Apolipoprotein B-100 production and cholesteryl ester content in the liver of developing chick

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Abstract In the chick, the large cholesteryl ester (CE) store present in the liver during the last period of embryonic life increases at hatching and is rapidly depleted after 2–7 days of postnatal life. In this study we asked whether these changes were associated with variations in the hepatic production of apoB-containing lipoproteins. Liver slices taken from chicks at −3, 0 (hatching), 2, 4, 7, and 10 days of development were incubated with [^{35}S]methionine in steady state incubations. ApoB production (cell + medium radioactivity) decreased from day −3 to day 0 (40%), increased at day 4 (54%), and decreased afterwards (45%). At day 4 the amount of ^{35}S-labeled apoB-containing lipoproteins (VLDL-LDL) secreted into the medium was 1.7- and 1.5-times that found at days 0 and 7, respectively; the radioactivity incorporated into medium HDL (containing predominantly apoA-I) was 1.7-times that found at days 0 and 7. The incubation of liver slices with [^{3}H]oleate showed that CE production at days 4 and 7 was 58% and 33%, respectively, of that found at day 0. The percentage of newly synthesized hepatic CE secreted into medium lipoproteins was 2.4%, 3.1%, and 2.2% at days 0, 4, and 7, respectively. The percentage of lipoprotein CE present in VLDL-LDL ranged from 38% at day 0 to 21% at day 7, and that present in HDL ranged from 62% at day 0 to 79% at day 7. To define whether the changes in the production of apoA-I- and apoB-containing lipoproteins were due to variations in apoB and apoA-I synthesis, the initial synthetic rate (pulse-labeling) and the mRNA content of these apolipoproteins were investigated. The initial apoB synthetic rate decreased 1.5-fold from day −3 to day 0, remained stable up to day 7, and decreased at day 10. Hepatic apoB mRNA followed a similar trend. The synthesis of apoA-I increased 2-fold from days −3/2 up to day 4 and did not change afterwards. In conclusion the increased hepatic CE content at hatching reflects a decreased production of apoB, while the depletion of CE observed from day 2 to day 7 is associated with an increased production of both apoB- and apoA-I-containing lipoproteins. The decreased apoB production at hatching is due to a decreased apoB synthesis whereas the increased apoB production at day 4 appears to be related to a post-translational event. —**Tarugi, P., S. Nicolini, L. Marchi, G. Ballarini, and S. Calandra.** Apolipoprotein B-100 production and cholesteryl ester content in the liver of developing chick. *J. Lipid Res.* 1994. **35:** 2019–2031.

Supplementary key words apoA-I • apoB mRNA • lipoproteins

The secretion of VLDL by the liver is a complex process requiring the coordinate synthesis of lipids and apoB and their assembly into lipoprotein particles (1, 2). The availability of apoB plays a major role in determining the capacity of hepatocytes to assemble and secrete VLDL (3, 4).

Numerous studies have demonstrated that hepatic apoB synthesis and secretion are metabolically regulated (2, 3, 5–11). Many reports have focused on the effect of free fatty acids on apoB production, as an increased supply of long chain fatty acids increases both triacylglycerol synthesis and VLDL triacylglycerol output (12, 13). In primary rat hepatocytes, oleate produces an increase in triacylglycerol loading of VLDL without secretion of additional apoB (12, 14), whereas the addition of oleate to hepatoma cells stimulates the secretion of apoB (15–19) without affecting the apoB synthetic rate or the apoB mRNA content (18, 20–22). The increased secretion of apoB by HepG2 cells exposed to oleic acid appears to be due to a reduction of the intracellular degradation of apoB (6, 19, 22–24).

Recently, it has become apparent that cholesteryl ester availability, which can also be influenced by fatty acids (13), may be important in regulating hepatic apoB secretion (25, 26). It has been reported that the increased secretion of apoB induced by the addition of oleate to HepG2 cells was not correlated to that of medium triacylglycerols but to that of medium cholesteryl esters (16, 20) and it was abolished by inhibiting cholesteryl ester bio-

Abbreviations: apoB, apolipoprotein B-100; apoA-I, apolipoprotein A-I; VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CE, cholesteryl ester; CH, cholesterol; HSS, high-speed supernatant.

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synthesis (26). Recently, Dashti (27) found that 25-hydroxycholesterol stimulated the synthesis of both cholesteryl esters and apoB and suggested that the synthesis and secretion of apoB and cholesteryl esters were tightly coupled. Furthermore, the increased intracellular availability of cholesteryl esters in rabbit hepatocytes was found to reduce the intracellular degradation of apoB and to increase its secretion (28). Taken together, these reports seem to indicate that cholesteryl ester synthesis is a primary stimulus for apoB secretion.

ApoB production, and VLDL assembly and secretion have been extensively investigated in the chick, especially because this model offers an advantage in that the synthesis of VLDL apolipoproteins (including apoB) is under the control of estrogens (29–36). There is, however, another reason that makes the chick a suitable model for the study of the regulation of hepatic apoB production, that is the dramatic changes in the hepatic content of cholesteryl esters that occur in late embryonic and early post-natal life of the chick. We have shown that the enormous hepatic cholesteryl ester store present at the time of hatching (37, 38) is depleted within the first week of post-natal life (39). In the present study we took advantage of this unique physiological situation to investigate whether the changes in the hepatic content of cholesteryl esters occurring post-natally were associated with variations in the hepatic production of apoB and an overproduction of apoB- and apoA-I-containing lipoproteins.

MATERIALS AND METHODS

Materials

[^{35}S]methionine (>37 TBq/mmol), [^{3}H]oleic acid (370 GBq/mmol), Pro-mix (L-[^{35}S]methionine and L-[^{35}S]cysteine, >37 TBq/mM) and [^{32}P]dCTP (3000 Ci/mmol), Hyperfilm-MP X-ray films, and Hybond-N membranes were obtained from Amersham (U.K.). Protosol and Omnifluor were obtained from DuPont NEN (Milano, Italy). Protein A-Sepharose was obtained from Pharmacia (Upsala, Sweden) and silica gel G plates for thin-layer chromatography were purchased from Merck (Darmstadt, Germany). Molecular weight protein standards were obtained from Bio-Rad (Richmond, CA). Polybrene, leupeptin, aprotinin, soy bean trypsin inhibitor, lima bean trypsin inhibitor, and glutathione were obtained from Sigma (St. Louis, MO). Ketalar (ketamine) was obtained from Parke-Davis (Milano, Italy).

Animals

Eggs (Warren strain) were obtained from a local poultry supplier and dates of fertilization were carefully noted. Male chicks born from the same batch of eggs were fed a standard diet (25% soya proteins, 4.5% lipids, and 5.5% fiber) ad libitum until the time of killing. The standard diet contained 0.045% (w/w) total sterols and 0.005% (w/w) cholesterol. Chicks were killed at the following stages of development: 3 days before hatching (day −3), at the time of hatching (day 0), and 2, 4, 7, and 10 days after hatching.

Blood and tissue samples

Chicks were anesthetized by peritoneal injection of Ketalar (5 mg/100 g body weight). Blood was collected by cardiac puncture using K_3 EDTA as anticoagulant. Livers were excised, washed in cold 0.154 M NaCl, and used for in vitro labeling or immediately frozen in liquid nitrogen and stored at −80°C for subsequent analysis (RNA and lipid extraction).

Plasma lipids and lipoproteins

Plasma cholesterol and triacylglycerols were measured by automatic enzymatic method (Ames Division, Miles, U.K.). Before the separation of plasma lipoproteins, equal aliquots of plasma taken from each animal (6–8 animals per group) at the various time points were pooled. Lipoproteins were isolated from the plasma pools by density gradient ultracentrifugation in an SW41 rotor (40). After centrifugation, aliquots of 500 μl (fraction 1) or 400 μl fractions 2–24 were collected (40) and their protein (41) and cholesterol concentrations were measured (see above).

Liver lipids and lipid droplets

Livers were finely minced, suspended in 10 ml 0.25 M sucrose per g of tissue and homogenized with three strokes of a Potter homogenizer. Lipids were extracted, separated, and measured as previously described (39). To isolate lipid droplets, 8 ml liver homogenate was overlayered with 4 ml distilled water and centrifuged at 9,000 *g* to sediment debris and mitochondria. The floating milky material was collected and adjusted to 0.5 M sucrose and 1 mM $MgCl_2$. An aliquot (8 ml) of this material was overlayered with 0.25 M sucrose (2 ml) and distilled water (2 ml) and centrifuged at 12,500 *g* for 30 min (42). The floating layer was gently collected and its protein (41) and lipid content (39) was measured.

Synthesis of apolipoproteins in vitro

Eight to ten chicks were killed at days −3 and 0, and five chicks at days 2, 4, 7, and 10. The right lobe of the livers was washed in cold 0.154 M NaCl, and cut into slices (43). For each time point, pools of equal amounts of liver slices (25–50 mg from each animal) were incubated in triplicate. In the steady-state incubations 250 mg of pooled liver slices was incubated for 4 h at 40°C in 3 ml Krebs-Ringer bicarbonate buffer containing [^{35}S]methionine (60 μCi/ml), 50 units/ml penicillin, 50 μg/ml streptomycin, and 100 kallikrein inhibitor units/ml of aprotinin, under an atmosphere of 95% O_2–5% CO_2. At the

end of the incubations, media were collected and supplemented with leupeptin (0.1 mM), polybrene (25 μg/ml), Na_2 EDTA (0.1 mM), soy bean trypsin inhibitor (20 μg/ml), lima bean trypsin inhibitor (20 μg/ml), glutathione (0.02%), phenylmethylsulfonylfluoride (PMFS) (0.2 mg/ml), and aprotinin (5 μg/ml). This material was exhaustively dialyzed against 10 mM NH_4HCO_3, 1 mM Na_2EDTA, Trasylol (100 kallikrein inhibitor units/ml), and 1 mM methionine, and lyophilized. Tissue slices were washed with ice-cold Krebs-Ringer bicarbonate buffer, 1 mM unlabeled methionine, and Trasylol (20 kallikrein inhibitor units/ml), and homogenized in 15 volumes (v/tissue weight) of 20 mM Na phosphate buffer, pH 7, 150 mM NaCl, 5 mM Na_2EDTA, 2% Triton X-100, and 200 μg/ml PMSF. This material was centrifuged at 226,000 *g* for 1 h at 4°C to prepare the high-speed supernatant (HSS) (44). In short-term incubations (pulse-labeling), pools of liver slices (250 mg) were incubated for 20 min at 40°C in 3 ml Krebs-Ringer bicarbonate buffer (see above) in the presence of 200 μCi/ml [^{35}S]methionine/[^{35}S]cysteine (Pro-Mix, Amersham) under an atmosphere of 95% O_2–5% CO_2. At the end of the incubation, liver slices were collected, washed with ice-cold Krebs-Ringer bicarbonate buffer containing 1 mM methionine, 1 mM cysteine, and Trasylol (100 kallikrein inhibitor units/ml), and immediately homogenized as specified above. Incubation media were dialyzed in the same buffer (see above) supplemented with 1 mM cysteine. Aliquots of incubation media and high-speed supernatants (HSS) were immunoprecipitated by anti-chick apoB (43, 44) and anti-chick apoA-I rabbit IgG or nonimmune rabbit IgG (39, 43–45). In preliminary experiments we titrated the HSS to define the conditions of antibody excess that could ensure the complete immunoprecipitation of apoB and apoA-I (44). Moreover, the further addition of specific IgGs to the supernatants of HSS immunoprecipitates as well as the direct analysis of these supernatants by SDS-PAGE (a study performed in every sample of each experiment) did not reveal the presence of labeled apoB or apoA-I. The apoB immuno-complexes were dissolved in 100 mM Tris-HCl, pH 7.3, 20% glycerol, 10% SDS, and 5% 2-mercaptoethanol, heated at 100°C for 5 min, and applied to a linear 5–10% gradient SDS-polyacrylamide gel. The apoA-I immuno-complexes were dissolved in 2% SDS, 3.5% 2-mercaptoethanol, 50 mM Tris-HCl, pH 7, heated at 95°C for 3 min, and applied to a linear 5–20% gradient SDS-polyacrylamide gel (39, 43). Gels were stained with Coomassie Blue R-250, destained, processed for fluorography, and exposed to Hyperfilm-MP X-ray films at −80°C. Radioactive bands corresponding to apoB and apoA-I were excised from the gel, solubilized in 30% hydrogen peroxide, and incubated overnight at 37°C. After the addition of 1 ml of Protosol, samples were shaken for 6 h at room temperature and counted in scintillation fluid (Omnifluor 3.8 g, Protosol 25 ml, and water 3.5 ml/l toluene) (44).

In both short and steady state incubations the amount of [^{35}S]methionine incorporated into cell and medium proteins was measured after trichloroacetic acid precipitation (44).

Isolation of ^{35}S-labeled lipoprotein secreted in vitro

In some experiments, incubation media of liver slices (taken at days 0, 4, and 7) that had been incubated with [^{35}S]methionine for 4 h as specified above, were dialyzed against 0.154 M NaCl, 10 mM Na_2EDTA, 1 mM methionine, and Trasylol (100 kallikrein inhibitor units/ml), and then subjected to density gradient ultracentrifugation to separate ^{35}S-labeled lipoproteins. Density fractions were collected as specified above for plasma lipoproteins and protein radioactivity was measured after precipitation of the samples with 10% trichloroacetic acid. The recovery of total protein radioactivity (24 density fractions) was >95% in all gradients. Aliquots of each density fraction were applied to a linear 5–20% gradient SDS-polyacrylamide gels. Gels were stained with Coomassie Blue R-250, destained, processed for fluorography, and exposed to Hyperfilm-MP X-ray films at −80°C.

[^{3}H]oleic acid incorporation into hepatic cholesteryl esters

In some experiments, quadruplicate samples of liver slices (250 mg), taken from chicks at days 0, 4, and 7, were pulse-labeled for 1 h in 3 ml Krebs-Ringer bicarbonate buffer in the presence of 70 μCi/ml of [^{3}H]oleic acid-albumin complex (46). At the end of the pulse period, two samples of liver slices were homogenized and extracted in chloroform–methanol 2:1 (v/v) (47); lipids were separated by thin-layer chromatography (39) and the spots corresponding to cholesteryl esters were scraped and counted in a liquid scintillation counter. The other two samples of liver slices were chased for 3 h in Krebs-Ringer bicarbonate buffer. At the end of chase period incubation media were collected, pooled, and ultracentrifuged to separate VLDL-LDL (d < 1.065 g/ml) and HDL (d 1.065–1.210 g/ml). Lipoprotein lipids were extracted in chloroform–methanol 2:1 (v/v) (47) and separated by thin-layer chromatography (39). The spots corresponding to cholesteryl esters were scraped and counted. Free fatty acids were extracted from liver homogenate according to Dole and Meinertz (48) and measured by the method of Novak (49). The analysis of free fatty acid composition was performed by gas–liquid chromatography after diazomethane methylation (50).

Northern blot analysis

RNA was extracted by the guanidine-HCl method, as previously described (51). At days −3 and 0, RNA was extracted from a pool of livers taken from ten animals; at days 2, 4, 7, and 10, RNA was extracted from single livers

(four animals at each developmental period). Equal amounts of total RNA (15 μg) were denatured and separated on 1% agarose-formaldehyde gels. Gels were stained with ethidium bromide, analyzed under ultraviolet light, and photographed just before transfer. The intensities of 28S ribosomal RNA bands as determined by densitometric scanning of photographs of ethidium bromide-stained gels were used to assess that the same amount of total RNA had been loaded onto the gels. RNA was electrotransferred to Hybond-N membranes that were either hybridized with a chick apoB or a chick apoA-I cDNA probe and subsequently reprobed with human β-actin cDNA. The chick apoB probe (clone CB12) was a partial chick apoB cDNA clone (52) that was a kind gift from Dr. D. L. Williams (Stony Brook, NY). The chick apoA-I probe was a full-length cDNA cloned in our laboratory (53). Conditions of pre-hybridization and hybridization were as previously described (51). ^{32}P-labeling of the cDNA probes was performed using the Multiprime Kit (Amersham, England). The specific activity of ^{32}P-labeled cDNA probes ranged from 1.5 to 2.5 $\times$ 10^8 cpm/μg DNA. Membranes were exposed to Hyperfilm MP for autoradiography at −80°C. Densitometric analysis of autoradiograms was performed using an LKB 2202 Ultroscan Laser Densitometer.

Chemical methods

Plasma estradiol was assayed using an enzyme immunoassay kit (NovaPath™ estradiol, Bio-Rad, Richmond, CA).

Statistical analysis

Differences among groups were compared by one-way analysis of variance (ANOVA). Differences were considered significant when the probability of the difference occurring by chance was less than 5 in 100 ($P < 0.05$).

RESULTS

Plasma and liver lipids during early post-natal development

Plasma cholesterol reached the highest levels in the first few days after hatching (days 0–4) and decreased sharply at day 7 (**Table 1**). The level of plasma triacylglycerols showed an opposite trend (Table 1). From late embryonic life (day −3) up to day 4 most of the plasma cholesterol (from 81% at day −3 to 55% at day 4) was carried in plasma lipoproteins of d < 1.065 g/ml (fractions 1–9 of the density gradient shown in **Fig. 1**) whereas, at the later stages, these fractions carried 21–17% of total plasma cholesterol (40).

The hepatic content of cholesterol (CH) and cholesteryl esters (CE) increased from day −3 to days 0–2 whereas from day 2 to day 7 CH decreased 2.5-fold and CE approximately 20-fold (Table 1). The isolation of "cytoplasmic lipid droplets" from the liver at some crucial stages of early post-natal development (i.e., days 0, 4, and 7) revealed that the total amount of lipids found in these droplets decreased by 42% and 95% at days 4 and 7, respectively, as compared to day 0 (**Table 2**). At days 0 and 4, lipid droplets were found to contain predominantly (90%) cholesteryl esters whereas at day 7 they contained predominantly triacylglycerols (92%) (Table 2).

[^{35}S]methionine incorporation into cell and medium apoB during steady-state incubations

Table 3 shows that the incorporation of [^{35}S]methionine into cell and medium proteins by liver slices in steady state (4 h) incubations decreased progressively from day −3 to day 7, with a trend to a transient increase at day 4.

^{35}S-labeled apoB immunoprecipitated from both cell and medium appeared as a single band comigrating with

TABLE 1. Plasma and liver lipids in developing chick

Age	BW	TC	TG	LW	CH	CE
days	*g*	*mg/dl plasma*		*g*	*mmol/g liver*	
−3	31.9 ± 2.6^a	201.4 ± 46.5^a	91.7 ± 25.6^{b,c}	0.5 ± 0.07^a	10.1 ± 0.9^a	73.7 ± 15.9^a
0	45.3 ± 3.0^b	388.5 ± 33.0^b	59.3 ± 11.0^a	0.9 ± 0.12^b	14.2 ± 1.8^b	103.3 ± 13.4^b
2	45.3 ± 2.9^b	353.5 ± 45.2^b	69.7 ± 9.4^{a,b}	1.5 ± 0.13^c	16.0 ± 0.7^b	93.1 ± 8.7^{a,b}
4	54.8 ± 4.1^c	377.0 ± 85.0^b	68.0 ± 33.0^{a,b,c}	1.8 ± 0.19^c	9.4 ± 0.8^{a,c}	39.1 ± 10.6^c
7	69.2 ± 1.6^d	186.4 ± 17.0^a	105.0 ± 16.5^c	2.2 ± 0.19^d	6.2 ± 0.5^d	4.7 ± 2.8^d
10	83.2 ± 8.2^e	204.4 ± 18.6^a	51.8 ± 9.5^a	2.6 ± 0.20^e	8.2 ± 0.2^e	1.9 ± 0.7^d

Each value represents the mean ± standard deviation for data obtained from five animals at each developmental period. Day 0, time of hatching. Values that differ significantly ($P < 0.05$) are denoted by different letters. Abbreviations: BW, body weight; TC, total cholesterol; TG, triacyglycerols; LW, liver weight; CH, free cholesterol; CE, cholesteryl esters.

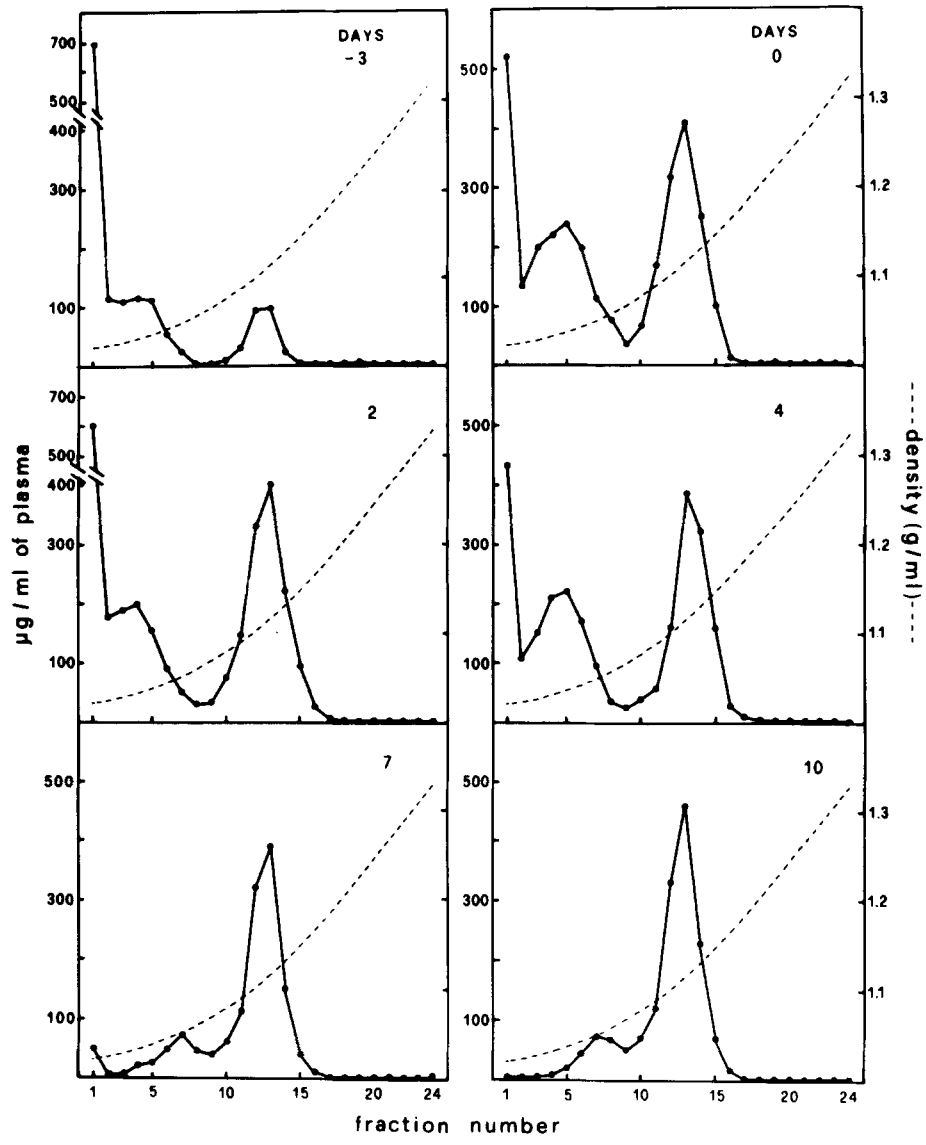

Fig. 1. Density profile of plasma lipoproteins in the developing chick. Plasma lipoproteins were separated by density gradient ultracentrifugation. Pools of plasmas taken from 6–8 chicks killed 3 days before hatching (day −3), at the time of hatching (day 0), and 2, 4, 7, and 10 days after hatching were used. VLDL + IDL, fractions 1–4; LDL, fractions 5–9; HDL, fractions 10–19. Lipoprotein concentration is given as lipoprotein-cholesterol (µg/ml of plasma).

TABLE 2. Composition of hepatic lipid droplets

Age	Protein	CH	CE	TG	PL
days	*mg/g liver*		*nmol/g liver*		
0	315.5	301.5	5982.4	202.8	31.6
4	66.4	215.9	3423.5	138.9	22.6
7	65.4	<2.0	<5.0	255.2	22.3

Each value is the average from a duplicate experiment. Abbreviations: CH, free cholesterol; CE, cholesteryl esters; TG, triacylglycerols; PL, phospholipids.

chick plasma apoB-100 in SDS-PAGE (**Fig. 2**) (43). No intermediate or low molecular weight bands were observed in the fluorograms, suggesting that no degradation of radioactive apoB occurred during our experimental manipulations. The radioactivity of apoB bands excised from SDS-gels (Table 3) was normalized by calculating the results relative to the radioactivity incorporated into total proteins (44). **Table 4** shows that the percentage of total protein radioactivity incorporated into cell and medium apoB decreased from day −3 to days 0–2, increased at day 4, and decreased at day 7. At day 10 the

TABLE 3. [^{35}S]methionine incorporation into cell and medium proteins by the liver of developing chick

Age	Total Protein Radioactivity		ApoB Radioactivity	
	Cell	Medium	Cell	Medium
days	*cpm* × 10^8/g liver		cpm × 10^6/g liver	
−3	2.34 ± 0.04^a	0.89 ± 0.06^a	0.73 ± 0.09^a	0.86 ± 0.18^a
0	1.81 ± 0.05^b	0.51 ± 0.08^b	1.42 ± 0.11^b	0.27 ± 0.08^b
2	1.68 ± 0.13^b	0.48 ± 0.04^{b,c}	0.35 ± 0.05^b	0.30 ± 0.03^b
4	1.86 ± 0.14^b	0.54 ± 0.08^b	0.64 ± 0.04^a	0.68 ± 0.15^a
7	1.34 ± 0.09^c	0.31 ± 0.05^c	0.32 ± 0.01^b	0.13 ± 0.01^b
10	1.29 ± 0.06^c	0.39 ± 0.03^{b,c}	0.25 ± 0.02^b	0.32 ± 0.01^b

Liver slices were incubated for 4 h (steady-state incubations) in the presence of [^{35}S]methionine as specified in Methods. Aliquots of medium and cell homogenate (high speed supernatant) were precipitated with trichloroacetic acid and counted (total protein radioactivity). ^{35}S-labeled apoB was immunoprecipitated from cell homogenates and incubation media and separated by 5–10% linear gradient SDS-PAGE. ApoB bands were excised from the gel and the amount of incorporated label was determined by scintillation counting (apoB radioactivity). Each value represents the mean ± standard deviation from three separate experiments. Values that differ significantly ($P < 0.05$) are denoted by different letters.

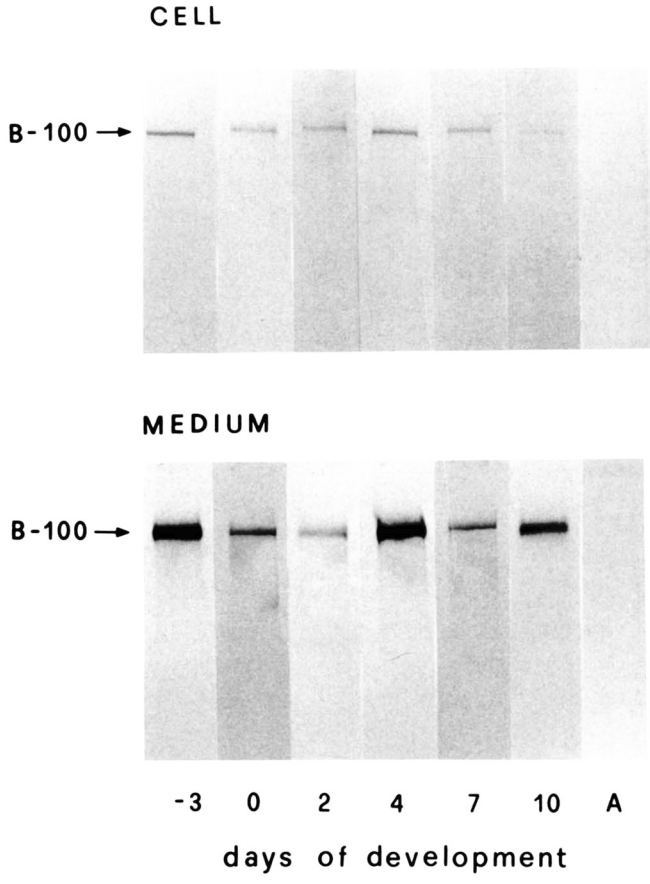

Fig. 2. Fluorograms of ^{35}S-labeled apoB immunoprecipitated from liver slices of the developing chick. Liver slices were incubated for 4 h (steady-state incubations) in the presence of [^{35}S]methionine as specified in Methods. ^{35}S-labeled apoB was immunoprecipitated from cell homogenates and incubation media and separated by 5–10% linear gradient SDS-PAGE. At each developmental period the same amount of total protein radioactivity (3 × 10^6 cpm cell proteins and 4.5 × 10^6 cpm medium proteins) was immunoprecipitated with polyclonal anti-chick apoB IgG. Lane A shows the nonspecific immunoprecipitation. The fluorograms are representative of three separate experiments.

percentage of total protein radioactivity incorporated into cell apoB remained stable, whereas that incorporated into medium apoB increased 2-fold as compared to day 7. The total apoB production (cell + medium ^{35}S-labeled apoB normalized for total protein production) (Table 4, right column) decreased from day −3 to days 0–2, increased at day 4, and decreased afterwards.

[^{35}S]methionine incorporation into cell and medium apoB during short-term incubations

In order to determine to what extent the variations of apoB production observed in the steady-state incubations (Tables 3 and 4) reflected variations in apoB synthesis, the initial synthetic rate of apoB was investigated in short-term incubations. Liver slices were pulsed for 20 min with

TABLE 4. Per cent of total protein radioactivity incorporated into liver apoB in developing chick

Age	Cell	Medium	Total Production
days			
−3	0.31 ± 0.03^{a,b}	0.95 ± 0.13^a	0.490 ± 0.07^a
0	0.23 ± 0.06^{b,c}	0.51 ± 0.07^c	0.295 ± 0.08^b
2	0.20 ± 0.01^c	0.62 ± 0.08^{b,c}	0.297 ± 0.01^b
4	0.34 ± 0.02^a	1.23 ± 0.09^d	0.547 ± 0.03^a
7	0.24 ± 0.02^{b,c}	0.43 ± 0.02^c	0.278 ± 0.02^b
10	0.19 ± 0.01^c	0.82 ± 0.05^{a,b}	0.341 ± 0.01^b

Liver slices were incubated for 4 h (steady-state incubations) in the presence of [^{35}S]methionine. ^{35}S-labeled apoB was immunoprecipitated from cell homogenates and incubation media and separated by 5–10% linear gradient SDS-PAGE. ApoB bands were excised from the gel and the amount of incorporated label was determined by scintillation counting. The radioactivity incorporated into cell and medium apoB is given as the percentage of total ^{35}S-labeled cell and medium proteins respectively (Table 3). Total apoB production = percentage of total protein radioactivity incorporated into cell plus medium apoB. Each value represents the mean ± standard deviation from three separate experiments. Day 0, time of hatching. Values that differ significantly ($P < 0.05$) are denoted by different letters.

TABLE 5. Initial synthetic rate of apoB and apoA-I in the liver of developing chick

Age	ApoB	ApoA-I
days		
−3	0.963 ± 0.08[a]	0.298 ± 0.01[a]
0	0.619 ± 0.09[b,c]	0.464 ± 0.11[a,b,c]
2	0.529 ± 0.14[b,c]	0.390 ± 0.08[a,b]
4	0.643 ± 0.12[b,c]	0.654 ± 0.04[d]
7	0.693 ± 0.04[b]	0.545 ± 0.02[b,c,d]
10	0.396 ± 0.03[c]	0.593 ± 0.04[c,d]

The initial synthetic rate of apoB and apoA-I was measured in liver slices incubated for 20 min in the presence of [^{35}S]methionine/[^{35}S]cysteine. ^{35}S-labeled apoB and apoA-I were immunoprecipitated from cell homogenates and separated by SDS-PAGE (see Methods for details). ApoB and apoA-I bands were excised from the gel and the amount of incorporated label was determined by scintillation counting. The radioactivity incorporated into apoB and apoA-I is given as the percentage of total ^{35}S-labeled cell proteins. Each value represents the mean ± standard deviation from three separate experiments. Day 0, time of hatching. Values that differ significantly ($P < 0.05$) are denoted by different letters.

[^{35}S]methionine/[^{35}S]cysteine, and cell and medium apoB and apoA-I were measured by immunoprecipitation (see Methods). As radioactive apoB and apoA-I were not detected in the medium at the end of the pulse period (data not shown), the initial rates of synthesis could therefore be reliably estimated from the amount of ^{35}S-labeled amino acids incorporated into cell apoB and apoA-I (32, 35, 36, 54). The percentage of total protein radioactivity incorporated into apoB decreased from day −3 to day 0, remained stable from day 0 up to day 7 (the increase observed at days 4–7 did not reach the level of significance) and decreased at day 10 (**Table 5**). Thus these results indicate that the variations of the initial synthetic rate of apoB are only in part superimposable to the changes of the apoB production observed in the steady state incubations (Table 4). The initial synthetic rate of apoA-I, measured in the same liver slices, increased from days −3/2 to day 4 and remained stable afterwards (Table 5).

Northern blot analysis

Figure 3 shows that there were substantial variations of apoB mRNA level from day −3 to day 10. To ascertain whether these variations were due to differences in RNA loading of the agarose gels, membranes were reprobed with ^{32}P-labeled β-actin cDNA. As the β-actin mRNA content was found to vary during development (Fig. 3 and

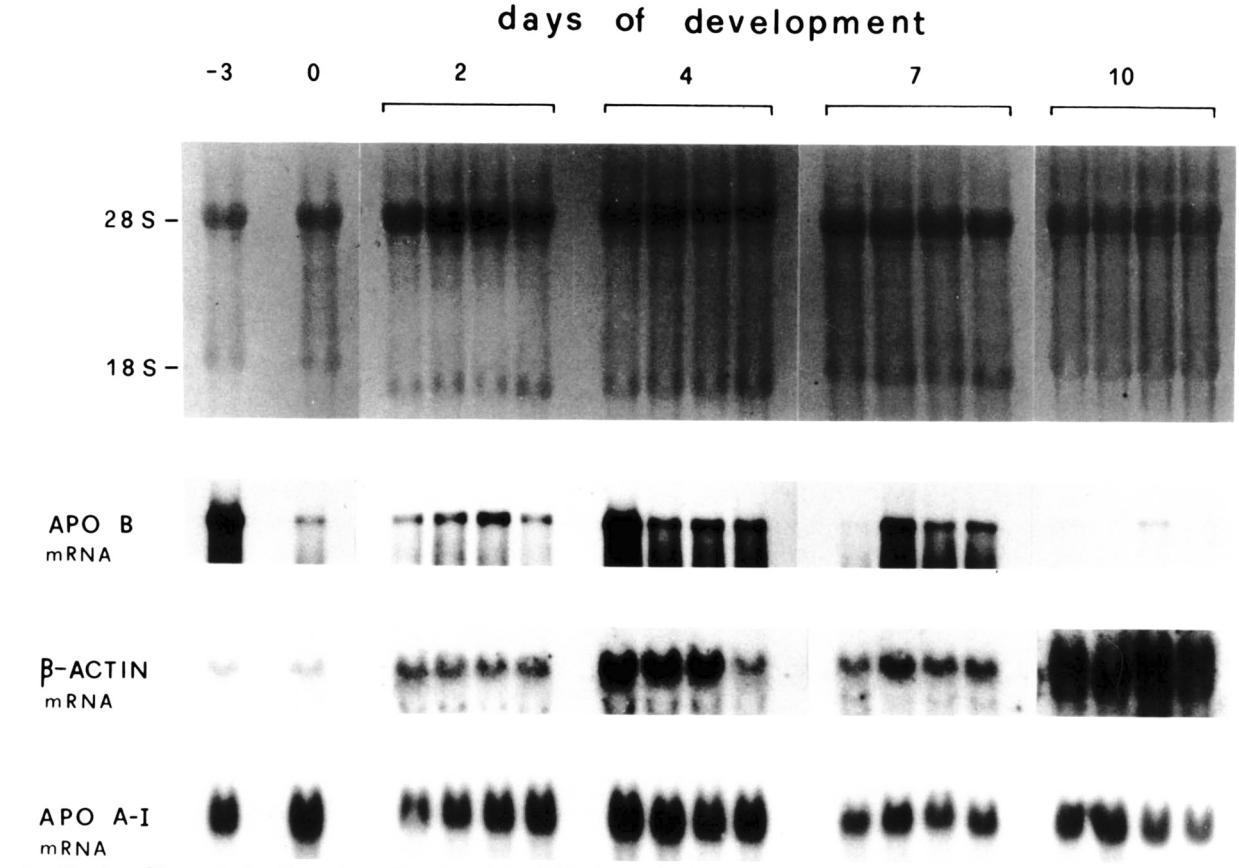

Fig. 3. Northern blot analysis of hepatic apoB and apoA-I mRNA in developing chick. Total RNA was extracted from chick liver at days −3, 0, 2, 4, 7, and 10 and applied to 1% agarose gel. At days −3 and 0, RNA was extracted from pools of livers taken from 10 animals; at days 2, 4, 7, and 10 each lane corresponds to RNA extracted from livers of single animals. RNA was hybridized with chick apoB, apoA-I, and β-actin ^{32}P-labeled cDNAs. Ethidium bromide-stained gels prior to transfer are shown in the upper panel.

TABLE 6. Hepatic apoB and apoA-I mRNA levels in developing chick

Age	ApoB	ApoA-I	β-Actin
days			
−3	14.56	7.97	0.91
0	1.92	9.45	1.16
2	2.48 ± 1.27[a,b]	6.88 ± 0.84[a]	2.74 ± 0.17[a]
4	8.82 ± 5.10[b]	9.41 ± 1.16[b]	6.13 ± 2.29[b]
7	6.98 ± 5.28[a,b]	6.52 ± 0.84[a]	2.99 ± 0.66[a]
10	0.39 ± 0.22[a]	7.43 ± 1.69[a,b]	4.85 ± 1.47[a,b]

Densitometric analysis of Northern blots of apoB, apoA-I, and β-actin mRNAs shown in Fig. 3. Data are expressed in arbitrary units. At days −3 and 0, RNA was extracted from pools of livers taken from 10 animals; at days 2, 4, 7, and 10, RNA was extracted from livers of single animals (n = 4). Values at days 2, 4, 7, and 10 represent the mean ± standard deviation; values that differ significantly ($P < 0.05$) are denoted by different letters.

Table 6) we performed the densitometric scanning of 28S ribosomal RNA on photographs of ethidium bromide-stained gels. As the densitometric signals of 28S ribosomal RNA were found to be the same in all samples, we concluded that differences in RNA loading could not account for the variations of apoB and apoA-I mRNAs. ApoB mRNA decreased approximately 7-fold from day −3 to day 0 but did not change significantly afterwards, even though the mean apoB mRNA level found at days 4–7 was approximately 4-fold higher than that found at day 0 or at day 10 (Table 6).

The level of apoA-I mRNA increased from day 0 to day 4, and decreased slightly at days 7–10 (Table 6).

Isolation of ^{35}S-labeled medium lipoproteins

^{35}S-labeled lipoproteins secreted by liver slices during steady-state incubations were separated by density gradient ultracentrifugation of the media taken at days 0, 4, and 7 (**Fig. 4**). The radioactivity incorporated into medium lipoproteins (fractions 1–19 of the density gradient shown in Fig. 4) was 21.5, 57.4, and 32.2 × 10^5 cpm/g of liver at days 0, 4, and 7, respectively. When these results were normalized for the total protein radioactivity of the media (Table 3), the percentage of total protein

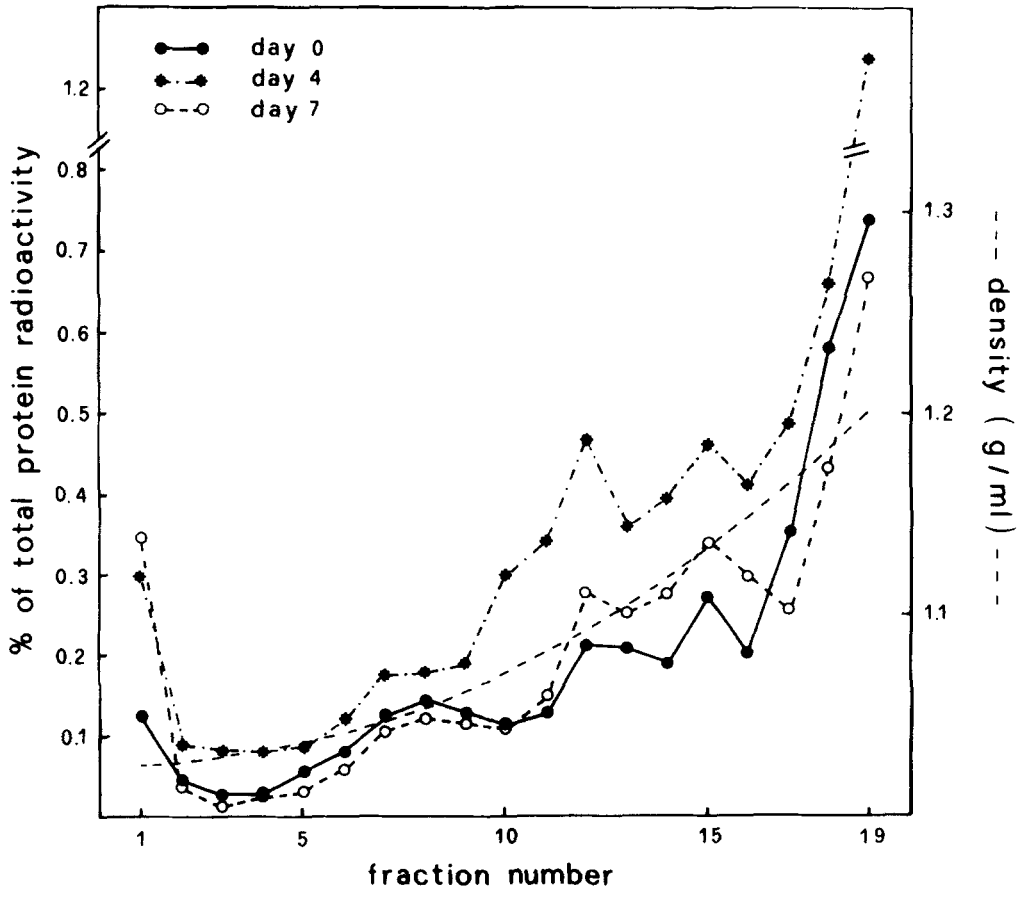

Fig. 4. Density profile of ^{35}S-labeled lipoproteins secreted into the medium by liver slices of developing chick. Liver slices taken at days 0, 4, and 7 were incubated in the presence of [^{35}S]methionine for 4 h (steady-state incubations) and pooled media from three incubations were subjected to density gradient ultracentrifugation. VLDL + LDL (fractions 1–9); HDL (fractions 10–19). The radioactivity of each fraction was measured after trichloroacetic acid precipitation. Values were adjusted for total protein radioactivity of the medium.

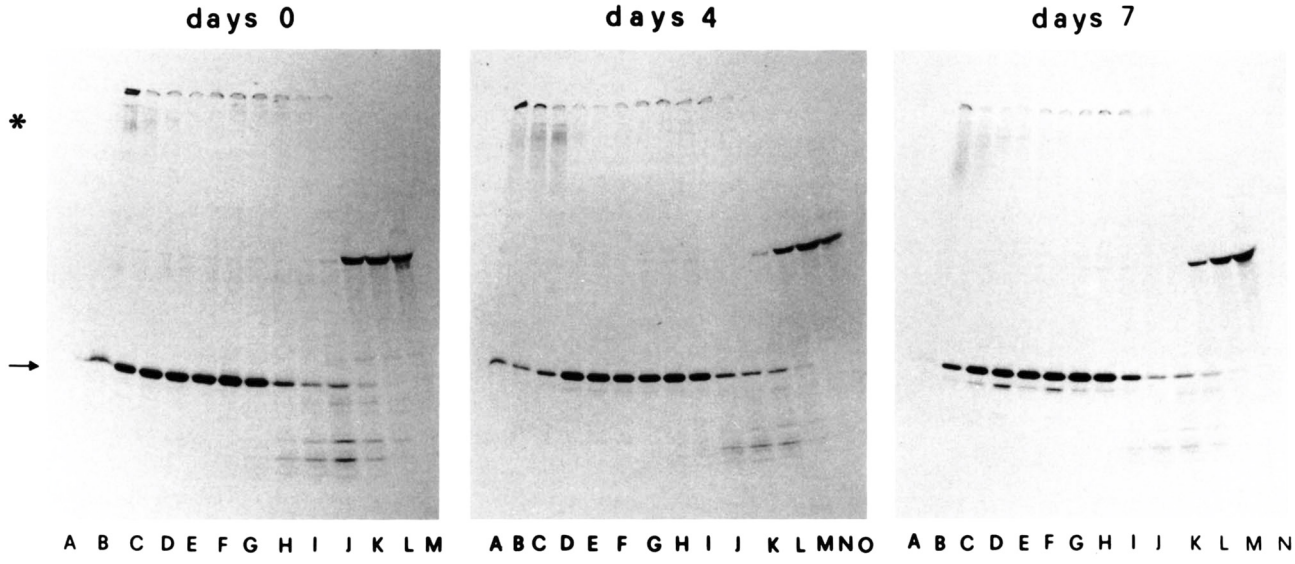

Fig. 5. Fluorograms of ^{35}S-labeled apolipoproteins of lipoprotein fractions isolated from the incubation media. ^{35}S-labeled lipoproteins separated from the incubation media by density gradient ultracentrifugation (as shown in Fig. 4) were analyzed on a 5–20% linear gradient SDS-PAGE. Approximately 50 × 10^3 cpm were applied on each lane. At day 0: lane A, density fractions (dfs) 1–9; lane B, dfs 10–11; lanes C–M, dfs 12–22. At day 4: lane A, df 1; lane B, dfs 2–5; lane C, dfs 6–9; lane D, dfs 10–11; lanes E–O, dfs 12–22. At day 7: lane A, dfs 1–5; lane B, dfs 6–9; lane C, dfs 10–11; lanes D–N, dfs 12–22. ApoB and apoA-I are indicated by a star and an arrow, respectively.

radioactivity found in medium lipoproteins was 3.76, 6.54, and 3.90 at days 0, 4, and 7, respectively (Fig. 4). More specifically, at day 4 the radioactivity found in apoB containing lipoproteins (density fractions 1–9) was 1.7- and 1.5-fold that observed at days 0 and 7, respectively. ^{35}S-labeled apoB was found predominantly in fractions of density <1.065 g/ml (fractions 1–9 of Fig. 4) (**Fig. 5**). Figure 4 also shows that there were differences in the radioactivity incorporated into HDL (fractions 10–19 of the density gradient; density range 1.075–1.220 g/ml). At day 4, HDL radioactivity was 1.7-fold that found at days 0 and 7.

The apolipoprotein analysis of labeled medium lipoproteins (Fig. 5) shows that radioactive apoA-I was present in all density fractions. In view of this finding and the fact that apoA-I is the main apolipoprotein of medium HDL, the results shown in Fig. 4 are consistent with the observation that the hepatic production of apoA-I was increased at day 4 post-hatching (Table 5).

[^{3}H]oleic acid incorporation into hepatic cholesteryl esters

To determine whether the accumulation and depletion of hepatic cholesteryl esters observed after hatching (Table 1) were associated with changes in the hepatic production of this lipid class, the incorporation of [^{3}H]oleic acid into cholesteryl esters (CE) was measured in liver slices taken at days 0, 4, and 7. At these stages of development the hepatic pool size of free fatty acids was the same (15.09 ± 1.15, 13.48 ± 1.84, and 15.99 ± 0.90 mmol/g of liver at days 0, 4, and 7, respectively), whereas the concentration of free oleic acid was 3.36 ± 0.25, 3.12 ± 0.42, and 4.60 ± 0.26 mmol/g of liver at days 0, 4, and 7, respectively. The value at day 7 was significantly higher ($P < 0.05$) than that found at days 0 and 4. Taking into account the oleic acid pool size of liver slices, we calculated the amount of CE synthesized by liver slices and secreted into medium lipoproteins (**Table 7**). At days 4 and 7 the amount of CE synthesized by liver slices was

TABLE 7. Incorporation of [^{3}H]oleic acid into cholesteryl esters

	Days of Development		
	0	4	7
Hepatic ^{3}H-CE dpm × 10^6/g/h	15.50 (86.5)	9.55 (50.78)	3.66 (28.67)
^{3}H-CE VLDL-LDL dpm × 10^3/g liver	147.38 (0.82)	94.46 (0.50)	17.49 (0.13)
^{3}H-CE HDL dpm × 10^3/g liver	235.72 (1.31)	207.33 (1.10)	64.28 (0.50)

Liver slices taken at days 0, 4, and 7 were pulse-labeled with [^{3}H]oleic acid-albumin complex for 1 h and chased for 3 h. At the end of the pulse period, hepatic ^{3}H-labeled cholesteryl esters (CE) were measured in liver slice homogenates (see Methods). At the end of the chase period the medium was subjected to ultracentrifugation to isolate labeled VLDL-LDL (d < 1.065 g/ml) and labeled HDL (d 1.065–1.210 g/ml). The content of ^{3}H-labeled CE in lipoprotein fractions was measured. Data represent the average from duplicate experiments. Numbers in parentheses indicate the amount of CE (nmol/g liver) synthesized in the pulse period and secreted in lipoproteins during the chase period (see Methods).

58.7% and 33.1%, respectively, of that found at day 0 (Table 7). To investigate to what extent labeled CE formed in the liver were incorporated into newly synthesized lipoproteins, liver slices were pulse-labeled with [^{3}H]oleic acid for 1 h and then chased for 3 h. At the end of the chase period, medium ^{3}H-labeled VLDL-LDL and ^{3}H-labeled HDL were isolated by ultracentrifugation. The percentage of hepatic CE (i.e., CE synthesized during the pulse period) found in total medium lipoproteins (VLDL-LDL + HDL) was 2.46%, 3.15%, and 2.19% at days 0, 4, and 7, respectively. The percentage of lipoprotein CE incorporated into VLDL-LDL was 38.5%, 31.2%, and 21.5% at days 0, 4, and 7, respectively.

Plasma estradiol

To ascertain whether the changes of apoB production observed in developing chick were associated with changes in plasma estrogen levels, plasma estradiol was measured at each time point. Plasma estradiol level decreased from day −3 to day 0 and remained stable up to day 10 (**Table 8**).

DISCUSSION

In the present study we confirmed that the enormous hepatic cholesteryl ester store present in late chick embryo increased at the time of hatching (days 0–2) but decreased sharply within a few days (days 2–7) of post-natal life (39). We also demonstrated that, at the time of hatching, part of this large amount of lipids was contained in cytoplasmic lipid droplets, where cholesteryl esters represented more than 90% of total lipid content. Cholesteryl esters were replaced by triacylglycerols after the first week of life (55).

It has been suggested that the hepatic accumulation of cholesteryl esters found in late embryo and around hatching derives from the uptake of remnants of cholesterol-rich lipoproteins secreted from yolk sac membrane as well as the inefficiency of the embryonic liver in disposing of this excess material (37, 38, 56). Our assumption was that the depletion of hepatic cholesteryl esters that occurs post-natally was accomplished (at least in part) via the incorporation of these lipids into apoB-containing lipoproteins. This hypothesis was tested by measuring the hepatic production of apoB and the secretion of apoB-containing lipoproteins (i.e., incorporation of [^{35}S]methionine into apoB during steady state incubations). The hepatic apoB production decreased from the embryo to the time of hatching, increased at day 4, and decreased afterwards (Table 4 and **Fig. 6**). In keeping with this finding, the amount of radioactive apoB-containing lipoproteins secreted into the medium was higher at day 4 than at days 0 and 7. Furthermore, VLDL, IDL, and LDL secreted by liver slices at day 4 contained 30% of the hepatic cholesteryl esters incorporated into total medium lipoproteins. As illustrated in Fig. 6, the hepatic CE content increases as the apoB production decreases (from day −3 to days 0–2) and, vice versa, it falls as the hepatic production of apoB increases (day 4). These findings are consistent with the idea that the availability of apoB plays an important role in the regulation of the hepatic content of cholesteryl esters during the early post-natal life of the chick. In this context the overproduction of apoB-containing lipoproteins by the liver would provide a vehicle for the rapid delivering of cholesterol to all peripheral tissues that require a large amount of cholesterol for growth.

Figure 6 also indicates that the depletion of cholesteryl esters (days 2–7) coincides with an increased production of apoA-I and an increased secretion of apoA-I-containing lipoproteins (Fig. 4). As newly secreted HDL (where apoA-I represents the main constituent peptide) were found to be the main carriers of cholesteryl esters secreted by liver slices (Table 7) our results indicate that the overproduction of apoA-I-containing lipoproteins by the liver plays a major role in the depletion of hepatic cholesteryl esters. Newly synthesized apoA-I was found to be present not only in HDL but also in VLDL, IDL, and LDL secreted into the medium (Fig. 5). As we have previously shown that in the plasma of newborn chicks apoB and apoA-I are carried on different VLDL-LDL particles (39), it is likely that VLDL-LDL particles containing apoA-I but no apoB are secreted directly by the liver.

It should be pointed out, however, that other mechanisms may be involved in the depletion of hepatic cholesteryl esters. A substantial amount of cholesteryl esters similar in fatty acid composition to those of the liver is found in the bile of late embryo, indicating that the bile may serve as a means to control the cholesterol accumulation in the liver (37, 38). On the other hand, during the first week of post-natal life, the uptake of cholesterol-rich lipoproteins produced by the yolk sac membrane decreases substantially as a consequence of the progressive atrophy of the yolk sac that takes place during that time (37).

TABLE 8. Plasma levels of estradiol in developing chick

Age	Plasma Estradiol
days	*pg/ml*
−3	33.75 ± 5.98^a
0	17.75 ± 3.57^b
2	23.50 ± 1.73^b
4	25.80 ± 7.30^{a,b}
7	19.25 ± 3.79^b
10	22.50 ± 1.91^b

Each value represents the mean ± standard deviation for data obtained from five animals at each developmental period. Day 0, time of hatching. Values that differ significantly ($P < 0.05$) are denoted by different letters.

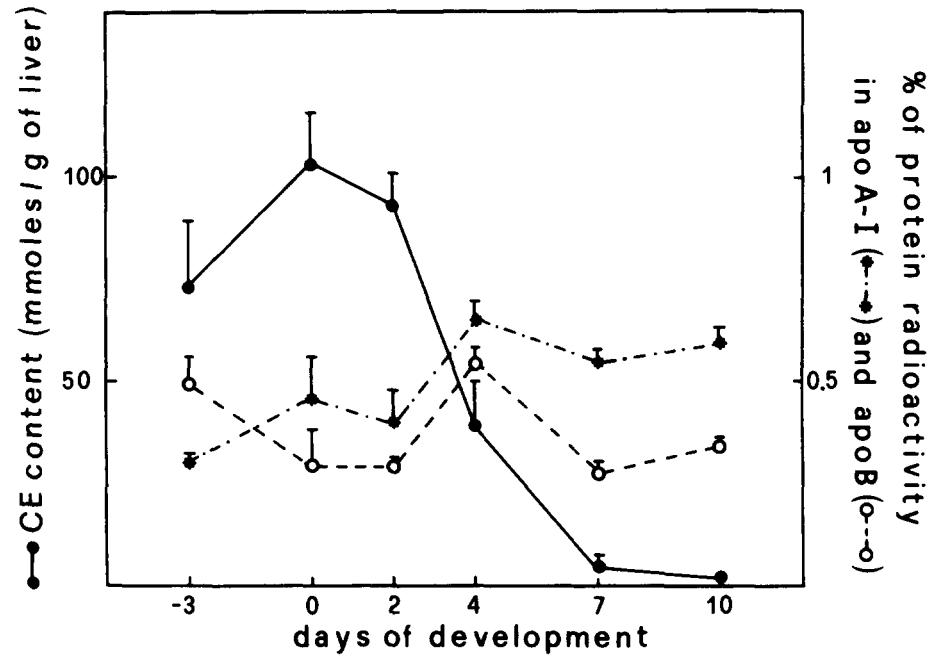

Fig. 6. Time-dependent changes of cholesteryl ester content, apoB production, and apoA-I synthesis in livers of developing chicks. The figure combines the results shown in Tables 1 (hepatic cholesteryl esters), 4 (total apoB production), and 5 (apoA-I synthesis).

In order to determine whether the variations of apoB production were due to changes in apoB synthesis, we measured the initial synthetic rate of apoB (20-min pulse with ^{35}S-labeled amino acids) and the hepatic level of apoB mRNA. Broadly speaking, these two parameters followed the same trend, i.e., a marked decrease from day −3 to days 0–2, followed by the tendency to increase at days 4–7 and to decrease at day 10 (see Tables 5 and 6). While these results clearly indicate that the decrease of apoB production (synthesis + secretion) observed in the steady-state incubations from day −3 to days 0–2 reflects a reduction in apoB synthesis, they do not provide a satisfactory explanation for the changes of apoB production observed in steady-state incubations at days 4–10 (Table 4). In fact, the increased or decreased apoB synthetic rate observed at days 4 and 10, respectively, did not reach the level of significance. This implies that the variations of apoB production observed post-natally (days 4–10) in steady-state incubations were mostly affected by post-translational processes, such as the intracellular degradation of apoB, the rate of assembly of apoB-containing lipoproteins, as well as their transit through the secretory pathway (6, 24, 36, 54, 57).

The mechanism(s) responsible for the decrease of apoB synthetic rate and apoB mRNA we observed from late embryonic to early post-natal period is unclear at present. In this study we considered the possibility that hepatic apoB synthesis might be influenced by the level of plasma estrogens, in view of previous observations indicating that, in the chick, hepatic apoB synthesis (58, 59) and hepatic apoB mRNA increase after the administration of estrogens (52). We found that the plasma estrogen level decreased from day −3 to day 0 and remained stable afterwards as did apoB mRNA and apoB synthetic rate. This finding raises the possibility that high levels of plasma estrogens could maintain the high rate of expression of the apoB gene observed in the liver of the late embryo and the fall of plasma estrogens, which occurs at the time of hatching, might account for the reduced hepatic content of apoB mRNA and apoB synthetic rate observed post-natally. This hypothesis, however, is in contrast with previous reports indicating that the administration of tamoxifen to 10- to 18-day embryos (58) or adult rooster (59) did not influence the basal level of hepatic apoB synthesis. Although the latter observations make our hypothesis less likely, we feel that the role of estrogens on apoB synthesis in developing chicks requires further studies. The incubation of hepatocytes from late embryo or newborn chick in the presence of estrogen levels comparable to those found in the plasma of these animals would provide the appropriate experimental setting to test our hypothesis.

In conclusion, we have shown that the changes in hepatic cholesteryl ester content observed during the late embryonic and early post-natal life of the chick are closely related to the changes in the hepatic production of apoB- and apoA-I-containing lipoproteins. We specifically demonstrated that the rapid and massive depletion of hepatic cholesteryl ester store that occurs around day 4 is facilitated by the increased secretion of a large spectrum

of lipoproteins containing either B, B/A-I, or A-I apolipoproteins as the major protein constituent(s). Although this study underlines the association between cholesteryl ester content and apoB and apoA-I production in the liver of newborn chicks, it does not clarify the mechanisms responsible for the effect (if any) of this lipid class on the transcription of apoB and apoA-I genes, the translation of the corresponding mRNAs, or the post-translational processes of the newly synthesized apolipoproteins. We reckon that the transient cholesteryl esters accumulation in the liver of newborn chick provides a new tool to investigate how preformed lipids stored in the cytoplasm may influence apolipoprotein synthesis as well as the assembly and secretion of nascent lipoproteins.

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