

# Plasma lipoproteins, tissue cholesterol overload, and skeletal muscle apolipoprotein A-I synthesis in the developing chick

Patrizia Tarugi, Daniela Reggiani, Enzo Ottaviani,\* Stefano Ferrari,† Roberta Tiozzo, and Sebastiano Calandra

Istituto di Patologia Generale, Dipartimento di Biologia Animale,\* and Istituto di Chimica Biologica,† Università di Modena, Modena, Italy

**Abstract** In the present study we investigated the changes of plasma lipids, lipoproteins, and tissue lipids that occur during the late embryonic life (5 days before hatching) and the postnatal period (0, 2, 7, 14, and 30 days after hatching) of the chick. The chick emerges from the egg with extreme hypercholesterolemia associated with a high level of cholesterol-rich VLDL + IDL. The density gradient profile of plasma lipoproteins showed that the concentrations of VLDL + IDL and LDL decreased during the first week of postnatal life, whereas HDL concentration increased sharply around hatching and remained stable afterwards. All plasma lipoprotein classes of the newborn chick (2 days from hatching) were enriched in cholesterol and cholesteryl esters; 2 weeks after hatching, the relative amount of cholesterol and cholesteryl esters decreased. In the newborn chick, plasma VLDL + IDL consisted of two populations of cholesteryl ester-rich lipoproteins: the main one (designated apoB-VLDL) contained apoB and no apoA-I; the other (designated apoA-I-VLDL) contained predominantly apoA-I. In the newborn chick there was an accumulation of free and esterified cholesterol in the liver and, to a lesser extent, in the skeletal muscle. These cholesterol deposits were depleted 2 to 7 days after hatching. The depletion in skeletal muscle was preceded by and associated with a striking increase in the synthesis of apoA-I in this tissue, as demonstrated by immunological methods and apoA-I mRNA measurements. In addition, apoA-I-containing HDL were secreted *in vitro* by explants of skeletal muscle of the newborn chick. The synthesis of apoA-I in the skeletal muscle decreased to the level found in the adult animal 1 week after hatching. It is likely that the rise of HDL and apoA-I in plasma observed 1-2 days after hatching reflects the production of apoA-I-containing HDL by skeletal muscle. We suggest that the cholesterol overload in skeletal muscle might stimulate the production of apoA-I which, in turn, would promote the removal of cholesterol from this tissue. The hypothesis that metabolic stimuli play a role in inducing apoA-I synthesis in skeletal muscle is supported by the observation that feeding the newborn chick a diet rich in proteins and lipids and free of carbohydrates delays the fall of apoA-I mRNA which normally occurs 1 week after hatching. — Tarugi, P., D. Reggiani, E. Ottaviani, S. Ferrari, R. Tiozzo, and S. Calandra. Plasma lipoproteins, tissue cholesterol overload, and skeletal muscle apolipoprotein A-I synthesis in the developing chick. *J. Lipid Res.* 1989. 30: 9-22.

**Supplementary key words** hypercholesterolemia •  $\beta$ -VLDL • anti-apoA-I immunoaffinity chromatography • plasma apoA-I • cholesterol stores in liver and skeletal muscle

The chick emerges from the egg with large deposits of cholesterol in three tissues: yolk sac, liver, and plasma (1, 2). Cholesterol deposits in liver and plasma appear to be related to a massive transfer of lipids from the yolk sac to the emerging chick (1, 2). It is also well established that within a few weeks after hatching there is a progressive depletion of hepatic and plasma cholesterol deposits (1, 2). These rapid changes of cholesterol content in yolk sac, liver, plasma, and possibly other tissues (such as the heart) (2) render the newborn chick an interesting model for the study of cholesterol transport among the different compartments of the body.

Although there are no systematic studies on the plasma lipoproteins of the developing chick, it was reported in the past that: *a*) the concentration of VLDL and LDL is elevated in late embryonic life and decreases after hatching whereas HDL shows an opposite trend (3); and *b*) the plasma concentration of apoB and apoA-I parallels the time-related changes of VLDL + LDL and HDL concentration, respectively (4). This would suggest that apoB-containing lipoproteins (VLDL and LDL) may be involved in the transfer of cholesterol from the plasma compartment to the tissues and its accumulation in tissues, whereas apoA-I-containing lipoproteins might play a cru-

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Abbreviations: VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; apoA-I, apolipoprotein A-I; apoB, apolipoprotein B; apo5C12, full length cDNA for chick apolipoprotein A-I; SDS, sodium dodecyl sulfate; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase.

cial role in the postnatal mobilization of cholesterol from tissue deposits.

In addition to these changes in lipid metabolism, recent studies indicate that in late embryonic and early postnatal period there are striking time-dependent variations of apoA-I gene expression in liver (5), intestine, and skeletal muscle (5-7). During embryonic life, apoA-I mRNA is present in high concentration only in the liver, while its level in the intestine is quantitatively relevant only after hatching. The switch from liver to intestine as primary site of apoA-I mRNA synthesis takes place about 10 days after hatching (5). In the skeletal muscle, both the apoA-I mRNA level and apoA-I synthesis are very high at hatching but decrease to the level found in the adult animal after the first week of postnatal life (5-10).

The present study was designed to investigate: *a*) the plasma lipoprotein changes underlying the transient hypercholesterolemia of the newborn chick; *b*) the time-dependent accumulation and depletion of lipids in liver and skeletal muscle; and *c*) the possible relationship between cholesterol accumulation in skeletal muscle and the local synthesis of apoA-I.

## MATERIALS AND METHODS

### Materials

[<sup>35</sup>S]Methionine (30 TBq/mmol) was obtained from Amersham (England); CNBr-activated Sepharose 4B and Blue Sepharose CL-6B were purchased from Pharmacia (Sweden). Peroxidase-antiperoxidase (PAP) complex and anti-rabbit IgG were purchased from Dakopatts (Denmark). Silica gel G plates for thin-layer chromatography were obtained from Merck (West Germany). GeneScreen and Hybond-N membranes were obtained from New England Nuclear (USA) and Amersham (UK), respectively.

### Animals

Eggs (Arbor Acres strain) were obtained from a local supplier and dates of fertilization were carefully noted. Chicks born from the same batch of eggs were fed a standard diet (25% soya proteins, 4.5% lipids, 5.5% fibers) or a carbohydrate-free, protein-rich diet (85% soya proteins, 5% maize germ oil, 1.7% D,L-methionine, 0.8% glycine, 0.2% choline, 6.3% salt mixture, 1% vitamins) (11) *ad libitum* until the time of killing. Chicks were killed 5 and 3 days before hatching (day -5 and -3), at the time of hatching (day 0), and 1, 2, 6, 7, 12, 14, and 30 days after hatching. Five animals were used at each time point. Blood was collected by cardiac puncture using K<sub>3</sub> EDTA as anticoagulant. Liver and leg muscle were excised, washed in cold 0.154 M NaCl, and immediately frozen in liquid nitrogen.

### Plasma lipids

Aliquots (50  $\mu$ l) of plasma were extracted in chloroform-methanol 2:1 (v/v) (12). The major lipid classes were separated by thin-layer chromatography and measured colorimetrically (13).

### Separation of plasma lipoproteins

Before the separation of plasma lipoproteins, equal aliquots of plasma taken from each animal at the various time points were pooled. Lipoproteins were isolated from the plasma pools by density gradient ultracentrifugation in an SW 41 rotor, according to Hermier, Forgez, and Chapman (14). After centrifugation, aliquots of 500  $\mu$ l (fraction 1) or 400  $\mu$ l (fractions 2-20) were collected (14) and their protein concentration was measured (15). In some experiments a pool of plasma obtained from 2-day-old chicks was brought to a density of 1.035 g/ml with solid KBr and centrifuged in a 60 Ti rotor at 150,000 *g* for 24 hr. The floating lipoprotein layer was collected and used for immunoaffinity chromatography.

### Lipid and apolipoprotein composition of plasma lipoproteins

Plasma lipoprotein fractions (50-100  $\mu$ g of protein) selected from the density gradients (Fig. 1) were extracted in chloroform-methanol 2:1 (v/v) (12). Lipid classes were separated by thin-layer chromatography and measured colorimetrically (13). Other aliquots (20-50  $\mu$ g of protein) of selected lipoprotein density fractions were precipitated in 10% trichloroacetic acid (TCA); the TCA-precipitate was extracted twice in ethanol-diethyl ether 3:2 (v/v), dissolved in 2% SDS, 3.5% 2-mercaptoethanol, 0.050 M Tris-HCl, pH 7, and heated at 95°C for 3 min. This material was applied to an SDS-polyacrylamide gel slab (140 mm  $\times$  200 mm  $\times$  1.5 mm). A linear polyacrylamide gradient (5-20%) was used as a running gel (16). Gels were stained with Coomassie Blue R-250.

### Immunochemical methods

ApoA-I was isolated from delipidated chick plasma HDL (d 1.080-1.210 g/ml) by Sephadex G-150 gel filtration (17); albumin was isolated from chick plasma by Blue Sepharose CL-6B affinity chromatography as described (17). Antisera against apoA-I and albumin were raised in New Zealand White rabbits (17). Anti-apoA-I and anti-albumin IgG were isolated according to a standard procedure (18). The plasma concentrations of albumin and apoA-I were measured by electroimmunoassay (17).

### Immunoaffinity chromatography

Anti-chick apoA-I IgG was covalently linked to CNBr-activated Sepharose 4B using a concentration of 14 mg of IgG protein per g of dry gel. A glass column (11  $\times$  0.7 cm)

was packed with anti-apoA-I CNBr-activated Sepharose 4B and washed exhaustively with the loading buffer (0.15 M NaCl, 0.015 M Tris, pH 7.4) (19). The column was loaded with 2.5 mg of 2-day-old chick d < 1.035 g/ml plasma lipoproteins that had been dialyzed against the loading buffer. The material bound to the column was eluted with 1 M acetic acid, pH 3. The eluted fractions were immediately neutralized with 2 M Tris solution. Both unbound and bound fractions were dialyzed in 0.01 M  $\text{NH}_4\text{HCO}_3$ , 1 mM  $\text{Na}_2\text{EDTA}$ , and lyophilized. This material was dissolved in 2% SDS, 3.5% 2-mercaptoethanol, 0.05 M Tris-HCl, pH 7, heated at 95°C for 3 min, and applied to a 5–20% polyacrylamide gradient gel slab for electrophoresis (16). Aliquots of both bound and unbound fractions were extracted with chloroform-methanol 2:1 (v/v) (12) for lipid analysis (13).

### Tissue lipids

Liver was finely minced, suspended in 10 ml of 0.25 M sucrose per g of tissue and homogenized with three strokes of a Potter homogenizer. Skeletal muscle was minced, suspended in 10 ml of 0.25 M sucrose per g of tissue and mechanically disrupted in a Polytron homogenizer. Tissue lipids were extracted, separated, and measured as previously described (12, 13).

### Histochemistry and immunohistochemistry

Fragments of liver and leg muscle were obtained from animals at -5, 0, and 7 days of development. Sudan Black B reaction for lipids was performed on unfixed and frozen tissues cut by cryostat.

For immunohistochemistry, fragments of liver and leg muscle were fixed in 10% formalin in phosphate buffer at pH 7.4 and embedded in paraffin. Tissue sections (5  $\mu\text{m}$  thick) were washed in methanol containing 1%  $\text{H}_2\text{O}_2$  for 30 min to inhibit endogenous peroxidase, rinsed in phosphate-buffered saline (PBS), and pre-incubated in swine normal serum (1 to 3 dilution) for 30 min at room temperature. Tissue slides were then incubated in either rabbit anti-chick apoA-I IgG (at 1 to 500 dilution) or rabbit anti-chick albumin IgG (at 1 to 500 dilution) for 24 hr at 4°C.

After three washes with PBS, tissue slides were incubated in swine anti-rabbit IgG (at 1 to 30 dilution) for 30 min at room temperature. After three washes in PBS, slides were incubated in peroxidase anti-peroxidase (PAP) complex (at 1 to 80 dilution) for 30 min at room temperature. After rinsing with PBS, the slides were incubated with PBS containing 0.05% diaminobenzidine and 0.02%  $\text{H}_2\text{O}_2$ , and then washed in water. Nuclei were counterstained with hematoxylin. Finally, tissue slides were dehydrated in a graded alcohol series, cleared with xylene, covered with a cover slip, and observed under light microscopy (20).

For control experiments, tissue sections were incubated with IgG isolated from nonimmune rabbit serum. All tissue samples isolated from animals at different stages of development were processed simultaneously using the same batch of reagents.

### Synthesis of apoA-I in skeletal muscle

Small fragments (5–10 mg each) of leg skeletal muscle of a 2-day-old chick were placed on a small sheet of silicone-treated lens paper and pre-incubated in 2 ml of Dulbecco's modified Eagle's medium (DMEM) in the presence or in the absence of 10% fetal calf serum (FCS) in 35-mm plastic Petri dishes for 24 hr. The type of pre-incubation medium (DMEM or DMEM supplemented with 10% FCS) did not influence the attachment of muscle slices to the lens paper and the synthesis and secretion of muscle lipoproteins. After this pre-incubation, the explants were washed with methionine-free DMEM, and then incubated overnight in the latter medium in the presence of [ $^{35}\text{S}$ ]methionine (50  $\mu\text{Ci}/\text{ml}$ ). At the end of the incubation, an aliquot of the medium was subjected to density gradient ultracentrifugation (14) while another aliquot was dialyzed in  $\text{NH}_4\text{HCO}_3$  and lyophilized. This material was dissolved in 100  $\mu\text{l}$  of 0.1 M NaCl, 0.01 M Tris-HCl, pH 7.5, 1 mM  $\text{Na}_2\text{EDTA}$ , and 0.5% Nonidet P 40.  $^{35}\text{S}$ -Labeled apoA-I and  $^{35}\text{S}$ -labeled albumin were immunoprecipitated with specific antisera as previously reported (21). A similar procedure was used to immunoprecipitate  $^{35}\text{S}$ -labeled apoA-I contained in some lipoprotein density fractions. Immunoprecipitated  $^{35}\text{S}$ -labeled apoA-I and albumin were separated by 5–20% polyacrylamide gradient gel slab electrophoresis. Gels were stained with Coomassie Blue R-250, destained, processed for fluorography, and exposed to Hyperfilm-MP films (Amersham) at -80°C.

### RNA extraction from skeletal muscle and Northern blot hybridization

RNA was extracted from pools of breast muscles (taken from five animals at each time point) by the guanidinium-HCl method, as previously described (10). Equal amounts of total RNA (10  $\mu\text{g}$ ) were denatured in 30  $\mu\text{l}$  of 50% formamide, 2.2 M formaldehyde, 1  $\times$  running buffer: 10 mM 3-(N-morpholino)-propane-sulfonic acid, pH 7; 2.5 mM Na acetate; 0.5 mM  $\text{Na}_2\text{EDTA}$ , and separated on a 1% agarose gel containing 2.2 M formaldehyde. Gels were stained with ethidium bromide, analyzed under ultraviolet light, and photographed to ensure that equal amounts of RNA had been applied to the gels. Electrophoretic transfer to GeneScreen or Hybond-N membranes was according to the manufacturer's instructions. Membranes were reexposed to the ultraviolet light to check the efficiency and homogeneity of the transfer. The membranes were prehybridized at 42°C in 50% formamide, 3  $\times$  SSC

(1 × SSC = 0.154 M NaCl, 0.015 M Na citrate, pH 7, 0.1% SDS) 50 mM Tris-HCl, pH 7.5, 5 mM Na<sub>2</sub> EDTA, 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinyl-pyrrolidone, 0.5% SDS, and 0.2 mg/ml denatured herring sperm DNA. Hybridization was in the same cocktail containing 2 × 10<sup>8</sup> cpm of apo5C12 DNA (10) labeled in vitro by nick-translation (22). Washes were sequential in 2 × SSC-0.5% SDS at room temperature (2 × 5 min) and in 0.5 × SSC-0.5% SDS at 60°C (4 × 30 min). Filters were finally exposed to Hyperfilm MP (Amersham) for autoradiography.

### Statistical analysis

Student's *t* test was used for statistical analysis.

## RESULTS

### Plasma lipids

**Table 1** shows that in late embryonic and early postnatal life (up to day 2) the concentration of total cholesterol in plasma was from 1.9- to 2.6-fold higher than that found at the later stages (14–30 days). The fall of plasma cholesterol to the level found in the adult animal was observed between day 2 and day 7. The ester:free cholesterol ratio did not change significantly during the whole time period investigated. The level of plasma triacylglycerols, which was elevated in late embryo, progressively decreased in the postnatal period.

### Characterization of plasma lipoproteins

The effect of development on the density profile of plasma lipoproteins is shown in **Fig. 1**. At each time point the density profile of plasma lipoproteins showed three peaks having the following density limits: *d* < 1.030 g/ml (VLDL + IDL); *d* 1.035–1.065 g/ml (LDL); *d* 1.070–1.150 g/ml (HDL). It should be pointed out that during postnatal development the density interval of the LDL peak shifted from 1.035–1.045 g/ml (day 0) to 1.045–1.065 g/ml

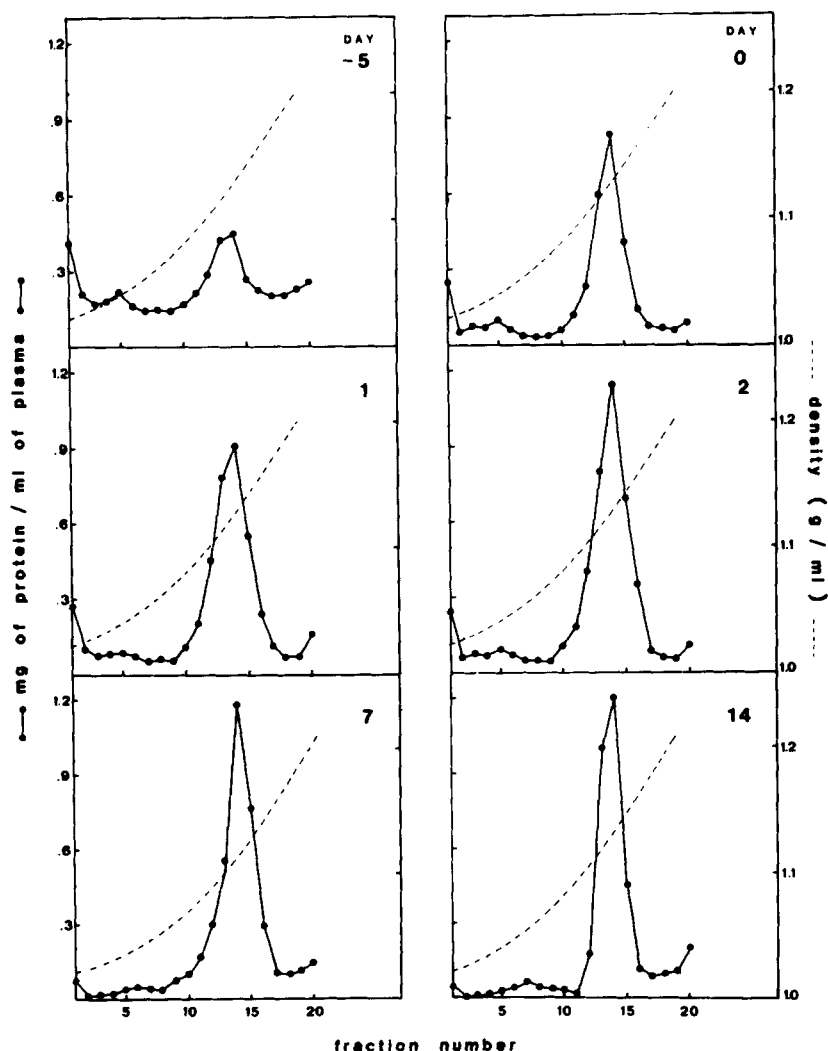
(day 14). On the other hand, the HDL peak became narrower during the first 2 weeks of the postnatal period. **Fig. 1** also shows that the VLDL peak progressively decreased in the post-natal period whereas that of HDL increased strikingly at hatching and remained stable afterwards. The lipoprotein density profile at day 30 was the same as that at day 14 (data not shown). **Fig. 2** shows the effect of development on the plasma concentration of lipoprotein cholesterol and triacylglycerols. In keeping with the data shown in **Fig. 1**, the concentration of VLDL-cholesterol (free and esterified) decreased dramatically 2 weeks after hatching; the concentration of VLDL-triacylglycerols followed the same trend, although less dramatically. The percentage of plasma cholesterol found in VLDL + LDL (fractions 1–8 of the density gradients shown in **Fig. 1**) was 51% at day -5, 42% at day 0, 31% at day 2, and 6% at days 14 and 30.

At each stage of development we investigated the apolipoprotein composition of the lipoprotein density fractions. The typical apolipoprotein patterns of VLDL, LDL, and HDL on days -5, 2, and 30, respectively, are shown in **Fig. 3**. In all stages of development the apolipoprotein pattern of VLDL and LDL was characterized by the presence of a major high molecular weight component (apoB) and a variable amount of minor components having the following relative molecular weights: 28 K (apoA-I), 22K, 14 K, and 8 K. At visual inspection, the relative content of the 22 K and 14 K bands in VLDL and LDL appears to be lower at day -5 than at later stages. In heavily loaded lanes, several additional bands were observed in the upper section of the gel below the apoB band. They presumably represent products of proteolytic degradation of apoB. In VLDL and LDL of day 2 and day 30, we also observed a band with a molecular weight of 38 K which might be the chick counterpart of mammalian apoE (14). HDL were characterized by the presence of apoA-I (which accounted for approximately 90% of total apoHDL), of the 14 K–8 K bands, and by the absence of the 22 K band. No band with the electro-

TABLE 1. Plasma lipid concentration in the developing chick

Days	Cholesteryl Esters	mmol/liter	
		Cholesterol	Triacylglycerols
-5	6.27 ± 0.93*	1.67 ± 0.69	1.66 ± 0.51*
0	7.18 ± 0.17	2.19 ± 0.59	0.95 ± 0.62
1	8.22 ± 1.24	2.73 ± 0.33	0.64 ± 0.29
2	7.18 ± 0.68	1.98 ± 0.45	0.52 ± 0.23
7	2.91 ± 0.55*	1.01 ± 0.10*	0.59 ± 0.25
14	3.19 ± 0.50*	0.99 ± 0.25*	0.53 ± 0.22
30	3.33 ± 0.66*	0.94 ± 0.07*	0.34 ± 0.005*

Values represent mean ± SD. Day 0 = time of hatching. \*Significantly different from the values found at day 0; *P* < 0.05.



**Fig. 1.** Density profile of plasma lipoproteins in the developing chick. Plasma lipoproteins were separated by density gradient ultracentrifugation (14). Pools of plasmas taken from five chicks killed 5 days before hatching (day -5), at the time of hatching (day 0), and 1, 2, 7, and 14 days after hatching were used. VLDL + IDL, fractions 1 and 2; LDL, fractions 5-8; HDL, fractions 11-16.

phoretic mobility of mammalian apoA-IV was detected at any stage of development.

#### Immunoaffinity chromatography of $d < 1.035$ g/ml lipoproteins

In order to ascertain whether the presence of apoA-I in VLDL and LDL of the developing chick (Fig. 3) was due to the presence of lipoproteins containing exclusively apoA-I, the  $d < 1.035$  g/ml lipoproteins of 2-day-old chicks were subjected to immunoaffinity chromatography. Two fractions were isolated using the anti-apoA-I affinity column. The bulk of the applied material passed directly through the column (unbound fraction); only 10% of the applied material was retained on the column and eluted

with 1 M acetic acid. **Fig. 4** shows that the unbound fraction contained apoB, its proteolytic products, and the 22 K band but no apoA-I. The bound fraction was found to contain predominantly apoA-I and the 14 K band. The unbound fraction contained 61% CE, 18% CH, 2% TG, and 19% PL; the bound fraction contained 59% CE, 25% CH, 2% TG, and 14% PL.

#### Concentration of apoA-I and albumin in chick plasma

**Fig. 5** shows the effect of development on the plasma concentration of apoA-I and albumin. Plasma apoA-I level increased from day -5 up to day 1, and remained stable from day 2 to day 30. By contrast, the concentration of plasma albumin steadily increased during the first week of postnatal life and reached a plateau at day 7.

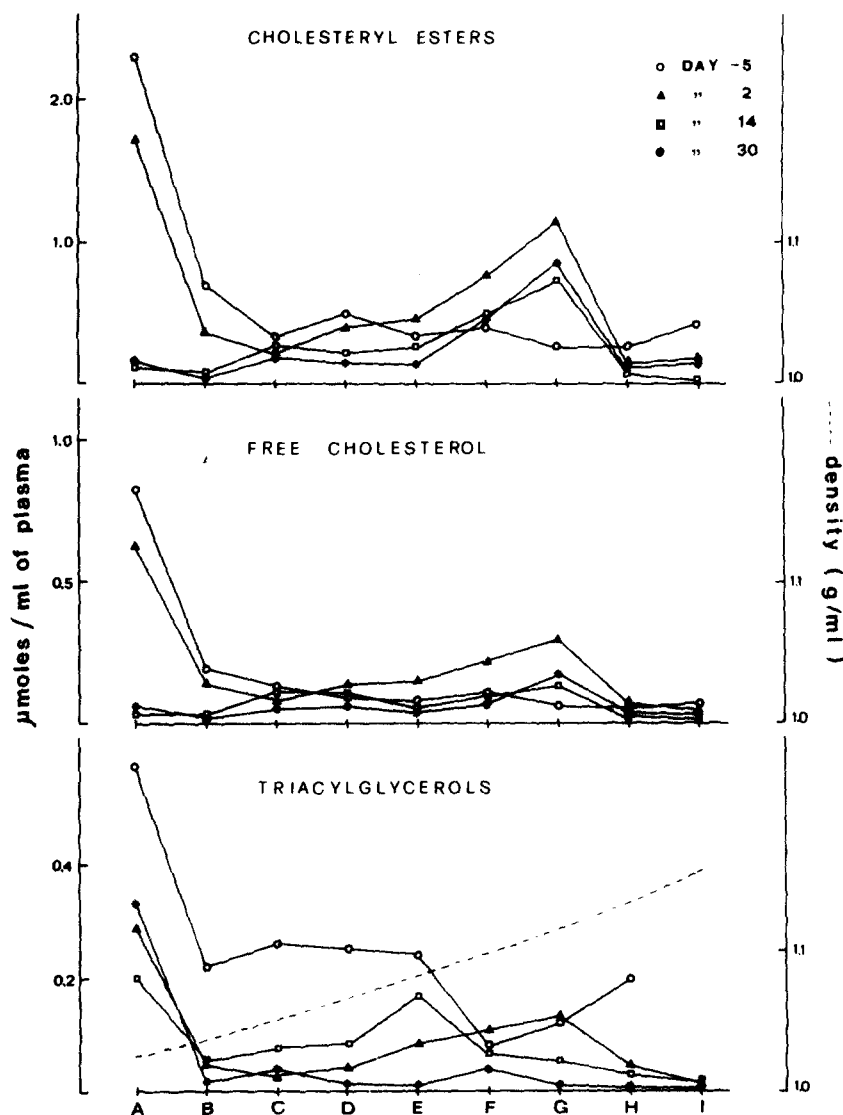


Fig. 2. Plasma concentration of lipoprotein lipids (cholesteryl esters, free cholesterol, and triacylglycerols) at various stages of development (day -5, 2, 14, and 30). Lipoproteins were separated from pooled plasma, by density gradient ultracentrifugation; the density fractions (corresponding to those shown in Fig. 1) were pooled as follows 1-4 (A); 5-6 (B); 7-9 (C); 10-11 (D); 12, 13, 14, and 16 (E, F, G, H); 17-18 (I).

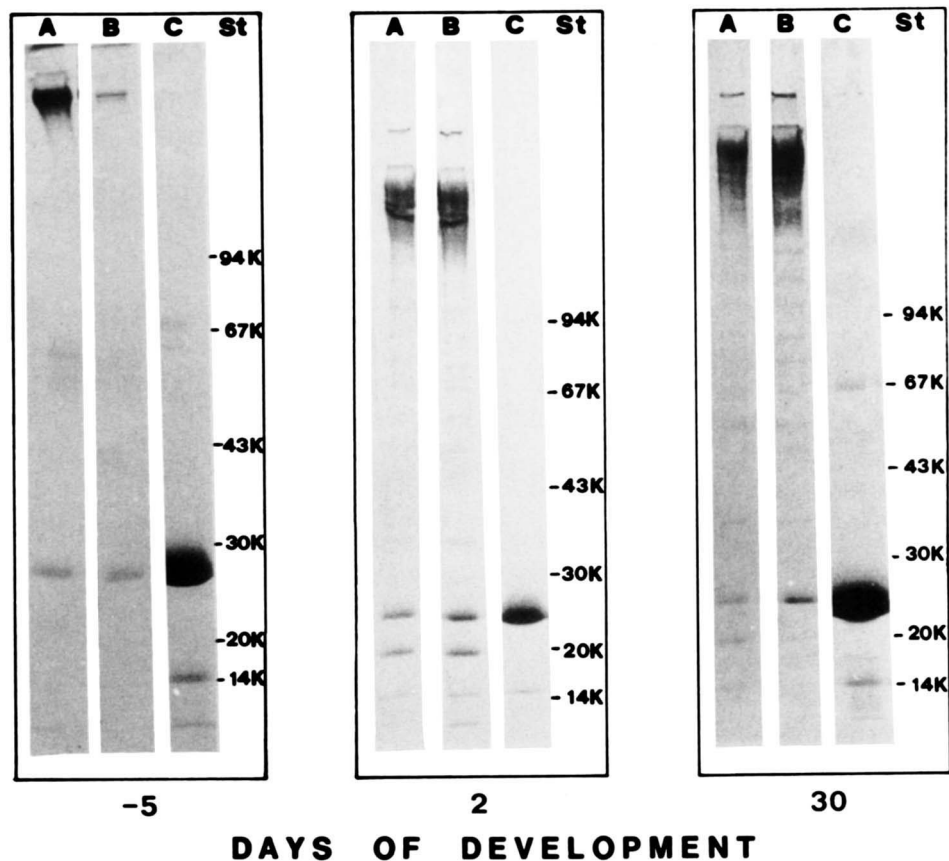
### Tissue lipids

A large accumulation of cholesteryl esters in the liver was observed as early as day -5. It reached a plateau around hatching and decreased very rapidly (15-fold decrease) from day 2 to day 7 (Fig. 6). Hepatic free cholesterol concentration followed the same trend but its decrease, after hatching, was much less remarkable. Hepatic concentration of triacylglycerols increased from day -5 to day 0 but decreased strikingly at day 1. In the skeletal muscle, the concentration of free cholesterol was higher than that of cholesteryl esters at all stages of development (Fig. 7). The highest levels of free and esterified

cholesterol were observed in the late embryonic period; in the postnatal period the concentration of cholesteryl esters decreased rapidly after day 2 whereas that of free cholesterol decreased more slowly. The level of triacylglycerols remained elevated up to day 7.

### Histochemistry and immunohistochemistry

Lipid accumulation was also demonstrated in sections of liver and skeletal muscle. At hatching time the cytoplasm of the hepatocytes was engulfed with Sudan Black B-positive material, which diminished significantly after the first week of postnatal life. In late embryonic stage



**Fig. 3.** SDS-polyacrylamide gradient (5-20%) gel electrophoresis of apolipoproteins of some plasma lipoprotein fractions (selected from the gradients of Fig. 1) isolated at day -5, 2, and 30. Thirty to 50  $\mu$ g of protein was applied to each lane. Molecular weight standards (St) were: phosphorylase b, 94 K; bovine serum albumin, 67 K; ovalbumin, 43 K; carbonic anhydrase, 30 K; trypsin inhibitor, 20 K; and lactalbumin, 14 K. Lane A: gradient fractions 1-2 (VLDL + IDL); lane B: gradient fractions 5-6 (LDL); lane C: gradient fraction 12 (HDL).

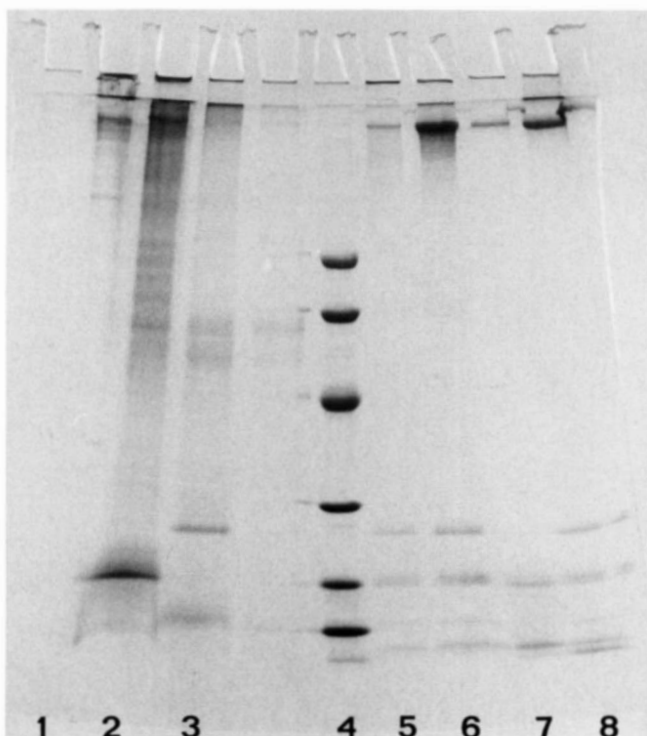
(day -5) and at day 0, Sudan Black B-positive material was found within and among the myoblasts. At day 7 the positive granules were present only in a few muscle fibers (data not shown).

We also studied the effect of development on the presence of immunoreactive apoA-I in liver and skeletal muscle. At day -5 and day 0 all hepatocytes were found to contain immunoreactive products in the cytoplasm. Positive material was also found around the plasma membranes facing the sinusoidal space. In the liver of 7-day-old chick positive products were found in the cytoplasm of only few hepatocytes (data not shown). The localization of apoA-I in the skeletal muscle is shown in **Figs. 8 A-D**. At day -5 very few positive areas were found within the myoblasts and around the capillary walls (data not shown). At the time of hatching, immunoreactive apoA-I was found in the cytoplasm of the muscle cells mainly adjacent to the sarcolemma (Fig. 8A); no reaction products were found when tissue slices were incubated with nonimmune serum (Fig. 8B). At day 7 reaction products were found only

around the capillary walls (Figs. 8C, D). As positive control we investigated the immunolocalization of albumin in liver and skeletal muscle. At all time points immunoreactive albumin was found in the cytoplasm of the hepatocytes; in the skeletal muscle, positive reaction products were observed only around the capillary walls (data not shown).

#### **Synthesis and secretion of apoA-I by the skeletal muscle**

To confirm that synthesis and secretion of apoA-I by the skeletal muscle occurred in early postnatal life, muscle explants of 2-day-old chick were incubated with [ $^{35}$ S]methionine and the labeled proteins secreted into the medium were immunoprecipitated with anti-apoA-I and anti-albumin rabbit IgG. **Fig. 9** shows that apoA-I but no albumin was secreted by the leg muscle explants. When the medium was subjected to density gradient ultracentrifugation (14), labeled apoA-I was found in the material floating in the HDL density range (1.120-1.180 g/ml). Since the isolation of  $^{35}$ S-labeled apoA-I in medium HDL



**Fig. 4.** SDS-polyacrylamide gradient (5–20%) gel electrophoresis of unbound and bound fractions separated from  $d < 1.035$  g/ml lipoproteins by anti-apoA-I immunoaffinity chromatography (see Methods). Lane 1: human LDL; lane 2: unbound fraction; lane 3: bound fraction; lane 4: molecular weight standards as in Fig. 2; lanes 5–6:  $d < 1.035$  g/ml lipoproteins (30 and 60  $\mu$ g of protein, respectively) of 2-day-old chick; lanes 7–8:  $d < 1.035$  g/ml lipoproteins (30 and 60  $\mu$ g of protein, respectively) of 30-day-old chick.

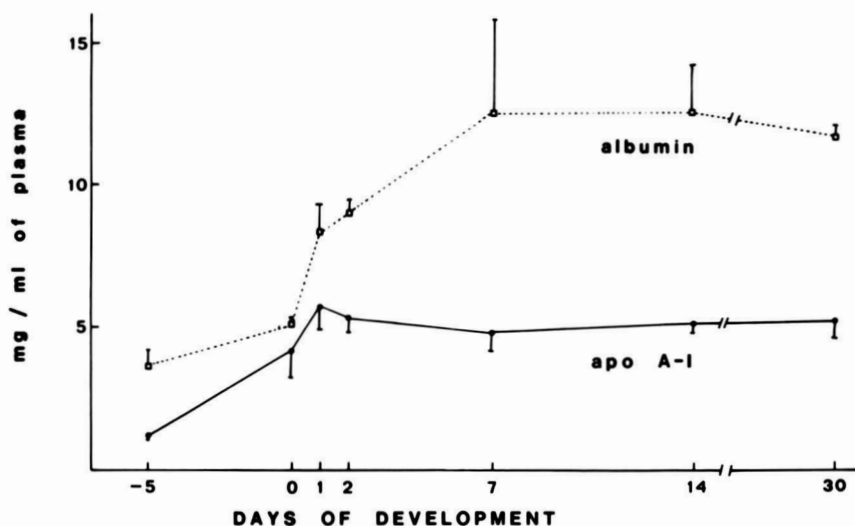
could, in theory, reflect the incorporation of lipid-free apoA-I into residual HDL of fetal calf serum, muscle explants were processed and incubated with [ $^{35}$ S]methionine in the absence of fetal calf serum (see Methods). **Fig. 10** shows that under these circumstances  $^{35}$ S-labeled apoA-I was isolated in the HDL density range. Furthermore, the addition of colchicine (25  $\mu$ M) completely abolished the secretion of labeled HDL (data not shown).

We have previously shown that the concentration of apoA-I mRNA in the skeletal muscle markedly increases around the time of hatching, being maximal at day 2 (5). The data shown in **Fig. 11** confirm this observation (panel A) and also indicate that the composition of the diet influences the development-related variations of apoA-I mRNA (panel B). When the chicks were fed a diet devoid of carbohydrates and rich in lipids and proteins, the apoA-I mRNA concentration remained elevated up to day 6 and was higher than controls at day 12.

## DISCUSSION

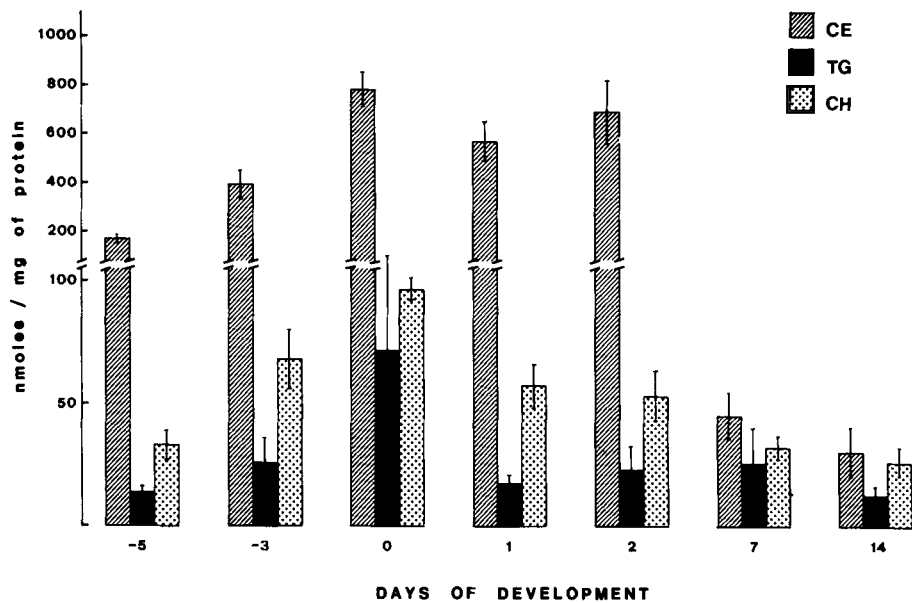
In the present study we have investigated the time-dependent changes of plasma lipoproteins and tissue lipids that occur in the chick during late embryonic and postnatal development.

One of the main features of the newborn chick is the presence of a transient hypercholesterolemia associated with complex changes of lipoproteins and an accumulation and then depletion of free cholesterol and cholesteryl esters in liver and skeletal muscle. The changes in cho-



**Fig. 5.** Effect of development on the plasma concentration of apoA-I and albumin. ApoA-I and albumin were measured by electroimmunoassay. Each value represents mean  $\pm$  SD of five animals in each group. The concentration of apoA-I at days -5, 1, and 2 was significantly different from that found at day 0;  $P < 0.05$ . The concentration of albumin at each time point was significantly different from that found at day 0;  $P < 0.002$ .

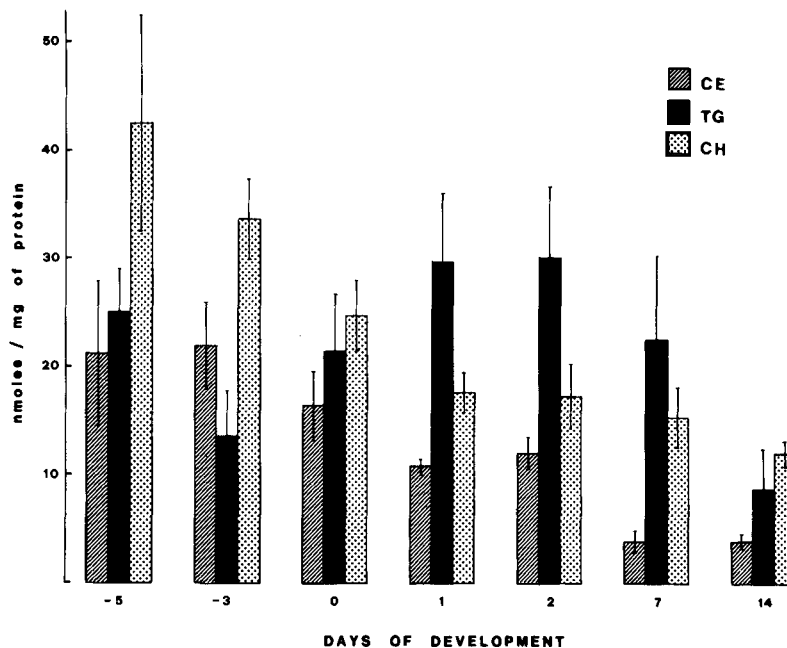




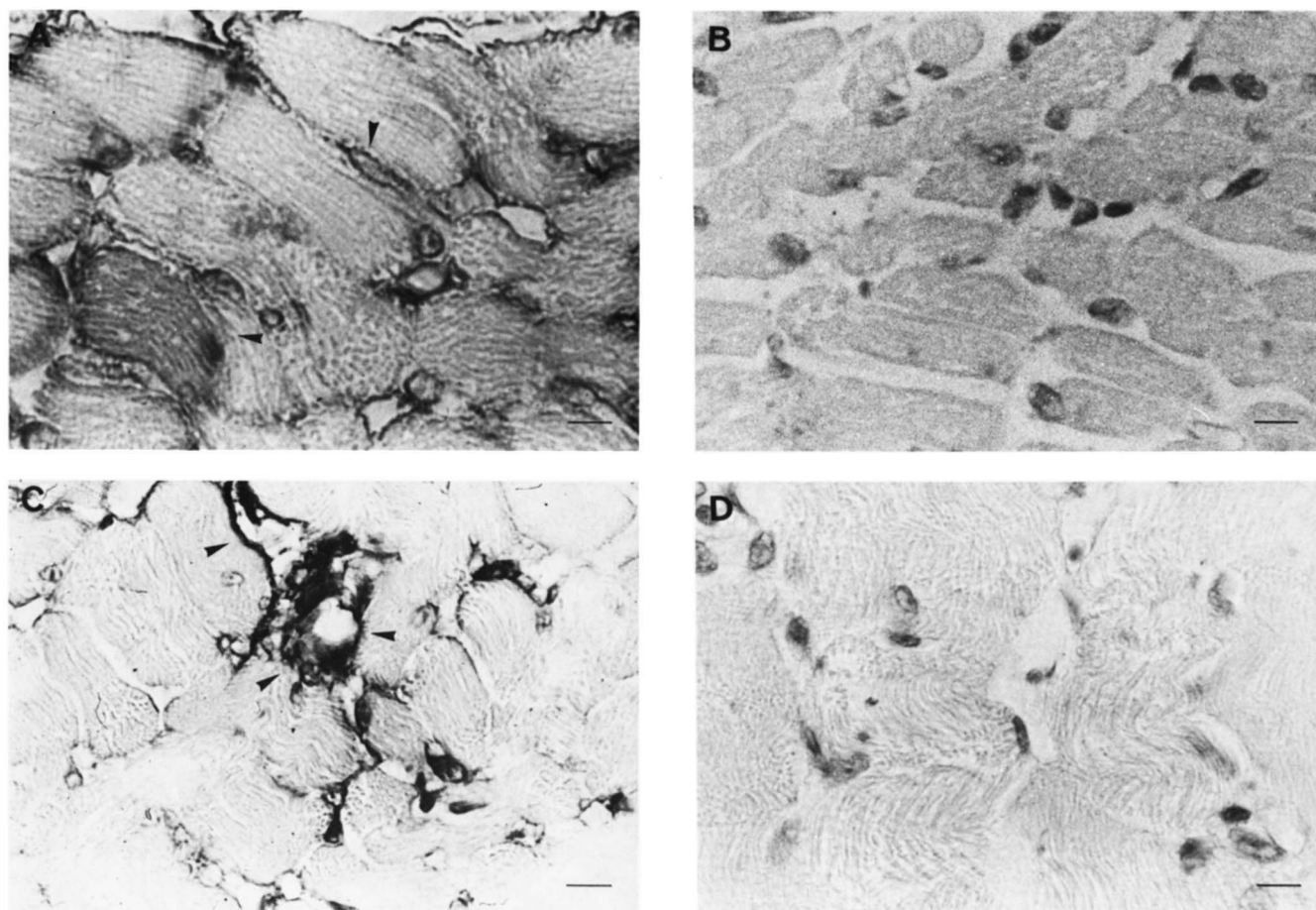
**Fig. 6.** Effect of development on the concentration of cholesteryl esters (CE), triacylglycerols (TG), and free cholesterol (CH) in chick liver homogenate. Each value represents mean  $\pm$  SD of five animals in each group. Similar results were obtained when lipid concentration was measured per gram of tissue wet weight. The concentration of CE and CH at days -5 and 7, was significantly different from that found at day 0;  $P < 0.001$ . The concentration of TG at days -5 and 7 was significantly different from that found at day 0;  $P < 0.02$ .

lesterol concentration in skeletal muscle were associated with variations in the local synthesis of apoA-I. The hypercholesterolemia of the newborn chick was due to an almost equal accumulation of cholesterol-rich VLDL +

IDL and cholesterol-rich HDL (Fig. 2). One week after hatching, plasma cholesterol, cholesterol-rich VLDL, and cholesterol-rich HDL decreased. Two weeks after hatching, HDL became the predominant lipoprotein class in



**Fig. 7.** Effect of development on the concentration of cholesteryl esters (CE), triacylglycerols (TG), and free cholesterol (CH) in chick skeletal muscle homogenate. Each value represents mean  $\pm$  SD of five animals in each group. Similar results were obtained when lipid concentration was measured per gram of tissue wet weight. The concentration of CE and CH at day 7 was significantly different from that at day 0;  $P < 0.005$ . The concentration of triacylglycerols at day 14 was significantly different from that at day 0;  $P < 0.005$ .



**Fig. 8.** Immunohistochemical localization of apoA-I in chick skeletal muscle at day 0 (A, B) and at day 7 (C, D). A: Immunoreactive products are seen in the cytoplasm and at the periphery of the fibers (arrows). C: Immunoreactive products are seen only around the capillaries and at the periphery of some fibers (arrows). B, D: Control experiments performed with nonimmune serum. Bar = 5  $\mu$ m.

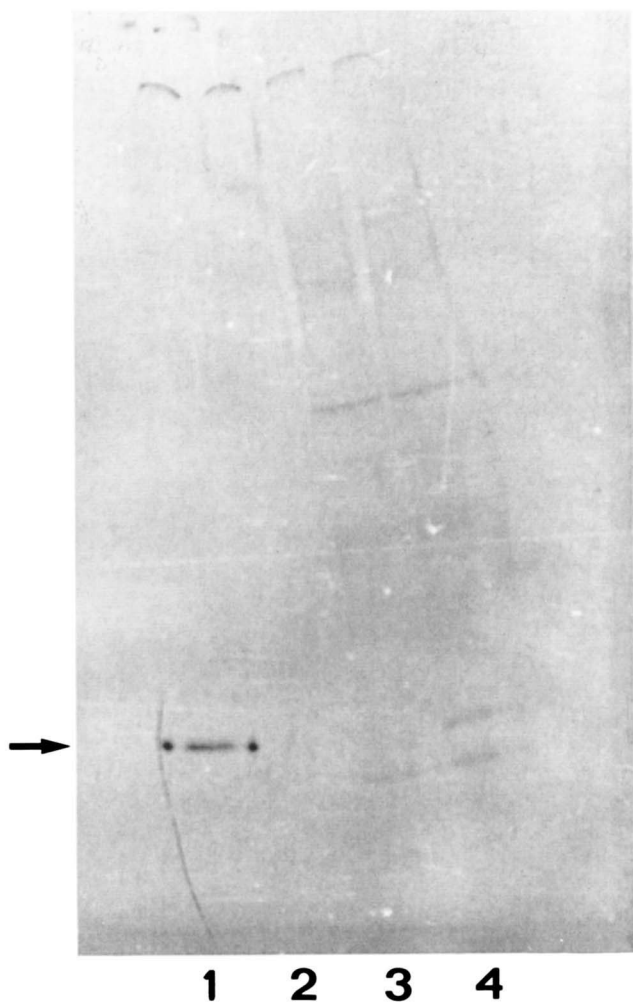
chick plasma (as found in the adult animal), while cholesterol-rich VLDL was replaced by triacylglycerol-rich VLDL (Fig. 2).

Cholesterol-rich lipoproteins isolated at  $d < 1.035$  g/ml (VLDL and IDL) from newborn chick resemble the  $\beta$ -VLDL found in plasma of cholesterol-fed pigeons which are cholesteryl ester-rich, contain apoB-100 and apoA-I as major apolipoproteins (23), and may be analogous to cholesteryl ester-rich  $\beta$ -VLDL found in various mammalian species after cholesterol feeding (24).

The analysis of the apolipoprotein composition of plasma lipoproteins showed that chick VLDL and LDL contained apoA-I in addition to apoB. The presence of this peptide in VLDL and LDL, which was not affected by the stages of development, appears to be a peculiar feature of avian VLDL and LDL (14, 23). In this study we demonstrated for the first time that the  $d < 1.035$  g/ml lipoproteins of chick plasma consist of two classes of lipoproteins, which possess a different apolipoprotein complement. The major class (designated in this study as

apoB-VLDL) contains predominantly apoB and a 22K unidentified polypeptide; the minor one (designated apoA-I-VLDL) contains almost exclusively apoA-I. In the newborn chick apoB-VLDL and apoA-I-VLDL are cholesteryl ester-rich and have the same lipid composition.

The tissue origin of apoB-VLDL and apoA-I-VLDL is uncertain. It is possible that apoB-VLDL are derived, at least in part, from the yolk sac membranes since recent reports indicate that in several mammalian species apoB is synthesized by the yolk sac in the late gestational period (25-27). Furthermore, the observation that the formation of cholesteryl esters is elevated in the yolk sac membranes of the chick during late embryonic development (28) is consistent with the hypothesis that cholesteryl ester- and apoB-rich lipoproteins may be released from the yolk sac directly into the circulation. With the reduction of the yolk sac and the absorption of residual yolk material by the intestine (29), which occurs during the first week of life, liver and intestine may replace the yolk sac as the major site of production of apoB-containing lipoproteins



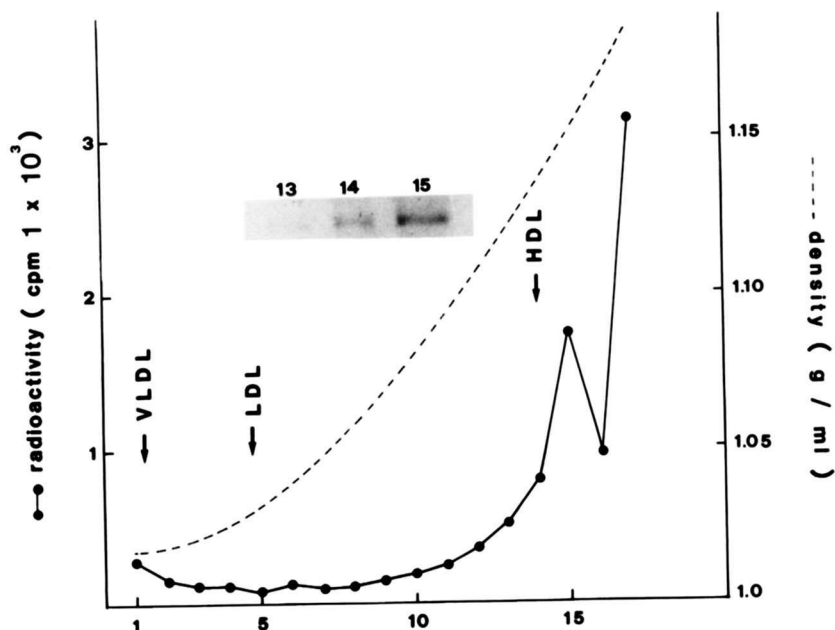
**Fig. 9.** Fluorogram of SDS-polyacrylamide gradient (5–20%) gel electrophoresis of  $^{35}\text{S}$ -labeled apoA-I and albumin secreted in vitro by explants of skeletal muscle of the 2-day-old chick. Explants of skeletal muscle were preincubated in DMEM and 10% FCS for 24 hr. After washing in DMEM, they were incubated in DMEM containing [ $^{35}\text{S}$ ]methionine for 12 hr. Lane 1, immunoprecipitated  $^{35}\text{S}$ -labeled apoA-I secreted into the incubation medium; lane 2, immunoprecipitated [ $^{35}\text{S}$ ]albumin secreted into the incubation medium; lanes 3 and 4,  $^{35}\text{S}$ -labeled proteins left in the supernatant after the immunoprecipitation of apoA-I (lane 3) and albumin (lane 4). The arrow indicates the position of  $^{35}\text{S}$ -labeled apoA-I (mol wt 28 K).

(apoB-VLDL and apoB-containing chylomicrons). The hypothesis that apoB-VLDL of newborn chick plasma is derived directly from lipoprotein-like material contained in the yolk sac seems rather unlikely, since the available data on lipid and apolipoprotein composition of yolk lipoproteins are different from those reported in this study for plasma VLDL and LDL (30–32).

ApoA-I-VLDL presumably represent “light HDL,” i.e., the less dense component of the HDL population. In the newborn chick the high cholesteryl ester content of these

“light HDL” renders them similar to HDL<sub>c</sub> which accumulate in plasma of some mammalian species following cholesterol feeding (24). It is likely that these “light HDL” originate in plasma from heavier HDL secreted from liver and/or peripheral tissues (such as the skeletal muscle). “Heavy HDL” containing apoA-I, phospholipids, and free cholesterol would be converted into “light HDL” by acquiring free cholesterol from peripheral tissues and by becoming enriched in cholesteryl esters through the action of lecithin:cholesterol acyltransferase (LCAT) (33). In this study we confirmed the previous observations by Entenman, Lorenz, and Chaikoff (1) and Wood (2) that from late embryonic to early postnatal life there is an enormous accumulation of cholesteryl esters in chick liver. It is conceivable that the hepatic cholesteryl ester store reflects a rapid and massive uptake of cholesteryl ester-rich VLDL and LDL which overwhelms the capacity of the liver to metabolize cholesterol by the various metabolic routes. In this respect the liver of the emerging chick would be similar to that of cholesterol-fed rabbits where the high uptake of cholesteryl ester-rich  $\beta$ -VLDL leads to a marked accumulation of cholesteryl esters in the hepatocytes. It is unlikely that the accumulation of cholesteryl esters in the liver reflects a high rate of cholesterol synthesis since the level of hepatic HMG-CoA reductase activity and the rate of acetate incorporation into nonsaponifiable lipids by liver slices is very low in the newborn chick; in fact both parameters increase sharply between 5 and 9 days after hatching (34) at the stage in which the depletion of cholesteryl ester store has already occurred. Within a few days from hatching the hepatic cholesteryl ester store is almost completely depleted. Taking into account the variation in liver weight that occurs between 2 and 14 days of postnatal life, approximately 70  $\mu\text{mol}$  of total cholesterol was removed from the whole liver within 12 days. The mechanisms involved in this removal may be an accelerated conversion of cholesterol to bile acids, an increased secretion of cholesterol into the bile or into the circulation, as a component of lipoproteins, or the utilization of cholesterol for the synthesis of the membranes of proliferating hepatocytes.

The pattern of accumulation of free cholesterol and cholesteryl esters in the skeletal muscle differs from that seen in the liver for two reasons: *a*) the level of free cholesterol always exceeds that of esterified cholesterol; and *b*) the concentration of both lipids is much lower than that in the liver. It should be pointed out that the accumulation of cholesterol and cholesteryl esters observed in the muscle of the developing chick resembles that found in the animals with muscular dystrophy (35–38). For example, the cholesterol content of the sarcolemma membranes and sarcoplasmic reticulum of the dystrophic chick muscle is 2.4- and 1.3-fold, respectively, that found in normal chick

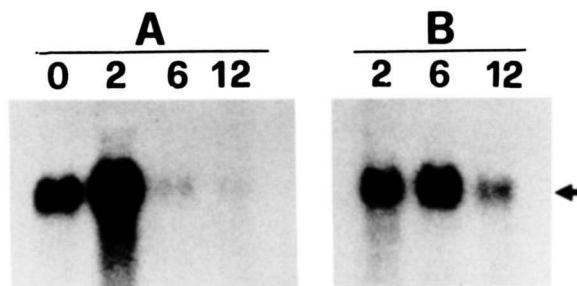


**Fig. 10.** Density profile of  $^{35}\text{S}$ -labeled lipoproteins secreted into the medium by explants of skeletal muscle of the 2-day-old chick. Explants of skeletal muscle were preincubated in DMEM for 24 hr in 35-mm plastic Petri dishes and then incubated in DMEM in the presence of [ $^{35}\text{S}$ ]methionine for 12 hr. Medium lipoproteins were separated by density gradient ultracentrifugation (14) without the addition of plasma carrier. Arrows indicate the fractions corresponding to chick plasma lipoproteins. The inset shows part of a fluorogram of  $^{35}\text{S}$ -labeled apoA-I immunoprecipitated from the density fractions 13, 14, and 15 (see legend of Fig. 9).

(37). That would suggest that the accumulation of cholesterol in dystrophic muscle reflects a de-differentiation process by which the skeletal muscle of the adult animal exhibits a lipid metabolic pattern typical of the embryonic or early neonatal period. The mechanism for this cholesterol accumulation in embryonic life is completely unknown. It is likely that during the embryonic period the myoblasts, which require cholesterol for growth and differentiation, have an efficient mechanism (for example a high number of apoB receptors) for taking up cholesterol from plasma VLDL and LDL. As the differentiation of the skeletal muscle proceeds, the uptake of exogenous cholesterol may decrease and the internalized cholesterol (stored in the cytoplasm as cholesteryl esters), which has not been used to fulfill the cellular needs, would be removed through the secretion of lipoprotein-like particles (see below). This hypothesis appears to be supported by our results which indicate that: *a*) the major depletion of cholesterol from the skeletal muscle occurs at the time when the level of apoA-I mRNA is highest in this tissue; and *b*) explants of skeletal muscle of newborn chick actively secrete apoA-I-containing HDL. Our results raise the possibility that the excess of free cholesterol and/or cholesteryl esters in the skeletal muscle might, at some specific stage of development, induce the expression of the

apoA-I gene, presumably at the transcriptional level (5). Support for this view is given by the observations that: *a*) cholesteryl esters accumulated in mouse peritoneal macrophages stimulate the synthesis of apoE (39, 40); and *b*) after the administration of a high-fat atherogenic diet, the level of apoA-I mRNA in rabbit liver and apoA-IV mRNA in mouse liver increases several-fold (41, 42).

The hypothesis that metabolic stimuli (such as cholesterol accumulation) play a role in inducing apoA-I synthe-



**Fig. 11.** Effect of a carbohydrate-free diet on the concentration of apoA-I mRNA in breast muscle during development. Total RNA was extracted at the indicated day (0, 2, 6, and 12 days after hatching) from the breast muscle of chicks raised on a standard diet (panel A) or carbohydrate-free diet (panel B). Northern blot analysis of apoA-I mRNA was carried out using  $^{32}\text{P}$ -labeled apoA-I cDNA as a probe.

sis in skeletal muscle is supported by the observation that feeding the chick a diet rich in proteins and lipids and devoid of carbohydrates influences the development-related variations of apoA-I mRNA. In the skeletal muscle of the newborn chick, the general trend is to shut off the apoA-I mRNA transcription as the animal grows (5). However, this process appears to be delayed when the animals are fed a carbohydrate-free diet in which the caloric intake is provided by lipids (11).

Finally, one can also speculate that the accumulation of cholesterol and cholesteryl esters in the muscle of the congenitally dystrophic chick might be responsible for the increased synthesis of apoA-I (43).

As a corollary to our findings, we suggest that the skeletal muscle may significantly contribute to the plasma pool of apoA-I-containing lipoproteins in the newborn chick. ■

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