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Effects of an Atherogenic Diet on Apolipoprotein E Biosynthesis in the Rat†

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ABSTRACT: The effects of an atherogenic diet on apolipoprotein E biosynthesis in the rat liver were studied by immunocytochemical and biochemical techniques. Two groups of rats were fed either a normal laboratory chow or a special atherogenic diet containing 5% lard, 1% cholesterol, 0.35% taurocholic acid, and 0.1% propylthiouracil for 25 days. The atherogenic diet fed animals developed increased plasma cholesterol concentrations (134 ± 22 vs. 70 ± 15 mg/dL) and apolipoprotein E (apoE) concentrations (42.5 ± 10.2 vs. 15.6 ± 4.8 mg/dL). Plasma albumin levels were unchanged (7.3 ± 3.0 vs. 7.4 ± 2.0 g/dL). When liver sections from these animals were studied by indirect immunoperoxidase staining, by using a rabbit anti-rat apoE serum, the number of apoE-positive grains increased from 5.45 ± 0.49 to 6.79 ± 0.54 per hepatocyte, and the intensity of staining of individual grains also increased. Rat liver slices were incubated in vitro in culture medium containing [^{35}S]methionine for 90 min at 37 °C. Radioactivity incorporated into immunoprecipitable apoE were 2.89 ± 0.2% of Cl_3AcOH-precipitable radioactivity in special diet fed animals compared to 1.32 ± 0.1% in normal controls. Again, radioactivity incorporated into immunoprecipitable albumin was unchanged (11.9% in special diet group vs. 11.5% in controls). Total RNA was isolated from the liver of both groups of animals. Poly(A) RNA was purified by oligo(dT)-cellulose chromatography. Translation of the poly(A) RNA in a wheat germ system in vitro indicates that the atherogenic diet fed rat liver contained significantly higher concentrations of apoE mRNA activity (3.17% of total Cl_3AcOH-precipitable cpm) when compared to that of the controls (1.5% of total activity). In contrast, albumin mRNA activity in these RNA samples stayed constant at 9.1% (atherogenic diet fed) and 8.9% (controls), respectively. Our observations have provided unequivocal evidence that feeding the rats the special atherogenic diet for 25 days leads to an increase in apoE synthesis in the liver. This increase was mediated at least in part by an accumulation of translatable apoE mRNA activity in this organ.

Hypercholesterolemia is an important factor in the development of atherosclerosis (Goldstein et al., 1973). Plasma cholesterol levels in man can be altered by supplementation of the diet with polyunsaturated or unsaturated fats, as well as with cholesterol (Shore et al., 1974). The rat has been used by a number of laboratories as an experimental model for diet-induced hypercholesterolemia (Mahley & Holcombe, 1977; Swaney et al., 1977). In this animal, the various lipoproteins are well characterized and the metabolic interconversions of these lipoproteins have also been studied extensively (Swaney et al., 1977; Wong & Rubinstein, 1977).

The rat does not develop atherosclerosis naturally. It is also relatively resistant to hypercholesterolemia. However, high serum cholesterol and lipoprotein levels and even atherosclerosis can be induced in this animal by feeding it a diet containing 5% lard, 1% cholesterol, 0.35% taurocholic acid, and 0.1% propylthiouracil (Mahley & Holcombe, 1977; Mahley, 1978). Rats as well as other animals fed such a diet (referred to below as atherogenic diet) develop characteristic changes in their lipoprotein patterns. These include the following: the occurrence of β-VLDL[1] (i.e., cholesterol-enriched

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[1] Abbreviations used: VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; apo, apolipoproteins; oligo(dT), oligothymidylate; EDTA, ethylenediaminetetraacetic acid; Gdn, guanidine; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; Cl_3AcOH, trichloroacetic acid.

0006-2960/81/0420-6474$01.25/0

β-migrating lipoproteins isolated at $d < 1.006$ g/mL), an increase in IDL ($d = 1.006$–1.02 g/mL), an increase in LDL, the appearance of HDL_c (i.e., cholesterol-rich α_2-migrating lipoproteins containing apoE and A-I as major apoproteins), and a decrease in HDL_2 (Mahley & Holcombe, 1977; Mahley, 1978). Many of these changes are caused by a redistribution of lipids and apolipoproteins among the various density classes, and in the rat, at least, some of the changes may be brought out by the concomitant hypothyroidism. Studies of Redgrave et al. (1976) and Ross & Zilversmit (1977) suggest that in the rabbit, cholesterol feeding significantly alters lipoprotein catabolism, resulting in some of these changes. Studies of apoE turnover in vivo (Wong & Rubinstein, 1979) suggest that there is increased production of this apolipoprotein. Experiments on isolated perfused livers as well as on liver slices in vitro (Roheim et al., 1963; Camejo et al., 1973) suggest that probably the synthesis of various apolipoproteins may be increased by cholesterol feeding of the animals. However, these studies have generally been indirect and do not address the question of the biosynthetic rate of specific individual apolipoproteins.

In this communication, we have examined the effects of atherogenic diet feeding on apolipoprotein E biosynthesis in the rat. The results indicate that such dietary manipulations increase the rate of synthesis of this apolipoprotein in the liver and that this effect is largely the result of an accumulation of specific translatable apolipoprotein E mRNA in this organ.

Materials and Methods

[^{35}S]Methionine (1600 Ci/mmol) was obtained from Amersham. Oligo(dT)–cellulose (T3) was purchased from Collaborative Research. Dithiothreitol, taurocholic acid, cholesterol, and propylthiouracil were from Sigma Chemical Co. Wheat germ was a gift of General Mills. Medium 199 and potassium penicillin G were purchased from GIBCO, New York.

Animals. Male Sprague–Dawley rats, weighing 250–300 g (Holtzman Farm, Wisconsin), were divided into two groups and housed in a 12 h light and temperature-controled room. One group was fed a normal commercial laboratory chow, and another group was fed a commercial laboratory chow supplemented with 5% lard, 1% cholesterol, 0.3% taurocholic acid, and 0.1% propylthiouracil (Mahley & Holcombe, 1977). After 25 days, the animals were killed by cervical dislocation. Blood was collected in 0.1% EDTA for lipoprotein preparation, and the liver was removed for RNA isolation.

Isolation of Lipoprotein. Rat plasmas from normal and hypercholesterolemic rats were centrifuged at 10000 rpm, 20 min, to remove chylomicrons. Lipoprotein isolation was carried out by sequential density flotation using the 60 Ti rotor in the Beckman L2-65B ultracentrifuge. Both VLDL ($d < 1.006$ g/mL) and LDL ($d = 1.006$–1.063 g/mL) were separated by ultracentrifugation of plasma at 59000 rpm for 18 h, while HDL ($d = 1.063$–1.21 g/mL) were separated by ultracentrifugation at 59000 rpm for 24 h. Appropriate amounts of KBr were added to adjust the density. The VLDL, LDL, and HDL fractions were recentrifuged once at their respective densities and then dialyzed against 0.15 N NaCl, 0.01% EDTA, pH 7, and 0.01% NaN_3 overnight.

Preparation of Rat ApoE and Rat Albumin. ApoE was purified from VLDL ($d = 1.006$ g/mL) by the method of Weisgraber et al. (1977) with a minor modification. Delipidation of VLDL was performed sequentially with tetramethylurea (TMU), chloroform–methanol (2:1 v/v), and ethanol–ether (3:1 v/v). Finally, delipidated apoproteins were further fractionated on a Sephacryl S-200 column (2.5 × 170 cm) in a 0.2 M Tris-HCl, pH 8, containing 4 M Gdn·HCl and 1 mM NaN_3. On this column, apoE was well separated from apoC proteins. Rat albumin purchased from Sigma was found to be contaminated with minor amounts of other proteins on $NaDodSO_4$–polyacrylamide gel electrophoresis. It was purified by filtration on a Sephacryl S-200 column (1.0 × 170 cm) in 10 mM Na_2HPO_4–NaH_2PO_4, pH 8, and 1 mM NaN_3. Purified apoE and albumin were found to be homogeneous by $NaDodSO_4$–polyacrylamide gel electrophoresis and urea–polyacrylamide gel electrophoresis and by immunoelectrophoresis against their respective antisera (Lin-Lee et al., 1981).

Preparation of Antibody. Purified rat apoE or albumin (0.04 mg/mL) was emulsified in an equal volume of Freund's complete adjuvant. Two male New Zealand white rabbits were injected at multiple subcutaneous sites on the back with 2.6 mL of emulsion containing 104 μg of apoE or albumin. After the initial immunization, they received subcutaneous injections (35 μg of the antigen each time) every 2 weeks. After 2 months sera were obtained and tested for anti-apoE or anti-albumin activity by immunodiffusion and immunoelectrophoresis. An immunoglobulin fraction was prepared by ammonium sulfate precipitation of total serum as previously described (Chan et al., 1976).

Agarose Gel Rocket Immunoelectrophoresis. ApoE and albumin levels in rat plasma or lipoprotein fractions were determined by agarose gel rocket immunoelectrophoresis according to the method of Laurell (1966). The gel was composed of 1.5% agarose, 1% dextran T10, 0.1 M sodium barbital, pH 8.6, 0.035% EDTA, 0.05% NaN_3, and either 0.1% albumin or 15% apoE antiserum (v/v). Two-microliter samples were applied to 1-mm wells punched out of the gel. Electrophoresis was performed in 0.05 M sodium barbital, pH 8.6, 0.017% EDTA, and 0.025% NaN_3 at 25 V for 16 h at 10 °C. At the end of the run, the gels were washed with 1 or 3 M NaCl for 2 h. They were stained with 0.5% Coomassie blue in 45% ethanol and 10% acetic acid and destained with 60% ethanol and 10% acetic acid. The distance from the center of the wells to the top of the peak was taken as the peak height. Appropriate amounts of purified apoE and albumin were used to generate a standard curve.

Immunocytochemical Method. The immunocytochemical localization of apoE in the normal and atherogenic diet fed rat livers was performed according to the procedure of Lin & Chang (1975) and Lin & Chan (1980) with some modifications. Since glutaraldehyde fixation blocks the antigenic sites of apoE, 4% formaldehyde, 0.1 M sodium phosphate buffer, sucrose, and 0.02% $CaCl_2$ were used for tissue fixation. Rat livers were excised (0.4 × 0.6 × 0.2 cm) and immersed immediately in the fixative for 3 h at room temperature. After being washed in the phosphate buffer for 30 min, the tissue fragments were dehydrated in ethanol and embedded in paraffin. Five-micrometer sections were obtained from each block and incubated at 57 °C overnight to allow attachment of the tissue to the glass slides. The sections were deparaffinized by xylene and toluene. Sections were incubated with 1% H_2O_2 in absolute methanol for 10 min to inhibit endogenous peroxidase activity. The sections were then rehydrated by washing with decreasing concentrations of ethanol to 50%. They were further washed in phosphate buffer for 20 min and incubated with 0.05% Pronase in distilled water at room temperature for 10 min (Huang, 1975) and subsequently rinsed in the same buffers. Sections were incubated with rat apoE anti-serum at 1:40 dilution for 60 min at room temperature. They were washed in the buffer for 20 min with two changes and reincubated with goat IgG-peroxidase con-

jugate against rabbit IgG (Miles Biochemicals, Elkhart, IN) at a dilution of 1:40 for 60 min at room temperature. Finally, the sections were washed with buffer and then incubated with 0.05% 3,3′-diaminobenzidine tetrahydrochloride in 0.05 M Tris-HCl, pH 7.6, and 0.01% H_2O_2 for 7 min at room temperature. Sections thus obtained were fixed with 0.05% OsO_4 in 0.1 M sodium phosphate, pH 7.4, for 2 min. The final preparations were dehydrated, mounted with coverbond xylene, and observed under a light microscope. As control experiments, rabbit antibody against apoE was either replaced by preimmune serum or preincubated with 5-fold molar excess of purified rat apoE.

Rat ApoE and Albumin Synthesis in Liver Slice in Vitro. Liver freshly removed from normal or atherogenic diet fed animals was sliced to 0.1 mm thick pieces by a Stadie Riggs tissue slicer. The tissue slices (400 mg/flask) were incubated in 2 mL of medium 199 (Grand Island Biological Co., Grand Island, NY), supplemented with only one-eighth the usual amount of amino acids normally present in medium 199, 1.2 mg/mL of $NaHCO_3$, 1 unit/mL penicillin, and 1 mg/mL streptomycin. One hundred microcuries of [^{35}S]methionine was added per flask, and the tissues were incubated at 37 °C in the presence of 95% O_2–5% CO_2 under continuous shaking. Under these conditions, incorporation of [^{35}S]methionine into Cl_3AcOH-precipitable protein was linear for at least 180 min. In the quantitation experiments, incubation was carried out for 90 min, at which time excess (0.5 mmol) unlabeled methionine was added. The medium was saved, and the tissues were removed, chilled in ice, and homogenized in 0.1 mM Na_2HPO_4, pH 7.6, and 0.15 M NaCl by using seven strokes in a glass–Teflon homogenizer. The homogenate was then centrifuged at 105000*g* for 60 min. Total Cl_3AcOH-precipitable radioactivity and immunoprecipitable radioactivity in apoE and in albumin were quantified in the supernatant fraction of the homogenate, as well as in the incubation medium.

Isolation of Total Liver mRNA. Total rat liver cellular RNA was purified by the Gdn·HCl method of Deeley et al. (1977) with some modifications. Livers were removed and immediately homogenized in 10 volumes of 20 mM sodium acetate, pH 5.0, containing 7.5 M Gdn·HCl and 0.1 M dithiothreitol. The homogenate was centrifuged at 16000*g* for 20 min at 10 °C, and the supernatant fraction was filtered through cheesecloth. RNA was precipitated with prechilled ethanol (0.5 volume of supernatant) at −20 °C for 1 h. The precipitate was collected and suspended in 20 mM sodium acetate buffer containing 7.5 M Gdn·HCl, 1 mM DTT, and 20 mM Na_2EDTA. The precipitation was repeated 3 times. Finally, the RNA was dissolved in 20 mM EDTA, pH 7.0, and extracted with an equal volume of chloroform–1-butanol (4:1). The aqueous phase was made 0.2 M in KCl and RNA was precipitated with two volumes of ethanol at −20 °C. DNA and low molecular weight RNA were removed by washing the RNA pellet with 3 M sodium acetate, pH 5.0, at 4 °C, and the washed RNA was finally recovered by ethanol precipitation overnight. Polyadenylate-containing mRNA was isolated from the total RNA by affinity chromatography on oligo(dT)–cellulose as described by Aviv & Leder (1972).

Preparation of Wheat Germ Extract. Wheat germ extract was prepared by the method of Davies et al. (1977). This method was found to result in a more active preparation, in our experience, than the original method of Roberts & Paterson (1973).

ApoE and Albumin Synthesis in Wheat Germ Cell-Free Protein Synthesizing System. A total incubation mixture of 50 μL contained 20 μL of wheat germ extract and 30 μL of a mixture composed of 10 mM Hepes, pH 7.6, 3 mM dithiothreitol, 1.6 mM ATP, 0.033 mM GTP, 13 mM creatine phosphate, 2 μg of creatine phosphokinase, 100 mM KCl, 0.16 mM magnesium acetate, 0.16 mM spermidine, 0.63 mM 19 L-amino acids (minus methionine), 8 μCi of [^{35}S]methionine, and various amounts of mRNA; H_2O was added to adjust the volume to 30 μL. The reaction was carried out at 25 °C for 90 min during which time protein synthesis was linear. At the end of the incubation, 5-μL aliquots were precipitated in 1 mL of 10% Cl_3AcOH, heated to 95 °C for 10 min, cooled in ice, and filtered on glass-fiber filters. The remainder of the reaction mixture was used for quantitation of immunoprecipitable apoE or albumin synthesized in vitro.

Quantitation of Immunoprecipitable ApoE Synthesized in Vitro. Inactivated *Staphylococcus aureus* Cowan I strain (Pansorbin; Calbiochem) were extensively washed and prepared according to the method of Kessler (1975). The wheat germ reaction products were first centrifuged at 105000*g* for 60 min at 4 °C. The supernatant fraction was then incubated with 30 μL of prewashed Pansorbin at room temperature for 5 min. Pansorbin was removed by low-speed centrifugation. Newly synthesized [^{35}S]apoE or [^{35}S]albumin was precipitated in a reaction mixture containing 0.5 μL of rabbit anti-rat apoE or anti-rat albumin IgG and 300 μL of TNENN buffer [50 mM Tris, pH 7.5, 0.15 M NaCl, 5 mM EDTA, 0.02% NaN_3, and 0.5% NP-40 (containing 10 mM methionine)] per 100 μL of the reaction product. The mixture was incubated at room temperature for 2 h and then at 4 °C overnight. Fifteen microliters of Pansorbin was added, and the mixture was gently shaken at 4 °C for 40 min. The Pansorbin was collected by low-speed centrifugation. It was layered on top of a 1 M sucrose cushion in TNENN buffer and centrifugated at 2000*g* for 20 min.

After another wash with TNENN buffer, immunoprecipitated product was released from the bacteria by incubation in 2.5% $NaDodSO_4$, 10% glycerol, 5% mercaptoethanol, and 65 mM Tris-HCl, pH 7.5, at 95 °C for 5 min. The bacteria were removed by low-speed centrifugation, and released radiolabeled antigen was recovered in the supernatant fraction. It was either counted directly by scintillation spectrophotometry or further analyzed by $NaDodSO_4$ gel electrophoresis.

Analytical Methods. Total cholesterol and triacylglycerol of rat serum were measured by autoanalyzer techniques. Protein was determined by the method of Lowry et al. (1951).

Results

Effects of Atherogenic Diet on Rat Plasma Lipid, Albumin, and Apolipoprotein E Levels. When Sprague–Dawley rats were fed the atherogenic diet for 25 days, there was a doubling of plasma cholesterol concentration, but no change in triacylglycerol concentration (Table I). For determination of whether such changes in plasma lipid concentrations were accompanied by concomitant changes in albumin and apoE levels, the two proteins were assayed by rocket immunoelectrophoresis by using monospecific antisera to the two proteins, respectively. The assays gave a very sensitive and accurate measurement of the two proteins in plasma, and quadruplicate samples measured by this technique gave highly reproducible values. In diet-induced hypercholesterolemic animals, total plasma apoE concentration increased 2–3-fold while plasma albumin concentration remained unchanged (Table I).

Effects of Atherogenic Diet on Intracellular ApoE Localization. Since the liver is the major, if not the sole, site of production of apoE, we have examined the distribution of apoE in normal and diet-induced hypercholesterolemic animals in

Table I: Dietary Effects on Plasma Lipids and Proteins in the Rat

diet	plasma lipids (mg/dL) [a] triacylglycerol	cholesterol	plasma proteins [b] apoE (mg/dL)	albumin (g/dL)
normal	90 ± 15	71 ± 12	15.6 ± 4.8	7.4 ± 2.0
atherogenic	70 ± 15 [c]	134 ± 34 [d]	42.5 ± 10.2 [d]	7.3 ± 3.0 [c]

[a] Plasma lipids were determined by autoanalyzer methods. Values represent $\bar{x}$ ± SD. [b] Plasma apoE and albumin concentrations were determined by quantitative immunoelectrophoresis (Laurell, 1966). Values represent $\bar{x}$ ± SD. [c] $p > 0.05$ ($n = 5$). [d] $p < 0.01$ ($n = 5$).

liver sections by immunocytochemistry. The double-antibody immunoperoxidase technique was used, with rabbit anti-apoE IgG as the first antibody and peroxidase conjugated goat anti-rabbit IgG as the second antibody. As shown in Figure 1, both normal and atherogenic diet fed rat hepatocytes contained grains of immunoreactive apoE reaction product. The number of grains per cell was quantified by direct counting under the high-power microscope. There were 5.45 ± 0.49 ($\bar{x}$ ± SD) grains per cell in normal animals and 6.79 ± 0.54 ($\bar{x}$ ± SD) grains per cell in atherogenic diet fed animals. (A total of eight sections from four animals in each group were thus studied.) This minor increase in apoE-containing granules probably underestimates the diet-induced increase in intracellular apoE content since the reaction product was much more intensely stained in the hypercholesterolemic than in the control animals. Control experiments using preimmune serum, or anti-apoE serum preadsorbed with excess apoE, failed to show any peroxidase reaction product in normal or hypercholesterolemic liver sections, indicating the specificity of the immunocytochemical staining.

Effects of Atherogenic Diet on ApoE Synthesis in Liver Slices. Since changes in apoE content induced by the atherogenic diet as observed by immunocytochemistry are difficult to quantify, we have measured the rate of incorporation of [^{35}S]methionine into immunoprecipitable apoE during in vitro incubation of liver slices freshly removed either from rats fed a normal diet or from animals fed an atherogenic diet. Liver slices were incubated at 37 °C in medium 199 in the presence of 95% O_2–5% CO_2. Under these conditions, incorporation of [^{35}S]methionine into Cl_3AcOH-precipitable protein was linear for at least 180 min. As shown in Table II, when immunoprecipitable apoE was quantified in the liver homogenate, there was a 2.2-fold increase in the rate of apoE synthesis in the atherogenic diet treated animals. However, during the 90-min incubation, there was no significant change in the amount of newly synthesized apoE secreted into the medium. In contrast, the rate of albumin synthesis was unchanged whether measured in liver homogenates or in the medium. To ensure that the immunoprecipitation was specific and free of other proteins coprecipitated with apoE or albumin, we analyzed the immunoprecipitated products on $NaDodSO_4$ slab gels. Upon fluorography, both products appeared as single bands of radioactivity of expected molecular weights uncontaminated by detectable amounts of other proteins (data not shown).

Effects of Atherogenic Diet on ApoE and Albumin mRNA Activities. As an alternative approach to measuring apoE synthesis under different dietary conditions, total polyA RNA was extracted from rat liver and translated in vitro in a wheat germ system. As shown in Figure 2, there was a mRNA-dependent increase in the Cl_3AcOH-precipitable radioactivity in the translation system. Furthermore, albumin and apoE

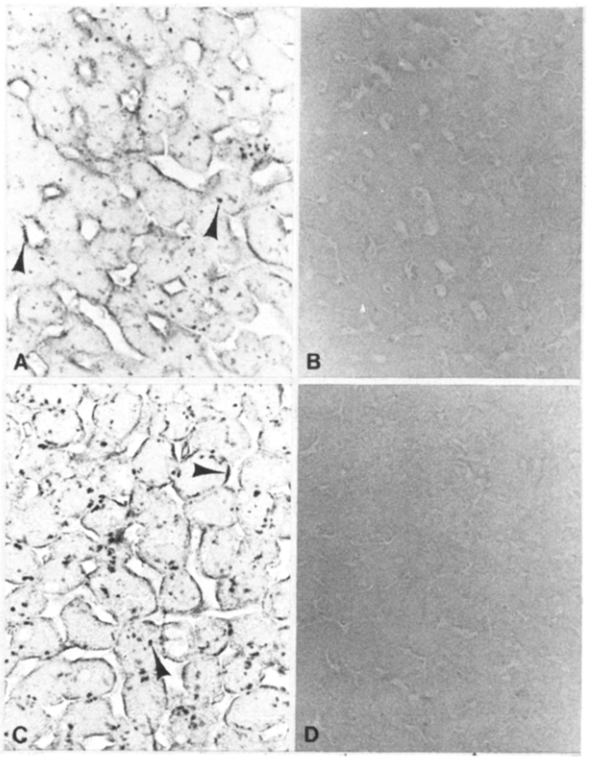

FIGURE 1: Localization of apoE in normal and cholesterol fed rat livers by immunocytochemical techniques. Sections were not counterstained. They were stained by the indirect immunoperoxidase technique as described under Materials and Methods. (A) Liver section from control rat. Grains of reaction product (arrowhead) were seen scattering in the cytoplasm of most hepatocytes. Note that the intensity of the reaction product is relatively weak. Some of the hepatocyte plasma membranes facing sinusoids are also stained (arrowhead) (×748). (B) Liver section from control rat stained with preimmune serum. No reaction product is found (×440). (C) Liver section from atherogenic diet fed rat. Most hepatocytes contain a number of reaction product positive grains (arrowhead) scattering in the cytoplasm. Reaction product is also evident on the plasma membrane facing sinusoids. The intensity of the reaction product is much stronger than that of the control section [compare with (A)]; the number of the grains per hepatocyte is also higher than that in the control section. Note that the cytoplasm of most hepatocytes contains numerous fat droplets. (D) Liver section from cholesterol fed rat stained with antiserum against apoE which had been preincubated with excess apoE. No reaction product is observed. The faint foamy appearance of cytoplasm is still recognizable (×440).

Table II: Dietary Effects on ApoE and Albumin Synthesis in Liver Slices [a]

diet	(immunoprecitable apoE cpm)/(Cl_3AcOH precipitable cpm) × 100	(immunoprecititable albumin cpm)/(Cl_3AcOH precipitable cpm) × 100
normal		
liver homogenate	1.32 ± 0.1 (49%) [b]	11.5 ± 0.1 (23%) [b]
culture medium	1.43 ± 0.4 (53%) [c]	11.8 ± 0.2 (77%) [c]
atherogenic		
liver homogenate	2.89 ± 0.2 (53%) [b]	11.9 ± 0.1 (28%) [b]
culture medium	1.27 ± 0.3 (47%) [c]	12.3 ± 0.3 (72%) [c]

[a] ApoE and albumin synthesis were determined in liver slices in vitro by the incorporation of [^{35}S]methionine into specific immunoprecipitable radioactivity as described under Materials and Methods. Four animals in each diet group were studied. Values represent $\bar{x}$ ± SD. [b] Represents percent of immunoprecipitable radioactivity in apoE or albumin in liver homogenates. [c] Represents percent of immunoprecipitable radioactivity in apoE or albumin in culture medium.

synthesized in vitro were also mRNA dependent. Appropriate competition experiments demonstrated that the in vitro

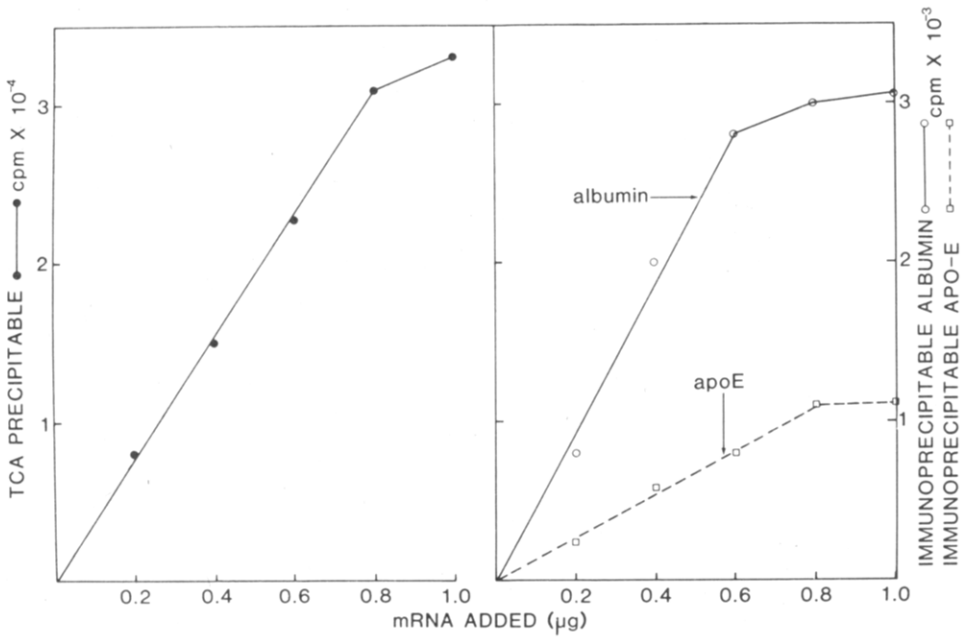

FIGURE 2: Translation of rat liver poly(A) RNA in wheat germ in vitro. Isolation of poly(A) RNA and preparation of the translation system are as described under Materials and Methods. [^{35}S]Methionine was the labeled amino acid used. (Left) Albumin and apoE mRNA activity. These were determined by quantitation of radioactivity incorporated into specific immunoprecipitable products by using specific anti-albumin and anti-apoE sera and *S. aureus* as the immunoadsorbent as described under Materials and Methods.

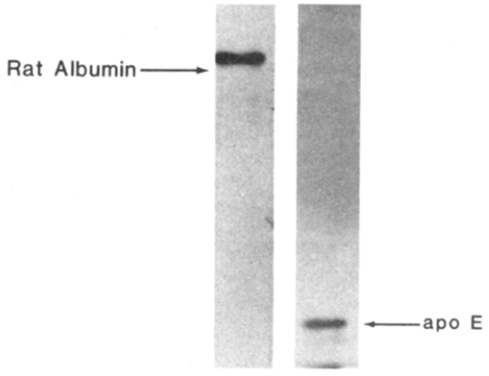

FIGURE 3: NaDodSO$_4$ gel analysis and fluorography of albumin and apoE synthesized in vitro. Specific ^{35}S-labeled immunoprecipitated products were analyzed on 10% acrylamide slab gels in NaDodSO$_4$. These were processed and fluorographed as described under Materials and Methods. (Left) Immunoprecipitable [^{35}S]albumin synthesized in vitro. Apparent M_r = 69 500. (Right) Immunoprecipitable [^{35}S]apoE synthesized in vitro. Apparent M_r = 37 000 (identical with the M_r of plasma apoE).

products were immunochemically identical with plasma rat albumin and apoE (Lin-Lee et al., 1981; Y.-C. Lin-Lee and L. Chan, unpublished data). When the translation products were analyzed on NaDodSO$_4$–acrylamide gels, both proteins synthesized in vitro migrated with the appropriate apparent molecular weights (Figure 3). Immunoprecipitable radioactivity in albumin had an apparent M_r of 69 500 compared to that of plasma albumin of 68 000, indicating that preproalbumin was synthesized in vitro (Strauss et al., 1978). ApoE synthesized in vitro migrated with an apparent M_r of 37 000, almost identical with that of rat plasma apoE. Our previous study had shown that the apoE synthesized in vitro did contain a signal peptide, but plasma apoE was glycosylated such that the apparent M_r of the latter was spuriously high on NaDodSO$_4$ gels (Lin-Lee et al., 1981). There was also no difference in the apparent M_r of apoE synthesized in vitro when using mRNA from control or hypercholesterolemic animals (data not shown).

Total mRNA was purified from the liver of a group of animals fed a normal diet and another group fed an atherogenic diet. Specific mRNA activities for apoE and albumin were quantified from the slopes of the linear part of mRNA saturation curves from these samples. As shown in Table III, atherogenic diet feeding resulted in a doubling of apoE mRNA content of total rat hepatic mRNA whereas albumin mRNA content was unchanged. The same results were obtained when absolute amounts of mRNA activities were measured or when mRNA activities were expressed as a ratio of total mRNA activities (Cl_3AcOH-precipitable cpm).

Table III: Dietary Effects on Rat Hepatic mRNA Activity

	immunoprecipitable cpm X 10^{-3}/μg of RNA [a]		(immunoprecipitable cpm)/(Cl_3AcOH precipitable cpm) X 100	
diet	apoE	albumin	apoE	albumin
normal	0.58 ± 0.06	3.28 ± 0.2	1.5	8.9
atherogenic	1.18 ± 0.1 [b]	3.72 ± 0.1 [c]	3.17	9.1

[a] Total rat liver poly(A) RNA was translated in a wheat germ system in vitro as described under Materials and Methods. Five different concentrations of RNA were used. Specific activity was determined from the linear portion of the activity curve (see Figure 2) for each individual RNA. Values represent $\bar{x}$ ± SD (n = 5). [b] $p < 0.01$. [c] $p > 0.05$.

Discussion

The rat is relatively resistant to the development of hypercholesterolemia. However, in this animal, markedly elevated plasma cholesterol levels and even atherosclerosis can be induced by a diet containing 5% lard, 1% cholesterol, 0.35% taurocholic acid, and 0.1% propylthiouracil. Animals fed such a diet have been used as a model for diet-induced hypercholesterolemia and hyperlipoproteinemia (Weisgraber et al., 1977).

Since the various plasma lipoproteins undergo complex interconversions, diet-induced hyperlipoproteinemias can be ascribed to changes in the various metabolic conversions. One

simple and important question has never been adequately answered: Is the rate of synthesis of the individual apolipoproteins changed by the dietary manipulation, and if so, is the site of alteration at the translational or pretranslational level? We have therefore concentrated our efforts on the effect of the atherogenic diet on the biosynthesis of a specific apolipoprotein. We have studied apoE in this communication since in the rat this protein is well characterized (Swaney et al., 1974, 1977). It is synthesized mainly in the liver and its plasma level is also known to be stimulated by the special diet (Mahley & Holcombe, 1977; Wong & Rubinstein, 1977). Furthermore, apoE might play an important role in the uptake of chylomicron remnants in the liver (Sherrill et al., 1980). Hence, the dietary regulation of this apoprotein is of considerable interest in the understanding of cholesterol homeostasis.

We found that plasma apoE concentration was consistently increased 2–3-fold when feeding the rat the special diet. Since the liver is the major site of synthesis of apoE, we have examined the apoE content of rat hepatocytes by immunocytochemistry. ApoE was localized in cytoplasmic granules of rat hepatocytes. The average number of such granules increased from a mean of 5.45 to that of 6.79 following atherogenic diet feeding. Though statistically significant, this represents a minor change. However, the numbers probably do not accurately reflect the true intracellular content of apoE since, as is apparent in Figure 1, the intensity of staining of apoE containing granules is much higher following atherogenic diet feeding. Unfortunately, this observation is highly subjective and cannot be quantified easily. Furthermore, the intracellular concentration of apoE reflects at least three separate events, namely, the rate of synthesis of apoE, the rate of secretion of the protein, and its rate of intracellular degradation. Since we are primarily interested in the rate of synthesis of this apolipoprotein, we proceeded to directly measure this parameter in liver slices from animals fed a normal or an atherogenic diet.

ApoE synthesis in rat liver slices was measured by the amount of [^{35}S]methionine incorporated into immunoprecipitable apoE during a pulse-labeling period in vitro. Authenticity of the product was verified by $NaDodSO_4$ slab gel electrophoresis which showed that under our conditions, only apoE was precipitated. Under these conditions, atherogenic diet feeding increased the rate of apoE synthesis in liver slices 2.2-fold. In contrast, the rate of albumin synthesis measured by the same technique was unchanged. Furthermore, when apoE or albumin synthesis was expressed as a ratio of total protein synthesis (Cl_3AcOH-precipitable radioactivity), again, only the relative rate of apoE synthesis was increased in the atherogenic diet fed animals. It is therefore unlikely that major changes in methionine pool size would account for our observations. It is interesting that the amount of immunoprecipitable apoE secreted into the medium during the 90-min labeling period was not different in diet-fed and control animals. This suggests that most of the newly synthesized protein was not actively secreted into the medium during this time and significant amounts of the protein were stored before release.

As an independent method for quantifying apoE biosynthetic activity, we have measured total mRNA activity in the rat liver. Such measurements would circumvent totally the effects of amino acid pool size changes. They would also provide additional information on the level of regulation of apoE synthesis. Total poly(A) RNA was isolated from the liver and translated in a wheat germ system. Radioactivity incorporated into Cl_3AcOH-precipitable material was not different in RNA samples isolated from the two groups of animals. However, when apoE mRNA activity was measured, it was found to be increased 2-fold in the atherogenic diet fed animals. This was true whether mRNA activity was expressed as absolute activity per microgram of RNA or as a ratio of the total (Cl_3AcOH-precipitable) activity. In contrast, albumin mRNA activity was unchanged by the dietary manipulation. Again, authenticity of our assay was verified by $NaDodSO_4$ slab gel electrophoresis which showed very distinct uncontaminated products.

Our experiments have demonstrated unequivocally that hepatic apoE synthesis is stimulated by feeding the rat a special atherogenic diet. Furthermore, the level of regulation appears to be largely pretranslational. This is important in view of the observation from RNA and protein synthesis inhibitor studies that oleic acid added to cultured rat hepatocytes might stimulate apoE synthesis at a translational level (Davis, 1980). It should be pointed out, however, that the effect of oleic acid was acute, lasting only 4 h, whereas in the present study, the animals received the atherogenic diet for 25 days.

Our observations are generally consistent with the results of previous studies which used very indirect techniques and which concluded that the atherogenic diet increased the hapatic secretion of apoE (Swift et al., 1980; Wong & Rubinstein, 1977, 1979). It should be noted that in our present study, we have fed our animals an atherogenic diet commonly used to induce hypercholesterolemia and atherosclerosis in the rat (Mahley & Holcombe, 1977). Since this diet contains multiple components, it is possible that each of these components might be effective in stimulating apoE synthesis in the rat liver. For example, hypothyroidism in rats has been reported to result in hyperlipidemia (Murase & Uchimura, 1980) and specifically in elevated plasma apoE (Dory & Roheim, 1981). However, a major effect of hypothyroidism in this animal appears to be the selective suppression of hepatic triacylglycerol lipase activity (Murase & Uchimura, 1980) as well as the decreased removal of lipoproteins from the circulation (Murase & Uchimura, 1980; Dory & Roheim, 1981) rather than any major change in apoE output. Nevertheless, it is possible that hypothyroidism acts synergistically with some components of the diet in the stimulation of apoE mRNA activity. The importance of the various components in the diet in the regulation of apoE synthesis will be the subject of further investigations.

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Physical Characteristics of the Reconstitution Intermediates (RI_{30} and RI_{30}*) from the 30S Ribosomal Subunit of *Escherichia coli*†

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ABSTRACT: The isolated reconstitution intermediates (RI_{30} and RI_{30}*) from the 30S ribosomal subunit of *Escherichia coli* were found to contain ten proteins. The sedimentation ceofficients, diffusion coefficients, density increments, extinction coefficients, and molecular weights were determined for the reconstitution particles and compared with those obtained from the 16S rRNA under identical buffer conditions. The results show that the binding of the proteins on the 16S rRNA at 4 °C does not markedly affect the folding of the RNA molecule. However, upon heating the RI_{30} particle at 40 °C to form the RI_{30}* particle, significant folding of the RNA took place, giving a structure considerably more compact than that of the 16S rRNA or the RI_{30} particle.

An active 30S ribosomal subunit from *Escherichia coli* can be reconstituted in vitro from its components, the 16S rRNA and the 21 proteins (Traub & Nomura, 1969). The assembly process has been studied extensively (Held & Nomura, 1973; Nomura, 1973), and the reaction follows the sequential steps

$$\text{16S rRNA + RI proteins} \xrightarrow{\text{0–10 °C}} RI_{30}\text{ particles} \quad (1)$$

$$RI_{30}\text{ particles} \xrightarrow{\text{heating}} RI_{30}^*\text{ particles} \quad (2)$$

$$RI_{30}^*\text{ particles + split proteins} \rightarrow \text{30S particles} \quad (3)$$

The "RI proteins" are defined as protins contained in the intermediate particle (RI_{30}), which has a sedimentation coefficient $s^0_{20,w}$ of 21.3 ± 0.7 in low-salt buffer (Traub & Nomura, 1969). The RI_{30}* particle is formed by heating the RI_{30} to 37–40 °C for 30 min. The sedimentation coefficient for the RI_{30}* particle has been reported to be 25–26 S in low-salt buffer on a sucrose gradient (Held & Nomura, 1973). These reports together with the observation by Hochkeppel & Craven (1977) strongly suggested that the heating step caused a folding in the conformation of the RI_{30} particle in order to form the RI_{30}* particle.

Recently, Dunn & Wong (1979) reported sedimentation coefficients for the RI_{30} and RI_{30}* particles to be 29.4 S and 26.5 S, respectively, and suggested that the RI_{30}* particles is more asymmetric and/or less compact than the RI_{30} particle. Their values were obtained in high-salt buffer and, in the case of the RI_{30} particle, in the presence of split proteins.

In order to better understand the molecular mechanism of the in vitro assembly process, we need to completely isolate and physically characterize the intermediates. We report here the sedimentation coefficients, diffusion coefficients, density increments, extinction coefficients, and the molecular weights of the isolated RI_{30}, RI_{30}*, and 16S rRNA in low-salt buffer. From these results it appears that the binding of the "RI proteins" on the 16S rRNA at low temperature is not responsible for the folding of the RNA molecule since the overall dimensions of the RI_{30} particle were found to be slightly larger than those of the RNA molecule itself. However, the heating step that converts the RI_{30} particle into RI_{30}* particle does cause massive folding of the RI_{30} particle. The RI_{30}* particle

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0006-2960/81/0420-6480$01.25/0