid · polyribosomes

The plasma lipoproteins represent the major carriers of cholesterol and lipid to which the arterial wall and peripheral tissues are exposed, and as such have been implicated in both the pathogenesis and the prevention of cardiovascular disease and as important elements in the pathobiology of various genetic and metabolic disorders in man (1–4).

Numerous reports have appeared concerning plasma lipoprotein production in both normal and altered physiologic states. It is generally thought that both the intestine and the liver are the major organs engaged in plasma lipoprotein synthesis. The intestinal biosynthesis of apolipoproteins has been demonstrated by the radiolabeling of several of these peptides in isolated and in situ perfused intestine (5, 6), by the immunofluorescence method for the detection of apolipoproteins A-I and B inside the mucosal cells of the rat intestine (7, 8), and by the identification of radiolabeled A-I in the mesenteric lymph after the intraduodenal administration of radioactive precursor (9). The evidence for the hepatic role in lipoprotein biosynthesis is far more extensive; it is based upon the consequences of hepatectomy (10), the labeling of lipoproteins secreted by perfused rat liver (5, 11–14) and rat liver slices (15), the electron microscopic visualization within the hepatic smooth endoplasmic reticulum and Golgi apparatus of particles having the characteristics of very low density lipoproteins (16–19), and the isolation from the hepatic Golgi apparatus of particles that have the size and apoprotein constituents of plasma VLDL (19–21). Antisera against LDL and HDL were used to identify these antigens among the products of cell-free protein synthesis by hepatic microsomes and ribosomes (22, 23) and among the secretory products of perfused livers (14). Quantitative considerations and morphological observations indicate that the biosynthetic activity is attributable to the hepatocytes rather than other cells of the liver.

Abbreviations: apoB, apolipoprotein B; ARP, arginine-rich peptides or apolipoprotein E; A_x, optical absorbance at x nm; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; DOC, sodium deoxycholate; HDL, high density lipoprotein; HDL_1, HDL of mean density 1.054 g/ml; HDL_3, HDL of $1.12 < d < 1.21$ g/ml; HEA, hen egg albumin (ovalbumin); HEPES, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid; IFA, incomplete Freund's adjuvant; LDL, low density lipoprotein; poly(A), polyriboadenylic acid; poly(U), polyuridylic acid; RSA, rat serum albumin; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; VLDL, very low density lipoprotein.

[1] Address correspondence to Dr. Godfrey S. Getz, The University of Chicago, Department of Pathology, 950 East 59th Street, Chicago, IL 60637.

This has been confirmed by the recent demonstration of VLDL secretion by isolated hepatocytes (24).

VLDL is thought to be a primary hepatic secretory particle as well as a precursor of other plasma lipoprotein classes (25, 26). The biosynthesis of its five or more distinct apoproteins and their assembly into a lipidated complex must be finely controlled by mechanisms yet to be determined. A few recent studies (27–29) have closely examined the production of individual VLDL apolipoproteins.

Of particular interest are the two major apoproteins of VLDL, arginine-rich peptides (ARP) and apolipoprotein B (apoB). These classes have been implicated as contributors to hyperlipidemic states (30) and atherosclerosis (31) in man. Neither is found exclusively in VLDL. ApoB is the major component of LDL in all species (32). Of the apolipoprotein classes, apoB is the least soluble in aqueous solution. ARP, while found at high levels in the lipoproteins of man only in some altered metabolic states (30, 33), is a major constitutive component of VLDL and HDL and of lipoprotein-free plasma in the rat (34).[2] In addition, we and others have recently identified in normal rat plasma an HDL_1 particle with flotational properties overlapping those of classically isolated LDL but in which about 60% of the protein mass is ARP (35–37).[3] Similar ARP-rich particles have been found in plasma of hypercholesterolemic animals (38, 39). ARP also comprises about 60% of the total apoprotein mass of nascent HDL, a second primary hepatic secretory particle (40, 41). The factors that channel ARP to VLDL or nascent HDL are entirely unknown. The ARP class of proteins in the rat has a mean molecular size, as judged by its electrophoretic mobility in sodium dodecyl sulfate (SDS) on polyacrylamide gels, of 31,000–35,000 daltons (34).[4]

Both apoB and ARP classes exhibit some degree of microheterogeneity,[4,5] the underlying basis of which is yet undetermined. ApoB heterogeneity is detectable by electrophoresis on low percentage polyacrylamide electrophoresis.[4] ARP heterogeneity is best seen upon isoelectric focussing (40, 42), the pattern of which appears to be at least partially genetically determined (43).

In approaching the study of the regulation of apolipoprotein biosynthesis at the molecular level, careful examination of the appropriate messenger RNA (mRNA) and the primary products of its translation is mandatory. Several procedures have been employed to isolate particularly abundant species of eukaryotic mRNA or those having an unusual size or peculiar nucleotide composition (44, 45). The immunological techniques developed by Shapiro et al. (46) and Shapiro and Schimke (47), however, presage a more general applicability for recovering less prominent species of mRNA. In this approach, antibodies reactive to a selected mature protein bind to the homologous nascent protein emerging from polyribosomes. These soluble antibody–polysome complexes are then aggregated with anti-antibody. Such large aggregates may be separated from the bulk of polysomes by centrifugation. Of the RNA extracted from immunoprecipitated polysomes, 1–3% (45) represents mRNA coding for the biosynthesis of the chosen protein. By virtue of the 20–200 adenylate residues comprising a 3′-terminal poly(A)-tract common to most eukaryotic mRNA species but absent in other classes of RNA (48), mRNA can be purified from polysomal RNA through reversible hybridization to an insoluble matrix such as polyuridylate–Sepharose (poly[U]–Sepharose).

In this report we describe the immunological identification of rat liver polysomes synthesizing presumed precursors of plasma VLDL apolipoproteins. We further show that the mRNA isolated from these polysomes is enriched for species directing the in vitro biosynthesis of protein immunologically and electrophoretically resembling ARP.

MATERIALS AND METHODS

Animals and materials

Male rats, 125–175 g, of the Sprague–Dawley strain were obtained from ARS–Sprague–Dawley (Madison, WI), or from Locke–Erickson (Melrose Park, IL) and were maintained with ad libitum Rockland–Purina Rat and Mouse Chow and water in a continuously lighted room. Swift or New Zealand white rabbits were used for antibody preparation. Rabbit anti-hen egg albumin (anti-HEA), rabbit anti-rat serum albumin (anti-RSA) (7S fractions from serum, lyophilized), and hen egg albumin (HEA) were purchased from ICN Pharmaceuticals, Inc. (Cleveland, OH). Goat anti-rabbit immunoglobulin (anti-antibody) was a generous gift of Dr. Frank Fitch. $Na^{125}I$ (carrier-free, 300–500 mCi/ml) was obtained from Industrial Nuclear Corporation (St. Louis, MO). Sucrose (free of RNase and ultraviolet chromogens) was obtained from Sigma Chemical Company (St. Louis, MO), or Schwarz/Mann (Orangeburg, NY). Sodium deoxycholate (DOC), Triton X-100, sodium heparin (Grade I, 160–170 U/

[2] L. F. Walker and G. S. Getz. Unpublished observations.
[3] L. Lusk, L. F. Walker, L. H. DuBien, and G. S. Getz. Submitted for publication.
[4] L. F. Walker, J. Ong, L. A. Pottenger, L. H. DuBien, K. V. Krishnaiah, and G. S. Getz. Manuscript in preparation.
[5] R. V. Hay, J. Ong, L. F. Walker, K. V. Krishnaiah, and G. S. Getz. Unpublished observations.

mg), rat serum albumin (RSA), reduced glutathione, polyriboadenylic acid (poly[A]), HEPES, dithiothreitol, ATP, GTP, creatine phosphate, creatine phosphokinase, and unlabeled L-amino acids were purchased from Sigma Chemical Company. Pancreatic RNase was obtained from Worthington Biochemicals (Freehold, NJ). α-Amylase was obtained from Sigma or Worthington. Reagent grade urea was recrystallized from 95% ethanol or Ultra Pure urea was purchased from Schwarz/Mann. Urea to be used for isolation and characterization of apolipoproteins was dissolved in water to a concentration of 6 M and immediately passed over an AG 501-X8D ion exchange column (Bio-Rad, Richmond, CA) to remove impurities. For desorption of antibody from affinity chromatography columns, Ultra Pure urea was used as supplied. Sodium dodecyl sulfate (SDS) was purchased from Pierce Chemicals (Rockford, IL), or from Schwarz/Mann. Sepharose 4B and 6B, CNBr-activated Sepharose 4B, poly(U)-Sepharose 4B, and a jacketed 3 × 90-cm column were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Acrylamide and bisacrylamide were purchased from Eastman Kodak Company (Rochester, NY).

L-[4,5-^{3}H]Leucine and [^{32}P]orthophosphate were purchased from Amersham/Searle Corporation (Arlington Heights, IL), as were NCS tissue solubilizer and PCS (phase-combining scintillant) solubilizer. Spermine (free base) was obtained from ICN Pharmaceuticals. Proteinase K was purchased in lyophilized form from EM Laboratories, Inc. (Elmsford, NY). Formamide (polarographic grade) was obtained from Eastman Kodak and was stored in tightly capped bottles at −20°C until just prior to use. Fresh commercial wheat germ, a gift of Dixie Portland Flour Mills (Chicago, IL), was stored dry and under vacuum at 4°C prior to extraction. Other chemicals were of the best grade commercially available. Solutions used in preparing or analyzing polysomes and RNA were autoclaved before use to minimize adventitious RNase activity. Sterile and disposable plasticware was used where possible. All other materials coming into contact with sterile solutions were autoclaved or boiled before use.

Polysome preparation and analysis

Total liver polysomes were prepared from postmitochondrial supernatant fraction by a procedure similar to that of Taylor and Schimke (49), differing chiefly in our use of α-amylase, as recommended by Gamulin, Gray, and Norman (50), to hydrolyze glycogen prior to treatment of the supernatant fraction with detergents. For some experiments, rats were injected intraperitoneally with 2–5 mCi of [^{32}P]orthophosphate 6–12 hr prior to killing in order to label polysomal RNA.

To display polysomal profiles, 1–2 A_{260} of polysomes in a total volume of 50–100 μl were layered over 3.9–4.2 ml of a continuous linear gradient of 0.5–1.5 M sucrose in polysome buffer (50 mM Tris–HCl, 25 mM NaCl, 5 mM $MgCl_2$, pH 7.2, containing 100–200 U/ml of heparin) and sedimented for 40–50 min at 56,000 rpm in the SW56Ti rotor. These gradients were collected using an ISCO gradient fractionator and monitored throughout the fractionation on an ISCO ultraviolet analyzer at 254 nm by means of a flow cell with a 2-mm light path.

Some polysomes were treated with pancreatic RNase (5–50 μg/ml) or with EDTA (pH 7.0, 50 mM) and held at 25–37°C for 30 min before they were layered over continuous sucrose gradients, as indicated in the figure legends, to display ribosomal monomers or subunits, respectively. Treatment with 500 mM KCl and puromycin to release nascent chains from ribosomal particles essentially followed the description of Blobel and Sabatini (51).

Preparation of lipoproteins and apoproteins

VLDL and LDL were obtained as previously described (52). LDL was isolated by flotation within the density range 1.020–1.050 g/ml. HDL and HDL_3 were isolated within the density ranges 1.063–1.21 and 1.12–1.21 g/ml, respectively. HDL_1, having an average buoyant density of 1.054 g/ml, was separated from LDL by rate-zonal flotation (53) of lipoproteins previously isolated within the density range 1.006–1.063 or 1.019–1.063 g/ml.[3]

For the isolation of ARP from VLDL, a procedure modified from that of Scanu et al. (54) by Dr. L. A. Pottenger was employed. Five milliliters of lipoprotein solution, adjusted by the addition of solid crystals to a final KBr concentration of 20% (w/v), was injected forcibly through a syringe into 20 ml of continuously agitated ethanol. Twenty milliliters of diethyl ether was added to this mixture during agitation, and the resultant mixture was shaken vigorously. After 5 min of centrifugation at 2000 rpm in an International centrifuge model UV, the protein formed a precipitate on the bottom of the tube and the supernatant solution was decanted and discarded. The precipitate was resuspended in 5 ml of ethanol. A glass rod was used to break up persistent clumps, and 35 ml of ethanol–ether 5:2 (v/v) was added. The mixture was again shaken vigorously. Centrifugation was repeated, and the supernatant portion was again discarded. The protein precipitate was rewashed with ethanol–ether 1:6

(v/v). After a third centrifugation and decantation, the precipitate was treated by three cycles of suspension, centrifugation, and decantation using 40 ml of ether for each cycle. Finally, 3 ml of ether was left to resuspend and disperse protein in the tube. The ether was removed by drying the mixture in a vacuum desiccator for 2 hr.

Protein was solubilized directly from the tube with 5 M urea buffered to pH 11.5 with 10 mM sodium borate. This solubilized apoVLDL was further fractionated into component apoproteins by one or two cycles of gel filtration on a 500-ml Sepharose 6B column in a buffer of 6 M urea, 50 mM Tris–HCl, 0.5 M NaCl, and 0.02% NaN_3, pH 8.6, at an elution rate of 10 ml/hour. The jacketed column was held at 5–7°C and was placed in series with a countercurrent flow dialyzer using 50 mM ammonium carbonate as dialysate.

ApoLDL protein was prepared from rat plasma LDL by delipidation according to Fless and Scanu (55).

Protein estimates

Protein concentrations were determined by the method of Lowry et al. (56) using bovine serum albumin (BSA) as a standard. To remove interfering lipid from hololipoprotein fractions, the samples were extracted with diethyl ether just prior to colorimetry.

Preparation of antibodies

Anti-ARP was prepared as follows: 200 μg of ARP, purified from VLDL and suspended in 0.15 ml of saline, was emulsified with an equal volume of complete Freund's adjuvant (CFA) and the emulsion was injected subcutaneously into a rabbit's hind foot pad. At subsequent intervals of 3–4 weeks, boosters of 200–400 μg of ARP in water were injected into the same site.

The anti-apoVLDL used in this study was prepared as follows: VLDL equivalent to 150 μg of protein in 0.4–1.0 ml of saline was emulsified with an equal volume of CFA and injected into a rabbit in equal aliquots in four sites—a hind foot pad (subcutaneously), both intradermally and subcutaneously on the ventral surface, and intraperitoneally. Subsequent boosters of an equal quantity of VLDL were emulsified with incomplete Freund's adjuvant (IFA) and injected at 4- to 6-week intervals in multiple (10 or more) intradermal sites dorsally or ventrally. Following the development of a strong anti-VLDL response as judged by Ouchterlony double immunodiffusion of this serum, the rabbit received three subcutaneous boosters of 3:1 ARP: VLDL (w/w protein, total protein 60–200 μg) suspended in Maalox, followed by several boosters of 350 μg of apoLDL suspended in water or in an equal volume of Maalox and injected subcutaneously into a hind foot pad.

Seven to fourteen days after an injection, rabbits were bled from a peripheral ear vein with a rabbit bleeding apparatus (Bellco Glass, Inc., Vineland, NJ). Serum was isolated from the blood and fractionated immediately or frozen at −20°C until future use. A crude antibody fraction was prepared by ammonium sulfate precipitation of whole serum essentially as described by Palmiter, Oka, and Schimke (57).

Antisera or antibody fractions were reacted with antigens at 0–4°C on Ouchterlony double immunodiffusion plates as previously described (52).

Preparation of affinity chromatography columns

HEA, RSA, LDL, and HDL_3 were suspended in or dialyzed against 0.1 M Na_2CO_3, pH 9.0, to a final protein concentration of 5–20 mg/ml. CNBr-activated Sepharose 4B, washed with 1 mM HCl as recommended by the manufacturer, was then added to each of these protein solutions in the ratio of about 1 ml of packed beads per 15–20 mg of protein. ARP, however, was suspended to a final concentration of 1–2 mg/ml in the above coupling buffer containing 1–2 M urea and then added to 1–1.5 volumes of packed, activated Sepharose. The mixtures were rotated end-over-end in capped tubes at room temperature for 1–4 hr and then at 0–4°C for at least 12 hr. The beads were reacted with ethanolamine and further washed and poured into small columns essentially as described by Shapiro et al. (46). In most cases the recommended alternate acidic and basic 1 M NaCl washes were followed by additional 50-ml washes of phosphate buffer (10 mM sodium phosphate, 15 mM NaCl, pH 7.2–7.6), of 6–8 M urea in phosphate buffer, and again of phosphate buffer before adsorption of antibody to the column. Under these conditions, 70–90% of the initially reacted protein was coupled to the Sepharose.

Affinity enrichment of antibodies

After a final washing of the columns with phosphate buffer, antibody-containing fractions were equilibrated with the columns at room temperature for at least 1 hr or at 0–4°C for at least 2 hr. Columns were washed thoroughly first with phosphate buffer and then with 1 M NaCl to remove the bulk of nonspecifically adsorbed protein. The remaining protein was further desorbed with one of several solutions, most frequently with 6–8 M urea in phosphate buffer. Eluted fractions were pooled, dialyzed extensively against water, and lyophilized.

Antibody fractions were freed of RNase activity by

tandem CM- and DEAE-cellulose chromatography as described by Palacios, Palmiter, and Schimke (58).

Radioiodination of antibodies and hololipoproteins

Antibody preparations were labeled under sterile conditions to a high specific activity (10^5–10^6 cpm/μg) with carrier-free $Na^{125}I$ by modifications of the method of Hunter and Greenwood (59). To 50–200 μg of RNase-free antibodies in 50 μl of 0.2 M sodium phosphate, pH 7.2, was added 0.5–2.0 mCi of $Na^{125}I$ (5–8 μl) and 20 μl of freshly prepared Chloramine-T solution (33 μg/ml in the same buffer). After this mixture was agitated on a vortex mixer for 3 min at room temperature, 5 μl of a solution of sodium metabisulfite (200 μg/ml) was added and the mixture was again agitated briefly. To this reaction mixture was added 0.1 ml of 0.01 M phosphate buffer, pH 7.2, containing 0.2% (w/v) BSA and 0.02% (w/v) NaN_3, and the resultant solution was agitated. To remove unbound iodine, this entire mixture was applied to a 10–12-ml Sephadex G-75 column previously equilibrated and subsequently washed with the BSA-containing buffer. Fractions of about 0.3 ml were collected, counted for ^{125}I activity, and the peak void volume fractions were pooled and frozen for up to 6 months before use.

HDL_3 was subjected to radioiodination and one or two cycles of Sephadex G-75 chromatography exactly as just described. LDL and HDL_1 were labeled as previously described for LDL (60), but were separated from free iodine by chromatography on Sephadex G-75 in borate–BSA buffer (0.13 M sodium borate, 0.07 M NaCl, 0.05% EDTA, 0.02% NaN_3, 0.05% BSA, pH 8.0). All radioiodinated lipoproteins were stored at 4°C.

Radioimmunoassays for hololipoproteins

Each radioimmunoassay was performed at 25°C in immunoprecipitation buffer (10 mM sodium phosphate, 150 mM NaCl, 0.2% [w/v] BSA, and 0.02% NaN_3, pH 7.2) in a total volume of 100–150 μl. Each assay ultimately contained the following in a 1.5-ml capped plastic hemiconical tube: a fixed level of ^{125}I-labeled lipoprotein within the range 2,000–20,000 cpm, representing <2 ng of apolipoprotein; different amounts of unlabeled lipoprotein as indicated in the figures; anti-apolipoprotein antibody at a final concentration of 0.2 A_{280}/ml; and anti-antibody at 5.6 A_{280}/ml. Labeled and unlabeled antigen and the appropriate volume of buffer were mixed thoroughly. After addition of anti-apolipoprotein antibody and incubation of the resulting mixture for 1 hr, anti-antibody was introduced and the reaction was continued for 2 hr. Immunoprecipitation buffer (0.9 ml) was mixed with the contents of each reaction vessel, and immunoprecipitates were pelleted by sedimentation in a Brinkmann Eppendorf Model 3200/30 microcentrifuge at 15,000 rpm (12,000 g_{max}) for 2 min. Supernatant fractions were carefully aspirated away from the pellets. The conical portion of each tube, containing the immunoprecipitate, was excised with a sharp razor blade and directly counted for ^{125}I activity.

Reaction of radioactive antibodies with polysomes

Radioactive antibodies were mixed with polysomes in a total volume of 0.3–1.0 ml and allowed to stand at 0–4°C for 45–60 min. Aliquots were then layered over continuous linear sucrose gradients and sedimented as described above. Gradients were fractionated and counted for ^{125}I activity.

Isolation of polysomes by indirect (double antibody) immunoprecipitation

Polysomes freshly isolated from normal rat liver were diluted in polysome buffer to a concentration of 10–16 A_{260}/ml and adjusted to contain 150–175 mM NaCl. To remove aggregates that might interfere with the immunoprecipitation of a small proportion of polysomes, the diluted polysomes were subjected to three centrifugations of 10 min each in the Sorvall SS34 rotor at 11,000 rpm at 0°C, with removal of the pellet after each spin. Affinity-enriched anti-apoVLDL, eluted from LDL–Sepharose with urea after washing the column with 1 M NaCl in phosphate buffer, was then added to a final concentration of 0.2 A_{280}/ml polysomes and the mixture was rotated in a capped tube at 4°C for 1 hr. Anti-antibody was then added (25 A_{280}/1 A_{280} of antibody) and the incubations were continued for an additional 2 hr at 4°C. Sedimentation of the immunoprecipitates twice through discontinuous sucrose gradients containing 0.15 M NaCl and 1% of both Triton X-100 and DOC to remove nonspecifically adsorbed polysomes was done essentially as described by Shapiro et al. (46).

Isolation of mRNA

The postmitochondrial supernatant fraction of rat liver was adjusted to contain 25 mM Tris–HCl (pH 7.4), 5 mM EDTA, and 1% each of Triton X-100 and DOC and then extracted in the presence of 0.2% SDS with an equal volume of a 1:1 mixture of buffer-saturated phenol (saturated with 50 mM Tris–HCl, 25 mM NaCl, 5 mM $MgCl_2$, pH 7.1) and chloroform as recommended by Shapiro and Taylor.[6] After thorough mix-

[6] Messenger RNA Workshop conducted by Drs. John Taylor and David Shapiro at the Given Institute of Pathobiology, Aspen, Colorado, August 17–22, 1975.

ing, the phases were separated by centrifugation and the aqueous phase was extracted thrice more with the solvent mixture. Polysome-containing immunoprecipitates were incubated for 1 hr at 25°C with 1–2% SDS, 5 mM EDTA, and 0.5 mg/ml of proteinase K, as suggested by the studies of Wiegers et al. (61, 62). RNA was precipitated from the extracts (adjusted to 0.2 M NaCl) with either ethanol or LiCl (61). The resulting precipitates were washed thoroughly and serially with LiCl, sodium acetate, and ethanol, basically as described by Shapiro et al. (46).

Purification of poly(A)-containing RNA by chromatography on poly(U)–Sepharose was conducted exactly as recommended by Shapiro and Taylor.[6] Briefly, polysomal RNA or postmitochondrial supernatant fraction RNA was dissolved in denaturation buffer (20 mM HEPES, 10 mM EDTA, 1% SDS pH 7.4) and was heat-denatured (10 min at 65°C), cooled in an ice bath, and diluted with an equal volume of 0.8 M NaCl to achieve conditions for binding to the column. The RNA was applied to the column in binding buffer (10 mM HEPES, 0.4 M NaCl, 5 mM EDTA, and 0.5% SDS, pH 7.4); the column was thoroughly washed with buffer consisting of 10 mM HEPES, 2 mM EDTA, and 0.5% SDS to remove ribosomal RNA; and poly(A)-rich RNA species were eluted with 70% formamide containing 1 mM HEPES and 1 mM EDTA, pH 7.4, and recovered by ethanol precipitation.

In vitro protein synthesis assays

Wheat germ S-30 supernatant extract was utilized in translating rat liver poly(A)-rich RNA by a combination of published procedures (63–65). The initial preparation of S-30 and its storage in liquid nitrogen was as described by Roberts and Paterson (64). Translation assays contained, in a final volume of 40 μl, 0.1 mg of wheat germ S-30 fraction protein, 0–250 ng of poly(A)-rich RNA, 28 mM HEPES (pH 7.0), 84 mM KCl, 3 mM magnesium acetate, 8 mM creatine phosphate, 8 μg/ml of creatine phosphokinase, 1 mM ATP, 20 μM GTP, 2 mM dithiothreitol, 20 μM of each of 19 unlabeled L-amino acids, 4 μCi of [^{3}H]leucine (2 μM), and 40 μM spermine. Incubation was at 25°C for 90 min. To assess total protein synthesis, reactions were terminated by the addition of 0.2 mg of BSA followed by 2 ml of 10% trichloroacetic acid (TCA) containing 10 mg/l of unlabeled leucine. These samples were heated at 90–95°C for 15 min, chilled, and filtered through Whatman GF/C filters to trap precipitated material. The filters were further washed six times with 3 ml of 5% TCA containing 10 mg/l leucine, dried in an oven at 100°C for 15 min, suspended in PCS, and counted for retained radioactivity as described below.

Immunoprecipitation of protein synthesized in vitro

Immunoprecipitation of protein synthesized in messenger-stimulated wheat germ extracts was by a procedure similar to that described by Taylor and Schimke (49). Protein synthesis assay incubations were halted by adjustment to a final concentration of 10 mM unlabeled L-leucine and 1% of both Triton X-100 and DOC. The samples were then centrifuged in 1.5-ml tubes in a Brinkman Eppendorf Model 3200/30 microcentrifuge at 15,000 rpm for 1 min to remove aggregates. To the supernatant portion was added 0.01 A_{280} of affinity-enriched anti-apoVLDL, anti-ARP, anti-apoB, or crude antibody obtained by ammonium sulfate precipitation of sera from non-immunized rabbits and the mixtures were allowed to incubate at 25°C for 1 hr. 0.35 A_{280} of anti-antibody was added to each of these mixtures, and the incubations were continued for another hour. Reaction mixtures were layered over a discontinuous gradient of 100 μl of 0.5 M sucrose layered over 100 μl of 1.0 M sucrose, both containing 10 mM sodium phosphate, pH 7.2–7.5, 150 mM NaCl, 10 mM unlabeled L-leucine, 1% Triton X-100, and 1% DOC, and then centrifuged at 12,000 g_{max} in the microcentrifuge for 1 min. The supernatant fraction and cushions were carefully aspirated, and the pelleted immunoprecipitates were suspended and washed with 1 ml of the same buffer used to make up the sucrose solutions with small glass stirring rods and vigorous vortexing. The immunoprecipitates were resedimented (without sucrose cushions) and washed three times with this same buffer; finally, each immunoprecipitate was suspended and transferred to a scintillation vial, mixed with 5–6 ml of PCS, and counted for radioactivity as described below.

Electrophoretic analysis of radioactive peptides in immunoprecipitates

Some immunoprecipitated material was resuspended, incubated, and subjected to polyacrylamide disc gel electrophoresis in the presence of SDS essentially as described by Weber and Osborn (66). Gels were fractionated for monitoring of radioactivity with a Gilson automatic gel fractionator, using NCS as a gel solubilizer. After overnight solubilization, 5 ml of toluene containing 4.9 g/l of PPO (2,5-diphenyloxazole) and 0.5 g/l of dimethyl-POPOP (1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene) was added to each vial and the samples were counted.

Monitoring of radioactivity

^{125}I activity was measured in a Searle Model 1185 gamma counter, a Nuclear-Chicago single tube counter, or a Packard Auto-Gamma counter. ^{3}H and

^{32}P were measured in a Searle Model 6872 Isocap/300 scintillation counter. The efficiency of counting ^{3}H in our system averaged 26%.

RESULTS

Apolipoproteins

The apoB component of VLDL and LDL could be resolved by SDS-electrophoresis in 3.5% polyacrylamide gels into two bands corresponding to particle sizes of approximately 3×10^5 and 2×10^5 daltons. The other protein components of VLDL, ARP and C-peptides, were also seen in varying amounts in LDL depending on the individual LDL preparation (34).

On Sepharose 6B column chromatography of VLDL, ARP emerged in a broad peak, the center of which was judged pure, giving a single band on SDS- or urea-electrophoresis on polyacrylamide gels (66, 67). It had an apparent molecular weight of 31,500 on SDS polyacrylamide gel electrophoresis. The amino acid composition of this fraction was indistinguishable from that reported in several other laboratories (28, 34, 37, 68, 69) and glutamic acid was its N-terminal amino acid. The examination of subsequent similar preparations by isoelectric focussing has revealed a profile of polymorphic forms similar to that observed for ARP in unfractionated apoVLDL.

Characteristics of radioiodinated fractions

In the case of ^{125}I-antibody, at least 90% of the ^{125}I recovered in the void volume fractions from chromatography on Sephadex could be precipitated with 10% TCA; at least 80% was precipitated by goat anti-rabbit immunoglobulin at the equivalence point.

TCA precipitated ≥90% of ^{125}I-LDL, ≥75% of ^{125}I-HDL_1, and ≥40% of ^{125}I-HDL_3. In the absence of unlabeled carrier hololipoprotein anti-apoVLDL precipitated 74% of ^{125}I-LDL, while 60% of both ^{125}I-HDL_1 and ^{125}I-HDL_3 were precipitated by anti-ARP. Less than 5% and 7%, respectively, of the ^{125}I in ^{125}I-LDL and ^{125}I-HDL_1 were lipid extractable.

Enrichment of antibodies by affinity chromatography

Although several solutions were tested, desorption of anti-apolipoproteins from affinity columns was best accomplished with urea. Elution of bound anti-ARP or anti-apoVLDL from the appropriate ligand–Sepharose with 8 M urea, following thorough washing of the column with 1 M NaCl, recovered 2–3% of the antibody initially applied to the column. Anti-apolipoproteins purified in this manner represented about 90% of the applied immunoreactivity as assessed by radioimmunoassay. Such fractions consistently bound to polysomes.

Anti-ARP eluted by this method from ARP–Sepharose and anti-apoVLDL likewise recovered from LDL–Sepharose were tested for their capacities to bind HDL_1 and LDL, respectively, in radioimmunoassays. Affinity-enriched anti-ARP was about 30-fold more effective in precipitating HDL_1 than an equivalent amount of antibody obtained by ammonium sulfate precipitation of antiserum reactive to ARP. The binding activity of anti-apoVLDL was similarly enhanced relative to the crude antibody fraction when tested against LDL (**Fig. 1**[*a*], [*b*]).

Enriched anti-ARP was strongly reactive in radioimmunoassay with HDL_3, a particle which contains all the apoproteins of HDL and VLDL excepting apoB. This anti-ARP was only about 1% as reactive toward LDL as was enriched anti-apoVLDL.

Characteristics of antibodies

The anti-ARP serum used in this study revealed prominent arcs of identity against VLDL, ARP, HDL and its subclasses, and normal unfractionated rat serum on Ouchterlony double immunodiffusion, but did not react against a mixture of C-peptides. The degree of reaction with LDL was related to the content of ARP as observed by SDS-electrophoresis. Multiple arcs were frequently seen when this anti-ARP serum, or anti-ARP serum from yet other animals, was reacted against ARP-containing lipoprotein fractions or whole rat serum. The relative intensities of these arcs varied with the lipoprotein fraction tested. Each arc formed against rat serum or other hololipoprotein fractions, however, shared complete identity with an arc seen against ARP purified from VLDL. In contrast to this crude antiserum, the affinity-enriched anti-ARP antibody used in this study exhibited a single arc of identity when diffused against serum, purified ARP, and the hololipoproteins VLDL and HDL_3 (**Fig. 2**). This latter preparation was the anti-ARP employed to characterize both rat liver polysomes and the wheat germ translation products.

The anti-apoVLDL serum and affinity-enriched anti-apoVLDL antibody used here gave one prominent diffusion arc of identity against VLDL, LDL, and unfractionated rat serum, and showed separate arcs of lesser but variable intensity on diffusion against C-peptides, purified ARP, and lipoprotein fractions containing these proteins. From the proportion of purified anti-apoVLDL antibody that bound to HDL_3–Sepharose, it was estimated that 20–30% of the antibodies were directed against antigens other than apoB.

A single passage of unfractionated anti-apoVLDL

serum through HDL_3–Sepharose was sufficient to completely adsorb the anti-HDL_3, anti-C-peptide, and anti-ARP reactivities previously demonstrable by immunodiffusion analysis. Antibodies subsequently adsorbed by LDL–Sepharose from the fraction excluded by HDL_3–Sepharose, however, retained some anti-HDL_3 immunoreactivity in the more sensitive radioimmunoassay. An additional exposure of the LDL-adsorbed components to HDL_3–Sepharose abolished this latter reactivity. Anti-apoVLDL freed in this manner of anti-HDL_3, anti-C-peptide, and anti-ARP immunoreactivities were designated anti-apoB. This preparation showed only a single arc of identity upon immunodiffusion against VLDL, LDL, and whole rat serum (Fig. 2).

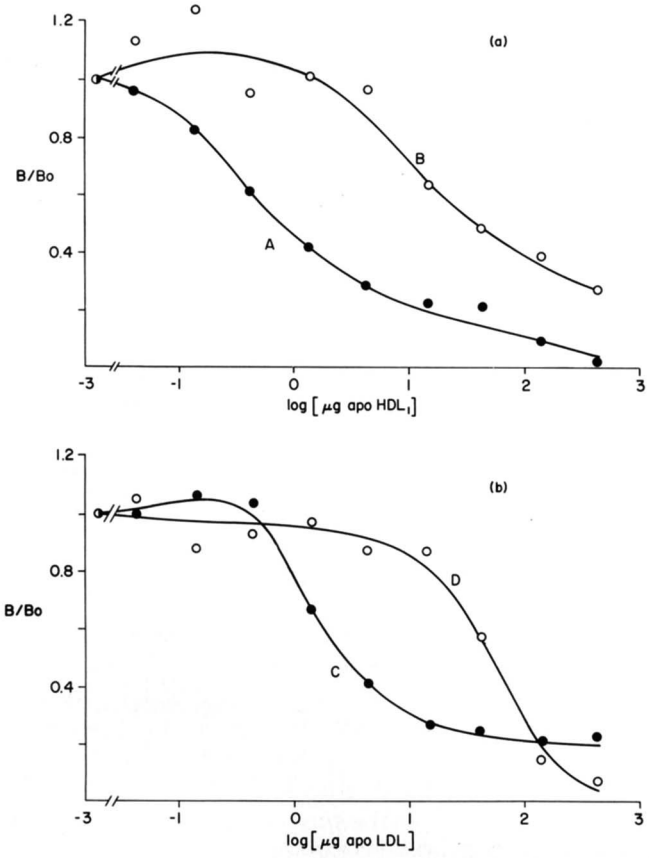

Fig. 1. Reaction of antibodies with hololipoproteins in radioimmunoassays. Radioimmunoassays were conducted with hololipoprotein fractions as described in the text. Various amounts of unlabeled HDL_1 (*a*) or LDL (*b*), expressed in terms of apolipoprotein mass as indicated on the abscissa, were combined with a constant amount of homologous ^{125}I-labeled lipoprotein and reacted with fixed amounts of antibody and anti-antibody. On the ordinate, B_0 represents the net radioactivity precipitated in the absence of, and B that precipitated in the presence of a given quantity of unlabeled antigen. Antibody fractions used to generate each curve were as follows: *A*, ammonium sulfate precipitate of antiserum reactive to ARP by immunodiffusion (crude anti-ARP); *B*, anti-ARP prepared as described in legend to Fig. 3; *C*, crude anti-apoVLDL; *D*, anti-apoVLDL prepared as described in legend to Fig. 3.

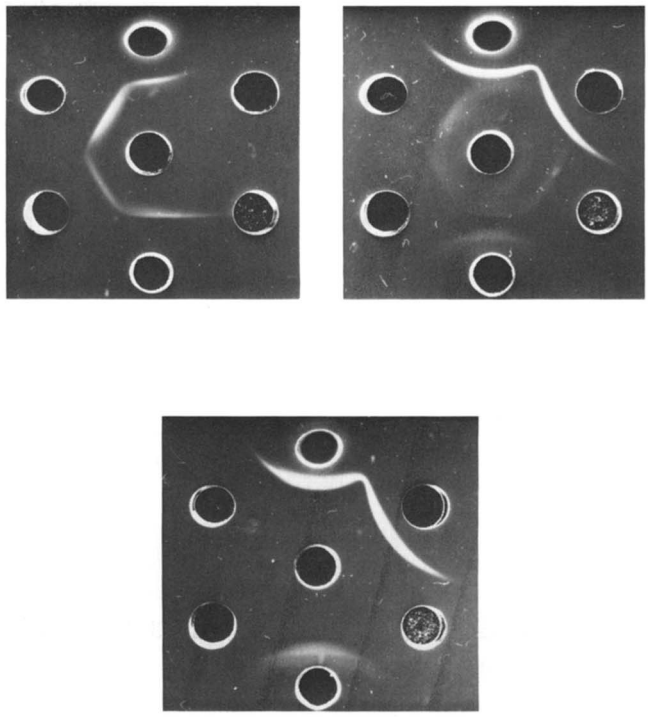

Fig. 2. Immunodiffusion analysis of antibodies. Antibodies were purified from crude antisera by selective affinity chromatography as described in the text. Central wells were filled as follows: upper left, anti-ARP; upper right, anti-apoVLDL; lower plate, anti-apoB. The outer wells of each array, proceeding from the top clockwise, were filled with the following antigens: VLDL; LDL (prepared by rate-zonal ultracentrifugation); C-peptide mixture; normal rat serum; HDL_3; ARP. The plates were photographed, without staining, after diffusion for 72 hr.

Thus, three enriched antibodies were employed for polysome binding studies and analysis of wheat germ translation products: *1*) anti-apoVLDL, purified from anti-apoVLDL serum by adsorption to and elution from an LDL–Sepharose column; *2*) anti-apoB, enriched from the same anti-apoVLDL serum by exclusion from HDL_3–Sepharose, followed by adsorption to and elution from the LDL–Sepharose, and finally, a second exclusion from HDL_3–Sepharose; *3*)anti-ARP, purified on ARP–Sepharose from a separately prepared anti-ARP serum (Fig. 2).

Reactivity of radioactive antibodies toward (poly)ribosomes

The specific binding characteristics of ^{125}I-anti-apoVLDL, -anti-ARP, and -anti-apoB to polysomes and polysomal derivatives are illustrated in **Fig. 3.** Several lines of experimental evidence indicate that the antibodies recognize and specifically bind to the nascent polypeptides of apolipoprotein antigens.

Antibodies purified by affinity chromatography showed enhanced binding to polysomes when compared in the same assay with crude antibody fractions

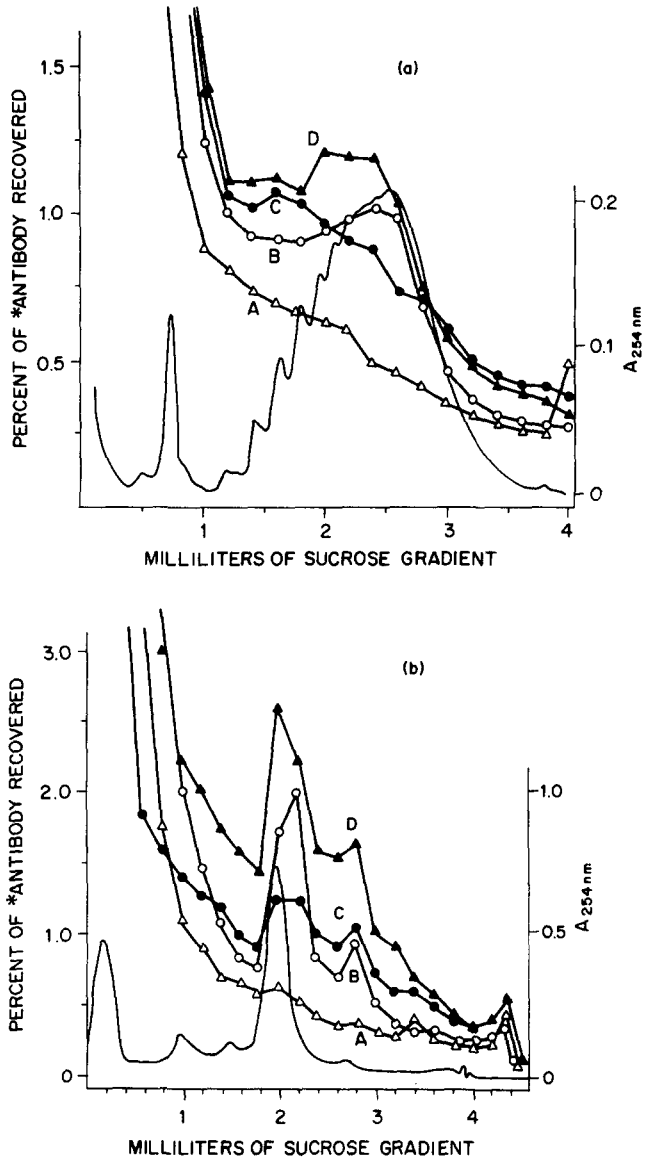

Fig. 3. Reaction of polysomes with various antibodies. 3.0 A_{260} of rat liver polysomes were reacted at 0°C in about 0.3 ml with antibody as detailed. In (*a*), 0.1 ml of each reaction mixture was layered onto a 3.9-ml linear gradient of 0.5–1.5 M sucrose in polysome buffer. In (*b*), each reaction mixture was first treated with 3 μg of RNase at 25°C for 15 min; 0.1 ml of each mixture was then layered onto a 4.4-ml linear gradient of 10–30% (w/v) sucrose in polysome buffer. All gradients were sedimented in the SW56Ti rotor at 56,000 rpm (408,000 g_{max}) and 2°C for 50 min. Sedimentation is from left to right. Gradients were fractionated and counted for ^{125}I activity. The species sedimenting at 2.0 and 2.7 ml in the tracing of (*b*) are ribosomal monomers and dimers, respectively. *A*, 30 μg of unlabeled anti-apoVLDL, 30 min, followed by incubation with 1.5 μg of ^{125}I-anti-apoVLDL for 60 min; *B*, 1.5 μg of ^{125}I-anti-ARP, 60 min; *C*, 1.5 μg of ^{125}I-anti-apoB, 60 min; *D*, 1.5 μg of ^{125}I-anti-apoVLDL, 60 min. Anti-apoVLDL and anti-ARP were eluted with urea from LDL–Sepharose and ARP–Sepharose, respectively, following a pre-elution with 1 M NaCl in phosphate buffer; anti-apoB was prepared from anti-apoVLDL serum by exclusion from HDL_3–Sepharose as described in the text. Distributions of radioactivity essentially identical to curves *A* were obtained when polysomes tested with ^{125}I-anti-ARP or -anti-apoB were preincubated with a 20-fold excess of unlabeled anti-ARP or anti-apoB, respectively, or when either group was preincubated with anti-apoVLDL. A binding curve nearly identical to curve *A* was also seen when polysomes preincubated with an equimolar mixture of unlabeled anti-ARP and anti-apoB were tested with ^{125}I-anti-apoVLDL.

of specific antisera. This enhancement paralleled the specific antibody enrichment as tested in radioimmunoassays with native plasma lipoproteins.

Antibodies prepared against rat plasma apolipoproteins did not react with monkey liver polysomes. Neither anti-HEA nor crude antibody from the sera of nomimmune rabbits bound to rat liver polysomes.

The distribution of radioactivity in the polysomal profile was characteristic for each type of antibody bound. Anti-ARP bound prominently in the 9- to 12-some region (Fig. 3[*a*], curve *B*). Anti-apoB bound over a broad region of the profile, most noticeably in the 4- to 9-some region (Fig. 3[*a*], curve *C*). Anti-apoVLDL bound broadly in what appeared to be an additive fashion (Fig. 3[*a*], curve *D*), with components of both anti-apoB and anti-ARP. By comparison, anti-RSA bound sharply in the 16- to 19-some region (data not shown). The distribution of bound radioactivity amongst reasonably intact polysomes was not dependent upon variations in the polysome size distribution between different preparations. This specificity of binding was demonstrated most convincingly in one experiment in which polysomes of cytoplasmic and membranous origin were separated by a modification of the method described by Venkatesan and Steele (70), and individually incubated with anti-ARP. Although the absorbance profile peak occurred at comparatively larger polysomes in the case of the cytoplasmic polysomes, the antibody-binding peak was in the same *n*-some region for both polysome preparations. This peak appeared on the ascending portion of the profile of free polysomes, but on the descending limb of the profile of membranous polysomes.

Treatment of polysomes with RNase, following or preceding their incubation with radioactive antibody, shifted the peak of radioactivity to positions coincident with the resultant ribosome monomer and dimer peaks (Fig. 3[*b*], curves *B*, *C*, and *D*). Conditions that release most nascent polypeptides from ribosomal particles, such as treatment with 50 mM EDTA or incubation with 500 mM KCl and puromycin, resulted in a shift of the radioactivity to the supernatant fractions. Treatment with 500 mM KCl alone was not sufficient to abolish antibody binding to polysomes. Some unreacted polysomes were treated with EDTA or 500 mM KCl and puromycin and then dialyzed to restore ionic conditions to those of the originally isolated polysomes. Such fractions, containing mostly subunits and some

ribosomal monomers, were incapable of binding radioactive antibody.

Binding of radioactive antibody to polysomes was competitively inhibited by homospecific or strongly cross-reactive antibodies. With a prior incubation of polysomes in a 20- to 50-fold excess of unlabeled homospecific antibody (Fig. 3, curves *A*), net binding of radioactive antibody was blocked. Unlabeled heterospecific or nonspecific antibody did not interfere with the binding of radioiodinated antibody. Anti-ARP and anti-apoB were not cross-reactive for binding to polysomes or monosomes. Incubation of polysomes with an excess of unlabeled anti-ARP followed by ^{125}I-anti-apoB, and with unlabeled anti-apoB followed by ^{125}I-anti-ARP, had no effect on the respective radioactive binding profiles and yielded curves indistinguishable from curves *C* and *B*, respectively, in Fig. 3. A prior incubation of polysomes with anti-apoVLDL, however, reduced the binding of either ^{125}I-anti-apoB or -anti-ARP to the level of curve *A*. Anti-ARP or anti-apoB alone only partially inhibited binding of ^{125}I-anti-apoVLDL (**Table 1**). Furthermore, when tested against intact polysomes, the residual binding curves for the competitive combinations anti-ARP/^{125}I-anti-apoVLDL and anti-apoB/^{125}I-anti-apoVLDL were nearly identical to curves *C* and *B*, respectively, of Fig. 3(*a*). An equimolar mixture of unlabeled anti-ARP and anti-apoB almost completely displaced the binding of ^{125}I-anti-apo-VLDL.

Immunoprecipitation of polysomes and extraction of polysomal RNA

Aliquots of polysomes whose RNA was labeled in vivo with ^{32}P were incubated with different amounts of affinity-enriched anti-apoVLDL or antibody from nonimmune rabbit serum and subsequently with an optimum amount of anti-antibody as illustrated in **Fig. 4.** This and other similar experiments showed

TABLE 1. Competitive binding of anti-apolipoprotein antibodies to rat liver polysomes

Unlabeled Antibody	^{125}I-labeled Antibody		
	−anti-apoVLDL	−anti-ARP	−anti-apoB
anti-apoVLDL	+	+	+
anti-ARP	+½	+	−
anti-apoB	+½	−	+

Affinity-enriched antibodies were reacted with rat liver polysomes; the mixtures were sedimented through continuous linear sucrose gradients, and the distribution of radioactivity within the gradients was determined by fractionation. In each case, the polysomes were first incubated with 30 μg of unlabeled antibody and then with 1.5 μg of ^{125}I-labeled antibody. The "+" symbol indicates complete displacement, "+½" partial displacement, and "−" nondisplacement, of the ^{125}I-labeled antibody binding to polysomes as seen in the absence of unlabeled antibody.

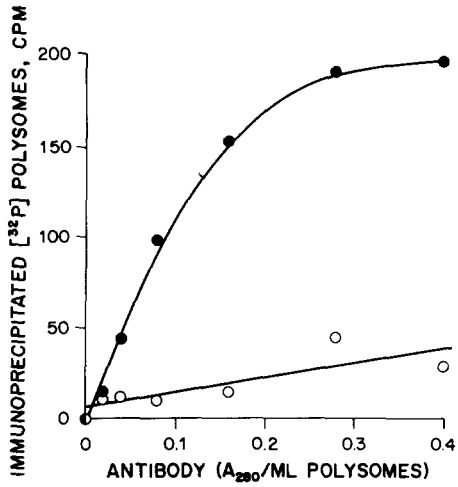

Fig. 4. Indirect immunoprecipitation of [^{32}P]polysomes. 3.5 A_{260} (15,000 cpm) of [^{32}P]polysomes prepared as described in the text were reacted in a final volume of 0.25 ml with various levels of affinity-enriched anti-apoVLDL (solid circles) or of crude immunoglobulin from the serum of a nonimmune rabbit (open circles) for 1 hr at 0–4°C. Anti-antibody was then added at a ratio to antibody of 25:1, and the incubations continued for an additional 2 hr. The reaction mixtures were layered onto discontinuous gradients of 3 ml of 0.5 M sucrose layered over 6 ml of 1.0 M sucrose, both in polysome buffer adjusted to contain 175 mM NaCl, 1% Triton X-100, and 1% DOC. Immunoprecipitates were pelleted for 20 min in the SM24 Sorvall rotor at 11,500 rpm (15,000 g_{max}) and 0°C. The gradients were carefully aspirated to the pellet; the pellets were resuspended and resedimented through the same discontinuous gradients and then assayed for ^{32}P activity.

that *a*) nonspecific trapping of polysomes in the immunoprecipitate constituted between 0.3 and 0.6% of the incubated polysomes; *b*) optimal specific immunoprecipitation of nascent apoVLDL-synthesizing polysomes could be achieved by reacting 0.16–0.28 A_{280} of antibody per ml of polysomes; and *c*) a net level of 1–2% of normal rat liver polysomes (i.e., in addition to the fraction precipitated nonspecifically) are engaged in the synthesis of proteins immunologically resembling apoVLDL components.

In order to prepare a sufficient quantity of RNA for the isolation of mRNA, 544 A_{260} units of polysomes freshly isolated from 26 g of rat liver were subjected to indirect immunoprecipitation with affinity-enriched anti-apoVLDL and anti-antibody as just described. Thirteen A_{260} (approximately 650 μg) of polysomal RNA, or a *net* of 1.8–2.1% of the starting material (excluding nonspecific precipitation, 0.3–0.6%), was obtained by proteinase K extraction of the immunoprecipitate and 5.8 μg of poly(A)-rich RNA was recovered from the polysomal RNA by poly(U)–Sepharose affinity chromatography.

Translation of poly(A)-rich RNA

Poly(A)-rich RNA from immunoprecipitated polysomes stimulated protein synthesis in wheat germ

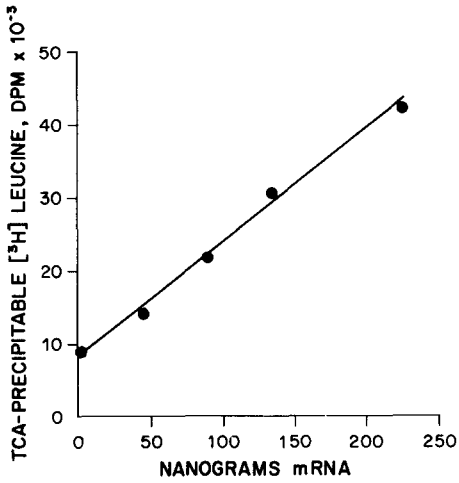

Fig. 5. Stimulation of protein synthesis in wheat germ extracts. Poly(A)-rich RNA was purified as described in the text, precipitated with ethanol, and rewashed with sodium acetate and ethanol. This RNA was dissolved in sterile water and aliquots were added to the mRNA-dependent wheat germ translational system. This figure illustrates stimulation by poly(A)-rich RNA obtained from polysomes immunoprecipitated with anti-apoVLDL. Similar results were obtained with total poly(A)-rich RNA derived from postmitochondrial supernatant. The level of incorporation of [³H]leucine into protein was determined by TCA precipitation.

extracts in a linear fashion over the range 0–250 ng of added RNA (**Fig. 5**). Similar data were obtained for poly(A)-rich RNA from rat liver postmitochondrial supernatant fraction (data now shown). The level of stimulation by exogenous RNA varied from 3 to 75 times that of endogenous synthesis.

The translation products of both poly(A)-rich RNA fractions were subjected to indirect immunoprecipitation by anti-apolipoproteins. Of the TCA-precipitable radioactivity present in extracts stimulated by total liver poly(A)-rich RNA, a net[7] of 2.3 ± 0.1% (mean and SEM) was precipitable by anti-apoVLDL, 1.7 ± 0.2% by anti-ARP, and roughly 0.3% by anti-apoB. Two other mRNA preparations were analyzed, with results of *a*) 2.1 ± 0.1% for anti-apoVLDL and 1.2 ± 0.2% for anti-ARP, and *b*) 1.5 ± 0.2% for anti-ARP, respectively. From those extracts stimulated by mRNA from immunoprecipitated polysomes, average net recoveries of 38 ± 4% (anti-apoVLDL), 28 ± 2% (anti-ARP), and 1.0 ± 0.2% (anti-apoB) were obtained. These results indicate that the poly(A)-rich RNA isolated from polysomes immunoprecipitated by anti-apoVLDL represents about a 17-fold purification of mRNA coding for proteins immunologically resembling both total apoVLDL antigens and ARP, but minimal enrichment of mRNA coding for proteins detectable by the purified fraction of anti-apoB. These data also suggest that the majority of normal rat hepatic protein synthesis devoted to antigens detectable by anti-apoVLDL is attributable to the translation of ARP-like proteins.

Part of the material immunoprecipitated by anti-ARP from wheat germ extracts stimulated by enriched mRNA was analyzed by electrophoresis in polyacrylamide disc gels in the presence of SDS. The gels were fractionated to determine the positions of radioactive polypeptides. The major species migrated identically with unlabeled ARP purified from plasma VLDL (**Fig. 6**), and was not affected by prior chemical reduction and alkylation of the immunoprecipitate (71). Essentially the same pattern is obtained upon electrophoresis of the anti-ARP precipitated products of a wheat germ extract primed with total liver poly(A)-rich RNA.[8] We have as yet been unable to satisfactorily analyze the small amount of radioactive material precipitated by anti-apoB.

DISCUSSION

The data presented here demonstrate that within rat liver there exist specific polysomes synthesizing presumed precursors of apoproteins found in plasma VLDL. At least two subpopulations bearing nascent chains immunologically recognizable as cross-reactive with apoB and ARP, respectively, can be distinguished. This population can be isolated by immunological means, and mRNA extracted from these polysomes is substantially enriched in species coding for the biosynthesis of proteins antigenically resembling apoVLDL and, more specifically, ARP.

During their glycosylation, lipidation, and association with other apolipoprotein species, the plasma apolipoproteins perhaps undergo more extensive posttranslational modification than do most other classes of proteins. In considering the relationship between initial translation products and their plasma counterparts, the many potentialities for both intra- and extrahepatic post-translational alterations must constantly be borne in mind. Therefore, it is probably most appropriate to regard the initial translation products present both on rat liver polysomes and in stimulated wheat germ extracts as developmental forms of plasma apolipoproteins. This situation poses obvious semantic dif-

[7] These numbers represent the net proportion of protein precipitated by the respective antibodies after subtraction of the proportion of protein precipitated by non-immune serum. In some experiments, anti-BSA has been used as a nonspecific control and essentially identical results were obtained. When wheat germ lysates were primed with globin messenger RNA, provided by Drs. Rabinowitz and Halbreich, there was no net precipitation of the translation products by anti-ARP.

[8] C. Reardon. Unpublished observations.

ficulties, at least. Recognizing this dilemma, we have designated the respective polysome-bound forms as "nascent apoB and ARP," and the immunoprecipitable products of in vitro mRNA translation as, e.g., "ARP-like proteins." Such labels are meant simply to distinguish each of these forms from the others and, in turn, from the mature plasma apolipoproteins. Although a broadly interpreted precursor–product correlation might be inferred from these designations, they are not intended to prejudge the complexity of relationships between developmental and mature apolipoprotein forms.

In light of critical reports questioning the specificity of antibody–polysome interactions (72, 73), rigorous criteria have been employed here to establish that anti-apolipoprotein antibodies are reactive toward specific nascent apolipoproteins on (poly)ribosomes rather than constituents of the ribosomes themselves or cellular proteins adventitiously adsorbed to the polysomes. These include observations that the antibodies tested do not react with polysomes of a foreign species; that binding levels are enhanced in proportion to antibody enrichment by affinity chromatography; that the antibodies bind to characteristic size classes of polysomes; that procedures that predictably shift the position of nascent polypeptides in sucrose gradients similarly affect the position of antibodies bound to polysomes; that antibody binding can be inhibited competitively by homospecific or cross-reactive species; and that mRNA purified from immunologically reactive polysomes is enhanced for the synthesis of similarly reactive proteins.

Although the polysome binding experiments described here are largely prefatory to the successful immunological isolation of mRNA, the binding profiles themselves provide some useful information regarding the biosynthesis of individual apolipoproteins. The average size of a newly synthesized polypeptide can be estimated by assuming that a protein of molecular weight $n \times 10^4$ is synthesized on polysomes containing about $3n$ ribosomes (45). Thus, the binding of anti-RSA to polysomes in the 16- to 19-some region and of anti-ARP in the 9- to 12-some region is consistent with estimated polypeptide sizes of about 60,000 and 35,000 daltons, respectively. Although the monomeric size and homogeneity of apoB have not been established, the binding of radioactive anti-apoB to polysomes yields two clues regarding the intrahepatic precursors of plasma apoB. Nascent immunoreactive apoB is prominent on small polysomes, in the 4- to 9-some region, suggesting that monomeric apoB could contain protein species within the range of about 12,000–30,000 daltons. The rather broad distribution of anti-apoB throughout the polysome profile raises

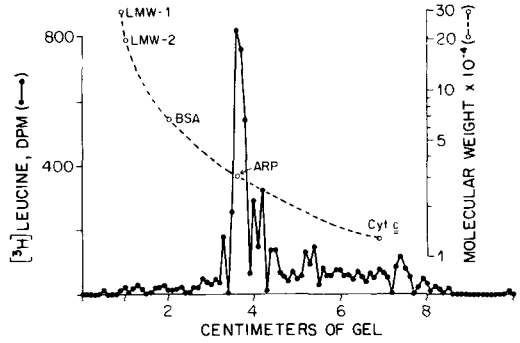

Fig. 6. Electrophoretic analysis of in vitro translation products. An anti-ARP immunoprecipitate of wheat germ extract stimulated by enriched mRNA was dissolved in electrophoresis buffer, incubated at 37°C for 2 hr, boiled for 2 min, and subjected to electrophoresis on a 12.5% acrylamide disc gel with a 0.7-cm, 2.5% acrylamide stacking gel. The gel was fractionated at 1-mm intervals, solubilized, and counted for ^3H activity. Protein markers are abbreviated as follows: LMW-1 and -2, the large molecular weight species of VLDL; BSA, bovine serum albumin; ARP, purified arginine-rich peptides; and cyt *c*, cytochrome *c*.

the possibility that apoB or its developmental forms could include several proteins with various molecular sizes.

The immunological approach has enabled us to make a relatively direct determination of the level of synthesis of developmental apolipoprotein forms in the liver. Close agreement of the level of in vivo-labeled polysomes precipitable with anti-apoVLDL, the proportion of total polysomal RNA recovered in a specific immunoprecipitate from a larger polysome preparation, and the amount of newly synthesized protein antigenically resembling components of plasma apoVLDL in a heterologous translation system programmed with unfractionated rat liver poly(A)-rich RNA provides an estimate that roughly 2% of total liver protein synthesis in the normal rat is devoted to apoVLDL-like proteins, and that about 1.5% is devoted to ARP-like proteins alone. The first estimate agrees with those reported for apoVLDL-like protein synthesis in slices of chicken liver (27), apoLDL-like protein synthesis by isolated rat liver ribosomes (23), and apoVLDL synthesis by isolated perfused rat liver (5).

Identification of the immunoprecipitated translation products deserves comment. The prominent species seen in Fig. 6 can be tentatively designated a precursor of plasma ARP on the basis of its immunoreactivity and its comigration with authentic plasma ARP. It should be stressed that, whereas the enriched mRNA fraction was derived from polysomes precipitated by anti-apoVLDL raised in one rabbit, the immunological recovery of the newly translated product of this mRNA was achieved by using an antibody induced with a separate, purified ARP antigen in a different animal. An electrophoretically similar product was also the

major species immunoprecipitated from the wheat germ extract by the purified anti-apoVLDL antibody.

The small proportion of total mRNA translation devoted in normal rat liver to producing protein immunologically detectable by anti-apoB, the heterodispersity of binding of anti-apoB to polysomes, and our relative ignorance about the structural characteristics of mature apoB all indicate how formidable is the task of further analyzing apoB translation at this time.

Further examination of ARP-like protein synthesis, on the other hand, would appear to be a fruitful endeavor. ARP is readily available in purified, highly immunogenic form, and possesses distinctive physical characteristics. A substantial proportion of mRNA-directed apoVLDL-like protein synthesis in vitro is devoted to the production of ARP-like proteins, and much of this translated protein corresponds electrophoretically to completed copies of ARP. Whether the ARP-like primary translation product contains sequences additional to those present in the mature circulating ARP is the subject of current investigation.

We are indebted to Dr. Ira G. Wool for his guidance and generous donation of rat plasma; to Mr. Lance Lusk, Ms. Laurie Walker, and Mr. Johnson Ong for their valuable assistance in processing the apoproteins; to Messrs. Howard Gershenfeld, Jack Ohringer, Robert Padley, Ken Tuman, Randolph Hughes, Laurence Frazier, James Foreman, Dr. Robert Heinrickson, Dr. Gunther Fless, Ms. Elizabeth Unger, Ms. Catherine Reardon, and Ms. Leila Aggarwal for technical advice and assistance; to Drs. Larry DuBien, David Sales, Robert Hunter, Jayme Borensztajn, Angelo Scanu, Joan Karlin, and Arthur Rubenstein for their criticisms and suggestions; to Drs. Larry Pottenger, Alan Permutt, John Taylor, and David Shapiro for their valuable suggestions and encouragement; to Dr. Robert Wissler for his continued interest and enthusiasm for this work; and to Ms. Lindy Guttman-Zass, Ms. Linda McGuire, and Ms. Pat Serrato for assistance in preparing the manuscript. We are grateful to Dr. Richard Havel for supplying a preprint of his paper (34) describing a radioimmunoassay for ARP. This work was supported by USPHS grants HL-15062 and 5 TO 5 GM-1939 (MSTP).

Manuscript received 21 April 1978; accepted 22 August 1978.

REFERENCES

1. Fredrickson, D. S., R. I. Levy, and R. S. Lees. 1967. Fat transport in lipoproteins—an integrated approach to mechanisms and disorders. *N. Engl. J. Med.* **276:** 34–42, 94–103, 148–156, 215–225, 273–281.
2. Partamian, J. O., and R. F. Bradley. 1965. Acute myocardial infarction in 258 cases of diabetes—immediate mortality and five-year survival. *N. Engl. J. Med.* **273:** 455–461.
3. Getz, G. S., D. Vesselinovitch, and R. W. Wissler. 1969. A dynamic pathology of atherosclerosis. *Am. J. Med.* **46:** 657–673.
4. Brown, M. S., and J. L. Goldstein. 1976. Receptor-mediated control of cholesterol metabolism. *Science.* **191:** 150–154.
5. Windmueller, H. G., P. N. Herbert, and R. I. Levy. 1973. Biosynthesis of lymph and plasma lipoprotein apoproteins by isolated perfused rat liver and intestine. *J. Lipid Res.* **14:** 215–223.
6. Wu, A-L., and H. G. Windmueller. 1978. Identification of circulating apolipoproteins synthesized by rat small intestine in vivo. *J. Biol. Chem.* **253:** 2525–2528.
7. Schonfeld, G., E. Bell, and D. H. Alpers. 1978. Intestinal apoproteins during fat absorption. *J. Clin. Invest.* **61:** 1539–1550.
8. Glickman, R. M., J. Khorana, and A. Kilgore. 1976. Localization of apolipoprotein B in intestinal epithelial cells. *Science.* **193:** 1254–1255.
9. Glickman, R. M., and P. H. R. Green. 1977. The intestine as a source of apolipoprotein A-I. *Proc. Nat. Acad. Sci. USA.* **74:** 2569–2573.
10. Roheim, P. S., L. I. Gidez, and H. A. Eder. 1966. Extrahepatic synthesis of lipoproteins of plasma and chyle: role of the intestine. *J. Clin. Invest.* **45:** 297–300.
11. Marsh, J. B. 1974. Lipoproteins in a nonrecirculating perfusate of rat liver. *J. Lipid Res.* **15:** 544–550.
12. Sales, D. J. 1976. Lipoprotein abnormalities in streptozotocin-induced diabetic rats. Ph.D. Dissertation. The University of Chicago.
13. Faloona, G. R., B. N. Stewart, and M. Fried. 1969. An intermediate density lipoprotein of rat serum. *Arch. Biochem. Biophys.* **130:** 680–683.
14. Marsh, J. B., and A. F. Whereat. 1959. The synthesis of plasma lipoprotein by rat liver. *J. Biol. Chem.* **234:** 3196–3200.
15. Radding, C. M., J. H. Bragdon, and D. Steinberg. 1958. The synthesis of low- and high-density lipoproteins by rat liver in vitro. *Biochim. Biophys. Acta.* **30:** 443–444.
16. Jones, A. L., N. B. Ruderman, and M. G. Herrera. 1967. Electron microscopic and biochemical study of lipoprotein synthesis in the isolated perfused rat liver. *J. Lipid Res.* **8:** 429–446.
17. Hamilton, R. L., D. M. Regen, M. E. Gray, and V. S. LeQuire. 1967. Lipid transport in liver. I. Electron microscopic identification of very low density lipoproteins in perfused rat liver. *Lab. Invest.* **16:** 305–319.
18. Glaumann, H., A. Bergstrand, and J. L. E. Ericsson. 1975. Studies on the synthesis and intracellular transport of lipoprotein particles in rat liver. *J. Cell Biol.* **64:** 356–377.
19. Claude, A. 1970. Growth and differentiation of cytoplasmic membranes in the course of lipoprotein granule synthesis in the hepatic cell. I. Elaboration of elements of the Golgi complex. *J. Cell Biol.* **47:** 745–766.
20. Mahley, R. W., T. P. Bersot, V. S. LeQuire, R. I. Levy, H. G. Windmueller, and W. V. Brown. 1970. Identity of very low density lipoprotein apoproteins of plasma and liver Golgi apparatus. *Science.* **168:** 380–382.
21. Mahley, R. W., R. L. Hamilton, and V. S. LeQuire. 1969. Characterization of lipoprotein particles isolated from the Golgi apparatus of rat liver. *J. Lipid Res.* **10:** 433–439.
22. Marsh, J. B. 1963. The incorporation of amino acids

into soluble lipoproteins by cell-free preparations from rat liver. *J. Biol. Chem.* **238:** 1752–1756.
23. Bungenberg de Jong, J. J., and J. B. Marsh. 1968. Biosynthesis of plasma lipoproteins by rat liver ribosomes. *J. Biol. Chem.* **243:** 192–199.
24. JeeJeebhoy, K. N., J. Ho, C. Breckenridge, A. Bruce-Robertson, G. Steiner, and J. JeeJeebhoy. 1975. Synthesis of VLDL by isolated rat hepatocytes in suspension. *Biochem. Biophys. Res. Commun.* **66:** 1147–1153.
25. Margolis, S., and D. Capuzzi. 1972. Serum-lipoprotein synthesis and metabolism. *In* Blood Lipids and Lipoproteins: Quantitation, Composition, and Metabolism. G. J. Nelson, editor. Wiley-Interscience, New York, N.Y. 825–880.
26. Sigurdsson, G., A. Nicoll, and B. Lewis. 1975. Conversion of very low density lipoprotein to low density lipoprotein: a metabolic study of apolipoprotein B kinetics in human subjects. *J. Clin. Invest.* **56:** 1481–1490.
27. Chan, L., R. L. Jackson, B. W. O'Malley, and A. R. Means. 1976. Synthesis of very low density lipoproteins in the cockerel: effects of estrogen. *J. Clin. Invest.* **58:** 368–379.
28. Marsh, J. B. 1976. Apoproteins of the lipoproteins in a nonrecirculating perfusate of rat liver. *J. Lipid Res.* **17:** 85–90.
29. Alexander, C. A., R. L. Hamilton, and R. J. Havel. 1976. Subcellular localization of B apoprotein of plasma lipoproteins in rat liver. *J. Cell Biol.* **69:** 241–263.
30. Havel, R. J., and J. P. Kane. 1973. Primary dysbetalipoproteinemia: Predominance of a specific apoprotein species in triglyceride-rich lipoproteins. *Proc. Nat. Acad. Sci. USA.* **70:** 2015–2019.
31. Hoff, H. F., C. L. Heideman, R. L. Jackson, R. J. Bayardo, H. S. Kim, and A. M. Gotto, Jr. 1975. Localization patterns of plasma apolipoproteins in human atherosclerotic lesions. *Circ. Res.* **37:** 72–79.
32. Morrisett, J. D., R. L. Jackson, and A. M. Gotto, Jr. 1975. Lipoproteins: structure and function. *Ann. Rev. Biochem.* **44:** 183–207.
33. Shore, B., and V. Shore. 1974. An apolipoprotein preferentially enriched in cholesteryl ester-rich very low density lipoproteins. *Biochem. Biophys. Res. Commun.* **58:** 1–7.
34. Fainaru, M., R. J. Havel, and K. Imaizumi. 1977. Radioimmunoassay of arginine-rich apolipoprotein of rat serum. *Biochim. Biophys. Acta.* **490:** 144–155.
35. Lusk, L. T., L. F. Walker, L. H. DuBien, and G. S. Getz. 1977. Characterization of a major rat lipoprotein containing predominantly arginine rich peptide. *Federation Proc.* **36:** 1142.
36. Danielsson, B., R. Ekman, B. G. Johannsen, P. Nilsson-Ehle, and B. G. Peterson. 1978. Isolation of a high density lipoprotein with high contents of arginine rich apoprotein (apo E) from rat plasma. *FEBS Lett.* **86:** 299–302.
37. Weisgraber, K. H., R. W. Mahley, and G. Assmann. 1977. The rat arginine rich apoprotein and its redistribution following injection of iodinated lipoproteins into normal and hypercholesterolemic rats. *Atherosclerosis.* **28:** 121–140.
38. Mahley, R. W., K. H. Weisgraber, and T. Innerarity. 1974. Canine lipoproteins and atherosclerosis. II. Characterization of the plasma lipoproteins associated with atherogenic and nonatherogenic hyperlipidemia. *Circ. Res.* **35:** 722–733.
39. Mahley, R. W., K. H. Weisgraber, T. Innerarity, H. B. Brewer, Jr., and G. Assmann. 1975. Swine lipoproteins and atherosclerosis: changes in the plasma lipoproteins and apoproteins induced by cholesterol feeding. *Biochemistry.* **14:** 2817–2823.
40. Felker, T. E., M. Fainaru, R. L. Hamilton, and R. J. Havel. 1977. Secretion of the arginine-rich and A-I apolipoproteins by the isolated perfused rat liver. *J. Lipid Res.* **18:** 465–473.
41. Hamilton, R. L., M. C. Williams, C. J. Fielding, and R. J. Havel. 1976. Discoidal bilayer structure of nascent high density lipoproteins from perfused rat liver. *J. Clin. Invest.* **58:** 667–680.
42. Gidez, L. I., J. B. Swaney, and S. Murnane. 1977. Analysis of rat serum apolipoproteins by isoelectric focussing. 1. Studies on the middle molecular weight subunits. *J. Lipid Res.* **18:** 59–68.
43. Utermann, G., M. Hees, and A. Steinmetz. 1977. Polymorphism of apolipoprotein E and occurrence of dysbetalipoproteinaemia in man. *Nature.* **269:** 604–607.
44. Brawerman, G. 1974. Eukaryotic messenger RNA. *Ann. Rev. Biochem.* **43:** 621–642.
45. Mathews, M. B. 1973. Mammalian messenger RNA. *Essays Biochem.* **9:** 60–102.
46. Shapiro, D. J., J. M. Taylor, G. S. McKnight, R. Palacios, C. Gonzales, M. L. Kiely, and R. T. Schimke. 1974. Isolation of hen oviduct ovalbumin and rat liver albumin polysomes by indirect immunoprecipitation. *J. Biol. Chem.* **249:** 3665–3671.
47. Shapiro, D. J., and R. T. Schimke. 1975. Immunochemical isolation and characterization of ovalbumin messenger ribonucleic acid. *J. Biol. Chem.* **250:** 1759–1764.
48. Greenberg, J. R. 1975. Messenger RNA metabolism of animal cells: possible involvement of untranslated sequences and mRNA-associated proteins. *J. Cell Biol.* **64:** 269–288.
49. Taylor, J. M., and R. T. Schimke. 1973. Synthesis of rat liver albumin in a rabbit reticulocyte cell-free protein-synthesizing system. *J. Biol. Chem.* **248:** 7661–7668.
50. Gamulin, S., C. H. Gray, and M. R. Norman. 1972. Comparison of methods for preparing polysomes free of glycogen. *Biochim. Biophys. Acta.* **259:** 239–242.
51. Blobel, G., and D. Sabatini. 1971. Dissociation of mammalian polyribosomes into subunits by puromycin. *Proc. Nat. Acad. Sci. USA.* **68:** 390–394.
52. Pottenger, L. A., and G. S. Getz. 1971. Serum lipoprotein accumulation in the livers of orotic acid-fed rats. *J. Lipid Res.* **12:** 450–459.
53. Kostner, G. M., J. R. Patsch, S. Sailer, H. Braunsteiner, and A. Holaske. 1974. Polypeptide distribution of the main lipoprotein density classes separated from human plasma by rate zonal ultracentrifugation. *Eur. J. Biochem.* **45:** 611–621.
54. Scanu, A. M., H. Pollard, R. Hirz, and K. Korathy. 1969. On the conformational instability of human serum low density lipoprotein: effect of temperature. *Proc. Nat. Acad. Sci. USA.* **62:** 171–178.
55. Fless, G., and A. M. Scanu. 1975. Physicochemical characterization of rhesus low density lipoproteins. *Biochemistry.* **14:** 1783–1790.
56. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J.

Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
57. Palmiter, R. D., T. Oka, and R. T. Schimke. 1971. Modulation of ovalbumin synthesis by estradiol-17β and actinomycin D as studied in explants of chick oviduct in culture. *J. Biol. Chem.* **246:** 724–737.
58. Palacios, R., R. D. Palmiter, and R. T. Schimke. 1972. Identification and isolation of ovalbumin-synthesizing polysomes. I. Specific binding of ^{125}I-anti-ovalbumin to polysomes. *J. Biol. Chem.* **247:** 2316–2321.
59. Hunter, W. M., and F. C. Greenwood. 1962. Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature.* **194:** 495.
60. Hay, R. V., L. A. Pottenger, A. L. Reingold, G. S. Getz, and R. W. Wissler. 1971. Degradation of ^{125}I-labelled serum low density lipoprotein in normal and estrogen-treated male rats. *Biochem. Biophys. Res. Commun.* **44:** 1471–1477.
61. Wiegers, U., and M. Hilz. 1972. Rapid isolation of undegraded polysomal RNA without phenol. *FEBS Lett.* **23:** 77–82.
62. Hilz, H., U. Wiegers, and P. Adamietz. 1975. Stimulation of proteinase K action by denaturing agents: application to the isolation of nucleic acids and the degradation of 'masked' proteins. *Eur. J. Biochem.* **56:** 103–108.
63. Roberts, B. E., M. Gorecki, R. C. Mulligan, K. J. Danna, S. Rozenblatt, and A. Rich. 1975. Simian virus 40 DNA directs synthesis of authentic viral polypeptides in a linked transcription–translation cell-free system. *Proc. Nat. Acad. Sci. USA.* **72:** 1922–1926.
64. Roberts, B. E., and B. M. Paterson. 1973. Efficient translation of tobacco mosaic virus RNA and rabbit globin 9S RNA in a cell-free system from commercial wheat germ. *Proc. Nat. Acad. Sci. USA.* **70:** 2330–2334.
65. Rao, M. S., B. C. Wu, J. Waxman, and H. Busch. 1975. Rigid structural requirement of the 5′ terminus of mRNA for translational activity. *Biochem. Biophys. Res. Commun.* **66:** 1186–1193.
66. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate–polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244:** 4406–4412.
67. Kane, J. P. 1973. A rapid electrophoretic technique for identification of subunit species of apoproteins in serum lipoproteins. *Anal. Biochem.* **53:** 350–364.
68. Koga, S., L. Bolis, and A. M. Scanu. 1971. Isolation and characterization of subunit polypeptides from apoproteins of rat serum lipoprotein. *Biochim. Biophys. Acta.* **236:** 416–430.
69. Swaney, J. B., F. Braithwaite, and H. A. Eder. 1977. Characterization of the apolipoproteins of rat plasma lipoproteins. *Biochemistry.* **16:** 271–278.
70. Venkatesan, N., and W. J. Steele. 1972. Free and membrane-bound polysomes of rat liver: separation in nearly quantitative yield and analysis of structure and function. *Biochim. Biophys. Acta.* **287:** 526–537.
71. Crestfield, A. M., S. Moore, and W. H. Stein. 1963. The preparation and enzymatic hydrolysis of reduced and *S*-carboxymethylated proteins. *J. Biol. Chem.* **238:** 622–627.
72. Holme, G., S. L. Boyd, and A. H. Sehon. 1971. Precipitation of polyribosomes with pepsin digested antibodies. *Biochem. Biophys. Res. Commun.* **45:** 240–245.
73. Eschenfeldt, W. H., and R. J. Patterson. 1975. Do antibody binding techniques identify polysomes synthesizing a specific protein? *Biochem. Biophys. Res. Commun.* **67:** 934–945.