Absence of apolipoprotein B-48 in the chick, *Gallus domesticus*

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Abstract This study was designed to investigate: *a*) whether multiple forms of apoB are present in chick plasma lipoproteins; and *b*) which forms of apoB are produced in vitro by liver and intestine at various stages of pre- and post-natal development. Plasma lipoproteins of $d < 1.019$ g/ml, isolated from fasted and nonfasted chicks, contained exclusively the high molecular weight apoB form (apoB-100) that comigrated with human and rat apoB-100 on SDS-PAGE gel. No apoB-48 was detected either in overloaded Coomassie blue-stained gels or after immunoblotting. ApoB-100 but no apoB-48 was found in portomicrons, the triglyceride-rich lipoproteins equivalent to chylomicrons, that in the chick are transported via the porto-mesenteric venous system. To ascertain whether a minute amount of apoB-48 was present in chick plasma, [^{35}S]methionine was injected intraduodenally and the ^{35}S-labeled $d < 1.019$ g/ml plasma lipoproteins were isolated 45 min later from the systemic and the porto-mesenteric circulation. Only apoB-100 was found to be labeled in these lipoproteins. Cholesterol feeding did not induce the appearance of apoB-48 in plasma despite a marked accumulation of cholesterol-rich $d < 1.040$ g/ml lipoproteins in the plasma. In vitro synthesis of apoB forms was studied in liver and intestinal slices isolated from chick embryos (8 and 5 days before hatching), newly hatched chicks (2 and 7 days after hatching), and young chicks (21 days old) that were incubated in the presence of [^{35}S]methionine. At each stage of development, liver slices secreted predominantly apoB-100. Intestinal slices of newly hatched and young chicks secreted two forms of apoB: apoB-100 and an additional form with an electrophoretic mobility similar to rat plasma apoB-95. No apoB-48 was synthesized or secreted by the intestine. Our results indicate that the absence of apoB-48 in chick plasma reflects the lack of synthesis of this peptide in the intestine. It is conceivable that in chick intestine the recently described molecular mechanism responsible for the co/posttranscriptional modification of apoB mRNA leading to the formation of apoB-48 is lacking or defective. —**Tarugi, P., L. Albertazzi, S. Nicolini, and S. Calandra.** Absence of apolipoprotein B-48 in the chick, *Gallus domesticus*. *J. Lipid Res.* 1990. **31:** 417–427.

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In humans and several mammalian species, apolipoprotein B (apoB) is a main protein constituent of chylomicrons, very low density lipoproteins (VLDL), and low density lipoproteins (LDL) (1). It is present in plasma primarily in two forms (1–6) designated apoB-100 and apoB-48 according to a centile nomenclature system proposed by Kane (3). Another minor apoB form designated apoB-95 has been documented in plasma of some mammalian species such as the rat and mouse (4, 6). In humans, apoB-100 that has a molecular mass of approximately 550,000 daltons in SDS-PAGE is found in plasma VLDL and LDL, whereas apoB-48 that has a molecular mass of approximately 250,000 daltons is found in intestinal lymph chylomicrons and in plasma chylomicron remnants (2, 3). It is generally accepted that in humans apoB-100 and apoB-48 are secreted by liver and intestine, respectively (3). There are remarkable species differences in the site of synthesis of the two major apoB forms. In the rat, for example, liver synthesizes apoB-100 and apoB-48, while intestine synthesizes only apoB-48 (7–9). ApoB-100 and apoB-48 have different biological functions: apoB-100 plays a central role in cholesterol and LDL metabolism by serving as ligand for the LDL receptors (1); apoB-48 plays a major role in the transport of dietary triglycerides (3).

The complete amino acid sequence of human apoB-100 and apoB-48 has been recently elucidated (10–17). Little is known about the presence of multiple apoB forms in nonmammalian species. A study conducted before the publication of the classical papers on the heterogeneity of mammalian apoB (2, 4, 5) indicated that, in estrogen-treated chicks, plasma apoB consisted of a single peptide with a molecular weight of 350,000 on SDS-PAGE gels (18). More recent reports suggest that plasma VLDL and LDL isolated from fasting chicks, pigeons, and geese contain only a high molecular weight

Abbreviations: VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; apoB, apolipoprotein B; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; MW, molecular weight; EDTA, ethylenediaminetetraacetic acid; TTBS, Tris-Tween-buffered saline.

apoB (19–21). It has also been consistently reported that chick hepatocytes secrete, in vitro, a single form of apoB resembling the high molecular weight apoB isolated from plasma VLDL (22, 23). There is only one report that indicates that chick intestine synthesizes in vitro an apoB form with a molecular weight of approximately 350,000 (24).

The main aim of this study was to verify the heterogeneity of plasma apoB in the chick, by addressing the following questions: *a*) whether VLDL and LDL isolated from plasma of fasting and nonfasting chicks contains multiple forms of apoB; *b*) whether cholesterol feeding is accompanied by an accumulation in plasma of apoB-48 as has been found to occur in cholesterol-fed rats (5); and *c*) which forms of apoB are synthesized by liver and intestine, respectively, during late embryonic and early postnatal development (25).

MATERIALS AND METHODS

Materials

[^{35}S]Methionine (50 TBq/mmol), [9, 10 (n)-^{3}H]palmitic acid (2 TBq/mmol), and Hyperfilm-MP X-ray films were obtained from Amersham (England); silica gel G plates for thin-layer chromatography were obtained from Merck (Darmstadt, West Germany); Zeta Probe membranes, colloidal gold conjugates Immun-Blot assay kit, and High Molecular Weight Protein Standard were purchased from Bio-Rad (Richmond, CA); polybrene, leupeptin, aprotinin, soybean trypsin inhibitor, lima bean trypsin inhibitor, and glutathione were obtained from Sigma (St. Louis, MO). Trasylol (aprotinin) was purchased from Bayer (West Germany). Ketalar (ketamine) was obtained from Parke-Davis (Italy); bovine thrombin (Topostatin) was from Roche (Italy). Trypsin was purchased from Millipore (Bedford, MA).

Animals and diets

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 (22.7% soy proteins, 3.5% lipid, and 4.5% fiber) ad libitum until the time of killing. This standard diet contained 0.045% (w/w) total sterols and 0.005% (w/w) cholesterol. In some experiments 7-day-old chicks were fed the standard diet supplemented with 2% (w/w) cholesterol for 2 weeks (26). Chicks were killed at the following stages of development: 8 and 5 days before hatching and 2, 7, and 21 days after hatching.

Blood and tissue samples

Overnight fasted and nonfasted chicks were anesthetized by peritoneal injection of Ketalar (5 mg/100 g body weight); systemic and porto-mesenteric blood samples were withdrawn by puncture of the left ventricle and the jejunal vein, respectively (27). K_3 EDTA was used as anticoagulant. Individual plasmas were separated by low speed centrifugation. Protease inhibitors, aprotinin (100 kallikrein inhibitor units/ml), leupeptin (0.1 mM), and a chelator of metal cations (1 mM Na_2EDTA), were then added to each plasma sample. All procedures were conducted at 4°C. Immediately after exsanguination, the liver and a segment of small intestine (2–10 cm in length according to the age of the animal, cut just below the duodenal loop) were removed and washed in Krebs-Ringer bicarbonate buffer. The intestine was flushed with a cold saline solution and opened longitudinally before being used for in vitro incorporation studies (see below).

Isolation of plasma lipoproteins

Before the separation of plasma lipoproteins, equal aliquots of plasma from either the systemic or the porto-mesenteric circulation of each animal were pooled. Plasma pools were made by using plasmas taken from 5 to 10 animals. In most experiments the density of each plasma pool was raised to 1.019 g/ml with solid KBr and centrifuged in a 50 Ti rotor at 150,000 *g* for 22 h. The same procedure was used to isolate the $d < 1.019$ g/ml lipoproteins from human and rat plasma (28). Isolated lipoproteins were dialyzed against 0.154 M NaCl, 0.1 mM Na_2EDTA, and Trasylol (100 kallikrein inhibitor units/ml) at 4°C. Aprotinin (100 kallikrein inhibitor units/ml) and leupeptin (0.1 mM) were added and the samples were processed immediately for protein and lipid analysis. Isolated chick lipoproteins ($d < 1.019$ g/ml) to be subjected to digestion with proteolytic enzymes (see below) were dialyzed against 0.01 M Tris-HCl, 0.3 mM Na_2EDTA, pH 8, stored at 4°C in the absence of protease inhibitors, and used within a few days. In some experiments lipoproteins were isolated from the plasma pools by density gradient ultracentrifugation in an SW 41 rotor (21, 25). After centrifugation, aliquots of 500 μl (fraction 1) or 400 μl (fractions 2–20) were collected and their total cholesterol and protein concentrations were measured.

Preparation of anti-chick apolipoprotein B antiserum

An aliquot of $d < 1.019$ g/ml plasma lipoproteins (1 mg of protein) was dissolved in sample buffer (100 mM Tris-HCl, pH 7.3, 20% glycerol, 10% SDS, and 5% 2-mercaptoethanol), heated at 100°C for 5 min (29), and loaded onto an SDS-polyacrylamide gel slab (140 mm × 200 mm × 1.5 mm). A linear 5–10% polyacrylamide gradient was used as running gel (30). The portion of the gel containing high molecular weight apoB (see results) was cut off and minced in 0.9% NaCl with a Potter-Elvehjem homogenizer and injected into New Zealand White rabbits (31). Anti-chick apoB IgG was

isolated from rabbit serum according to a standard procedure (32). Anti-chick apoB IgG showed a single precipitation line against whole plasma and plasma VLDL and LDL of the chick (double immunodiffusion). No precipitation line was found with chick HDL. As judged by double immunodiffusion and Western blotting, our anti-chick apoB IgG did not cross-react with rat or human plasma and apoB containing lipoproteins of both species. Anti-rat apoB rabbit IgG (prepared as specified above) and anti-human apoB-100 sheep polyclonal antiserum (a kind gift from Dr. Gabelli, University of Padua) did not cross-react with whole serum or apoB-containing lipoproteins of the chick.

Enzymatic digestion of chick apolipoprotein B

Plasma $d < 1.019$ g/ml lipoproteins (40–60 μg of protein) dialyzed as specified above were digested with either 5 μg of trypsin or 6 units of thrombin at 37°C for 48 h; proteolytic fragments were dissolved in sample buffer (see above), heated at 100°C for 5 min, and separated by electrophoresis on a linear 5–10% polyacrylamide gradient gel in the presence of SDS (29). For each sample, duplicate gels were run; one gel was stained with Coomassie Blue R-250, the other was used for immunoblotting.

Immunoblotting

Apolipoproteins of the $d < 1.019$ g/ml chick plasma lipoproteins, as well as proteolytic fragments of apoB, were separated by SDS-polyacrylamide gradient gel electrophoresis (as specified above) and then electrotransferred from the gels to Zeta Probe membranes according to the manufacturer's instructions (Bio-Rad). The membranes were then incubated with anti-chick apoB rabbit IgG in TTBS buffer (20 mM Tris, 500 mM NaCl, 0.05% Tween-20, pH 7.5), and 1% gelatin, for 2 h at room temperature. After several washes in TTBS, the membranes were incubated with the goat anti-rabbit IgG gold conjugate solution according to the manufacturer's instructions.

In vivo labeling of plasma apolipoprotein B

Nonfasted chicks (21 days old) maintained on either the standard or the cholesterol-rich diet were used. The chicks were lightly anesthetized with Ketalar and an oblique 3-cm incision was made in the right abdominal wall. [^{35}S]methionine (300 μCi) dissolved in 1.2 ml of 0.9% NaCl was injected into the exposed duodenum and the incision was sutured. After 45 min the abdominal wall was reopened and the intestine was exposed (33). Porto-mesenteric blood was withdrawn by puncturing the jejunal vein and, immediately after, systemic blood was withdrawn from the left ventricle (27). ^{35}S-Labeled $d < 1.019$ g/ml lipoproteins were isolated from pooled plasmas as previously described. ^{35}S-Labeled apoB in whole plasma was immunoprecipitated by anti-chick apoB rabbit IgG (see below) whereas apoB present in the ^{35}S-labeled $d < 1.019$ g/ml plasma lipoproteins was analyzed directly by SDS-PAGE (see below).

Synthesis of apolipoprotein B in vitro

Chicks were killed 8 and 5 days before hatching and 2, 7, and 21 days after hatching. Eight to ten chick embryos, four to six nonfasted newborn chicks, and three nonfasted young chicks (21 days old) were used. The right lobe of the livers and the intestinal segments isolated from embryos and newborn chicks were washed as previously specified, cut into slices, and pooled before the in vitro incubation experiments. Liver and intestine isolated from 21-day-old chicks were processed as specified above, but incubated separately. Tissue slices (200–300 mg wet weight) were incubated for 4 h at 37°C in 3.5 ml of Krebs-Ringer bicarbonate buffer containing 50 μCi of [^{35}S]methionine, 50 units/ml of penicillin, 50 μg/ml of streptomycin, 100 kallikrein inhibitor units/ml of aprotinin under an atmosphere of 95% O_2–5% CO_2. At the end of the incubation, media were collected and supplemented with leupeptin (0.1 mM), polybrene (25 μg/ml), Na_2EDTA (0.1 mM), lima bean trypsin inhibitor (20 μg/ml), soybean trypsin inhibitor (20 μg/ml), and glutathione (0.02%). This material was exhaustively dialyzed against 10 mM NH_4HCO_3, 1 mM Na_2EDTA, and Trasylol (100 kallikrein inhibitor units/ml), and then lyophilized. Tissue slices were washed with 5 ml of ice-cold Krebs-Ringer bicarbonate buffer and then homogenized in 1 ml of 100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM Na_2EDTA, and 0.5% Nonidet-P40. This material was centrifuged at 12,000 *g* at 4°C for 30 min; the 12,000 *g* supernatant was collected and stored at −20°C. Incubation media and 12,000 *g* tissue homogenate supernatants were immunoprecipitated by either anti-chick apoB rabbit IgG or nonimmune IgG (34). The immuno-complexes were dissolved in sample buffer (see above) and applied to a linear 5–10% gradient SDS-polyacrylamide gel. Gels were stained with Coomassie Blue R-250, destained, processed for fluorography, and exposed to Hyperfilm-MP X-ray films at −80°C (34).

Isolation of apoB-containing lipoproteins secreted in vitro

In some experiments, incubation media of three samples of liver and intestinal slices incubated with [^{35}S]methionine (see above for details) were collected and pooled. After the addition of 300 μl of fresh chick plasma as a carrier, the incubation media were ultracentrifuged to separate ^{35}S-labeled lipoproteins of density less than 1.019 g/ml. The latter were exhaustively dialyzed against 10 mM NH_4HCO_3, 1 mM Na_2EDTA, and Trasylol (100 kallikrein inhibitor units/ml) and then lyophilized. This material (corresponding to 30–60 × 10^4

cpm) was dissolved in sample buffer and separated by SDS-polyacrylamide gel electrophoresis as specified above. Gels were stained with Coomassie Blue R-250, destained, processed for fluorography, and exposed to Hyperfilm-MP X-ray films at −80°C (34).

In vivo labeling of plasma triglycerides

In order to label triglycerides secreted by the intestine, 21-day-old nonfasted chicks were anesthetized as previously described. An oblique 3-cm incision was made in the right abdominal wall. [^{3}H]Palmitic acid (350 μCi) dissolved in 1.2 ml of 0.9% NaCl containing 0.02 M Na-taurocholate was injected into the exposed duodenum and the incision was sutured. After 45 min the abdominal wall was reopened, the intestine was exposed, and blood was collected from the porto-mesenteric vein and the systemic circulation as previously described.

Chemical methods

Proteins were measured according to the method of Lowry et al. (35). Total plasma cholesterol was measured colorimetrically (28). In some experiments aliquots of the $d < 1.019$ g/ml lipoproteins (corresponding to 100 μg of protein) were extracted with chloroform–methanol 2:1 (v/v) (36). The major lipid classes were separated by thin-layer chromatography and measured colorimetrically (37). ^{3}H-Labeled plasma and/or ^{3}H-labeled lipoproteins isolated from animals that had received an intraduodenal infusion of [^{3}H]palmitic acid, were extracted with chloroform–methanol 2:1 (v/v) (37). The major lipid classes (phospholipids, triglycerides, fatty acids, and cholesteryl esters) were separated by thin-layer chromatography (37). The areas corresponding to the lipid classes were scraped and counted in a liquid scintillation counter.

Statistical analysis

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

RESULTS

Plasma apolipoprotein B in chicks fed a standard diet

Fig. 1A shows that $d < 1.019$ g/ml plasma lipoproteins isolated from the systemic circulation of nonfasted 21-day-old chicks contain only a high molecular weight form of apoB that has the same mobility in SDS-PAGE as human and rat apoB-100. Under strict control of proteolysis, no bands migrating in the position of human apoB-75, apoB-48, or apoB-26 could be detected, even in overloaded gels, under circumstances in which a minute amount of human apoB-48 could be easily detected (Fig. 1B). The presence of a single apoB form in the $d < 1.019$ g/ml lipoproteins isolated from the systemic circulation was a consistent finding at all stages of pre- and postnatal development of the chick (data not shown). A single apoB form was consistently found also in fasting conditions (data not shown). In Western blotting, material immunoreactive to anti-chick apoB rabbit IgG was found exclusively in the area corresponding to apoB-100 (**Fig. 2A, B**). Despite the high sensitivity of the immunodetection method we adopted (see below) no apoB-48 could be visualized on the blotting membrane (Fig. 2A, B). In order to establish whether our anti-chick apoB IgG raised against intact chick apoB-100 reacted with portions of chick apoB-100 (including the one corresponding to the whole apoB-48 or some part of it) we performed immunoblotting analysis of trypsin- and thrombin-generated fragments of chick apoB-100. Fig. 2A shows that all trypsin-generated fragments with a molecular weight higher than 38,000 to 40,000 cross-reacted with anti-chick apoB-100 IgG. Fig. 2A also demonstrates the high sensitivity of our immunodetection method which allowed the identification of fragments that were hardly detectable on the Coomassie-stained gel (see, for example bands a and d of Fig. 2A). Thrombin digestion produced two major high molecular weight fragments that were recognized by our anti-chick apoB-100 IgG (Fig. 2B).

Since it is generally accepted that in the chick dietary lipids absorbed by the intestine are transported mainly via the porto-mesenteric venous system (27, 33, 38), $d < 1.019$ g/ml plasma lipoproteins were isolated directly from the jejunal vein of nonfasted 21-day-old chicks. In preliminary experiments we had found that 45 min after the intraduodenal injection of [^{3}H]palmitic acid, the lipid radioactivity of plasma isolated from the porto-mesenteric system was distributed as follows: 66% in triglycerides, 32% in fatty acids, 1.3% in cholesteryl esters, and 0.5% in phospholipids. The lipid radioactivity of plasma isolated from the systemic circulation of the same animals was distributed as follows: 50% in triglycerides, 48% in fatty acids, 1.2% in cholesteryl esters, and 0.5% in phospholipids. Fig. 1C shows that $d < 1.019$ g/ml plasma lipoproteins isolated from the porto-mesenteric system contain only apoB-100 as the corresponding lipoproteins isolated from the systemic circulation.

Plasma apolipoprotein B forms in chicks fed a cholesterol-rich diet

Since in the rat cholesterol feeding is accompanied by an increased plasma concentration of apoB-48-containing lipoproteins (chylomicron-remnants) (5) the analysis of plasma apoB forms was conducted in 21-day-old chicks that had been fed a cholesterol-rich diet for 2 weeks. After this dietary treatment, total plasma cholesterol concentration increased from 2.8 ± 0.4 to 5.0 ± 0.6 mmol/l ($P < 0.001$). This hypercholesterolemia reflected a marked elevation of VLDL and, to a lesser extent, of IDL and LDL (data not shown). As shown in Fig. 1D, in both control and cholesterol-fed chicks, $d < 1.019$ g/ml plasma

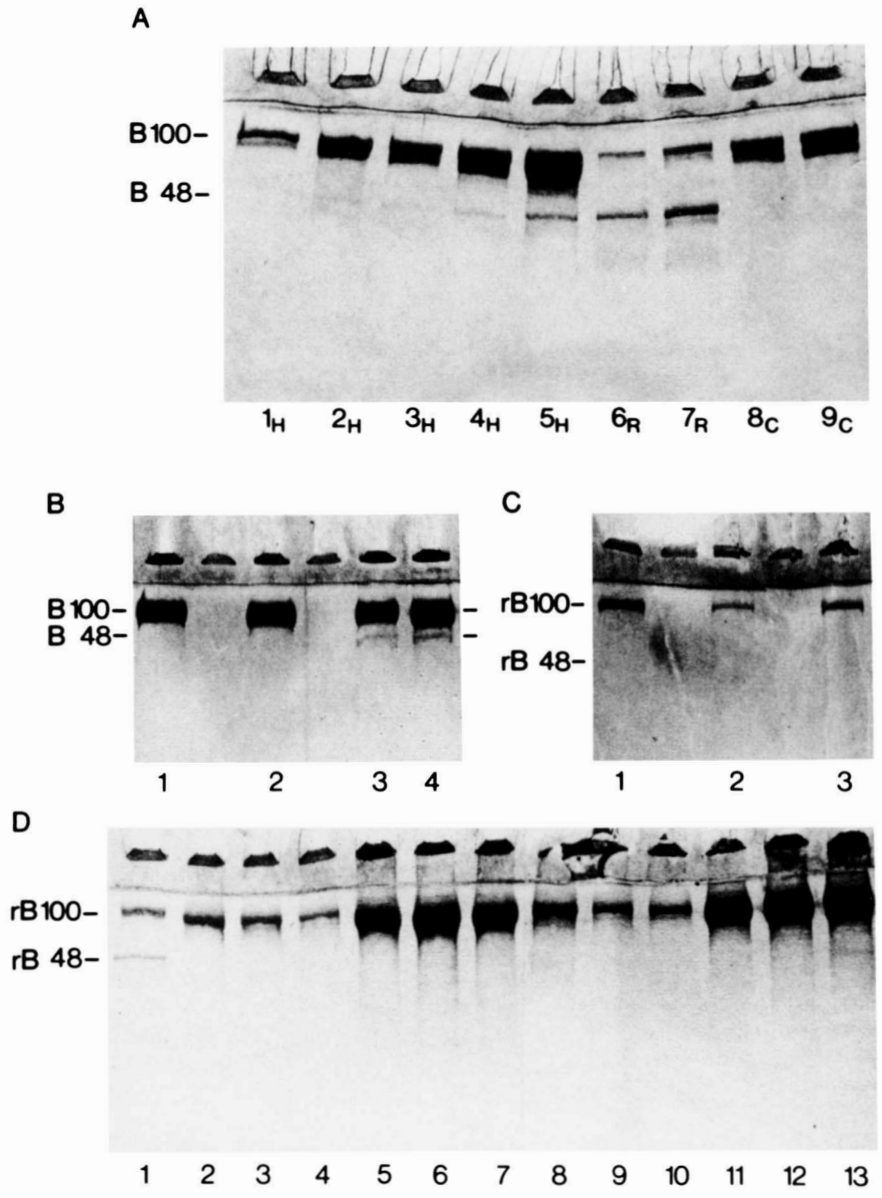

Fig. 1. SDS-polyacrylamide gradient (5–10%) gel electrophoresis of the apolipoproteins of d < 1.019 g/ml plasma lipoproteins. In all figures, rB-100 and rB-48 indicate the mobility of rat apoB-100 and apoB-48, respectively. Numbers in brackets indicate the amount of protein applied to the gels. A: Human (H), rat (R), and chick (C) lipoproteins isolated from the systemic circulation; lanes 1 and 2: fasted human lipoproteins (10–20 μg); lanes 3–5: nonfasted human lipoproteins (10–40 μg); lanes 6 and 7: fasted rat lipoproteins (10–20 μg); lanes 8 and 9: nonfasted 21-day-old chick lipoproteins (10–20 μg). B: Chick and human lipoproteins (d < 1.019 g/ml) isolated from the systemic circulation; lanes 1 and 2: nonfasted 21-day-old chick lipoproteins (30–40 μg); lanes 3 and 4: fasted human lipoproteins (30–40 μg). C: Chick lipoproteins (d < 1.019 g/ml) isolated from the systemic circulation (lanes 1 and 2) (20 and 10 μg) and porto-mesenteric circulation (lane 3) (15 μg). D: Chick lipoproteins (d < 1.019 g/ml) isolated from the systemic (lanes 2–7) and porto-mesenteric (lanes 8–13) circulation in control and cholesterol-fed 21-day-old chicks; lane 1: d < 1.019 g/ml rat plasma lipoproteins (30 μg); lanes 2–4 and 8–10: lipoproteins (20–30 μg) isolated from three control chicks; lanes 5–7 and 11–13: lipoproteins (40 and 60 μg) isolated from three cholesterol-fed chicks.

lipoproteins isolated from the systemic and the porto-mesenteric circulation contained only apoB-100. Faint bands of lower molecular weight moving just below apoB-100 found in cholesterol-fed chicks (Fig. 1D, lanes 5–7 and 11–13) presumably represent proteolytic degradation of apoB-100 since their number and intensity tended to increase with storage of lipoprotein samples (25). These bands are more easily seen when gels are overloaded.

In vivo labeling of plasma apolipoprotein B

In order to ascertain whether chick plasma contained such a minute amount of apoB-48 to be undetectable in Coomassie-stained gels, the analysis of plasma apoB

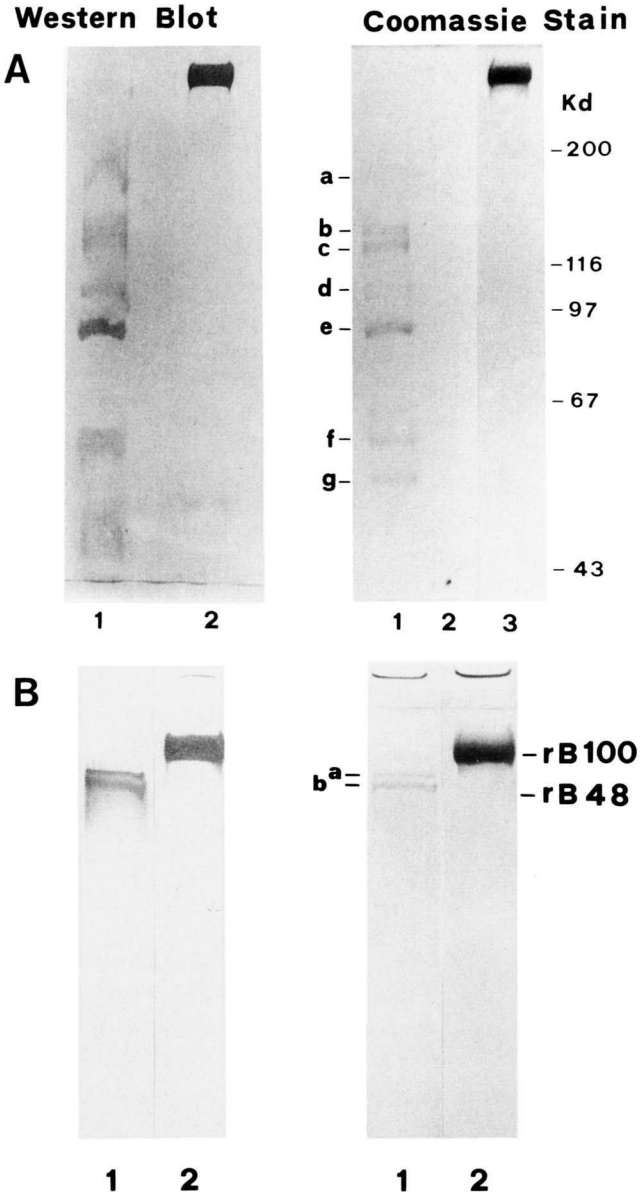

Fig. 2. Immunodetection of apolipoprotein B in chick plasma lipoproteins after digestion with trypsin (A) and thrombin (B). Plasma lipoproteins ($d < 1.019$ g/ml) of nonfasted 21-day-old chicks (25 μg of protein) were digested with trypsin (2.5 μg) and bovine thrombin (3 units) and then applied to SDS-PAGE gels. Proteolytic fragments visually detectable in the Coomassie-stained gels are indicated by small letters. A: Western blot analysis of chick apoB by using an anti-chick apoB rabbit IgG; lane 1: trypsin-generated fragments of chick apoB-100; lane 2: intact chick apoB-100. Coomassie-stained gel of the same plasma lipoprotein sample; lane 1: trypsin-generated fragments of chick apoB-100; lane 2: trypsin; lane 3: intact chick apoB-100. The migration of molecular weight standards is shown on the right. B: Western blot and Coomassie stain of chick apoB-100 before and after digestion with thrombin. Lane 1: thrombin-generated fragments of chick apoB-100; lane 2: intact chick apoB-100.

forms was repeated after in vivo protein labeling. ^{35}S-Labeled $d < 1.019$ g/ml plasma lipoproteins were isolated from animals that had received [^{35}S]methionine intraduodenally. Only apoB-100 was found to be labeled in $d < 1.019$ g/ml plasma lipoproteins isolated from either the systemic or the porto-mesenteric circulation of 21-day-old chicks fed a standard diet (**Fig. 3**, lanes 1 and 2). This result was confirmed by the immunoprecipitation of ^{35}S-labeled apoB from the whole plasma which showed the presence of a single radioactive band comigrating with apoB-100 (Fig 3, lanes 3 and 4). A similar study conducted in cholesterol-fed chicks demonstrated once again that the only form of ^{35}S-labeled apoB found in ^{35}S-labeled $d < 1.019$ g/ml plasma lipoproteins was apoB-100 (**Fig. 4A**). Even in overloaded and overexposed gels we failed to detect other radioactive apoB forms with a lower molecular weight (Fig. 4B).

In vitro synthesis of apolipoprotein B forms in liver and intestine

ApoB forms synthesized and secreted in vitro by slices of liver and intestine were analyzed at different stages of chick development. The rationale for this study originated from the observation that late embryonic and early post-natal life of the chick are accompanied by marked qualitative and quantitative changes of plasma apoB-containing lipoproteins (25). In late embryonic life (8 and 5 days before hatching) liver slices secreted only apoB-100 (**Fig. 5**). The same form of ^{35}S-labeled apoB was immunoprecipitated from liver slice homogenates (data not shown). Immunoreactive ^{35}S-labeled apoB found in the incubation medium of intestinal slices consisted of five to six bands migrating in the 100,000–400,000 molecular weight range in SDS-gels (data not shown). They presumably result from an extensive degradation of newly secreted apoB by gut-associated proteases. However ^{35}S-labeled intracellular apoB (i.e., apoB

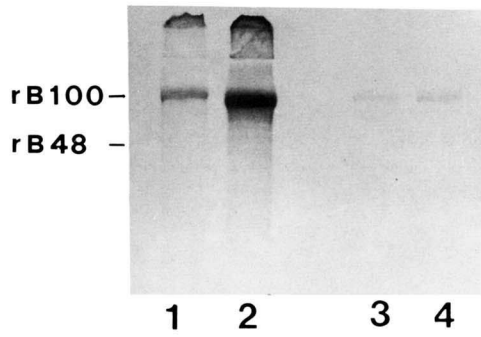

Fig. 3. In vivo labeling of plasma apolipoprotein B in normally fed 21-day-old chicks. Fluorogram of an SDS-polyacrylamide gradient (5–10%) gel electrophoresis of $d < 1.019$ g/ml plasma lipoproteins isolated from the porto-mesenteric and systemic circulation after the intraduodenal injection of [^{35}S]methionine. Lane 1: ^{35}S-labeled $d < 1.019$ g/ml lipoproteins (1×10^4 cpm) isolated from the porto-mesenteric circulation; lane 2: ^{35}S-labeled $d < 1.019$ g/ml lipoproteins (3×10^4 cpm) isolated from the systemic circulation; lanes 3 and 4: ^{35}S-labeled apoB immunoprecipitated with anti-chick apoB rabbit IgG from the whole plasma of the porto-mesenteric and systemic circulation, respectively. The figure is representative of two experiments.

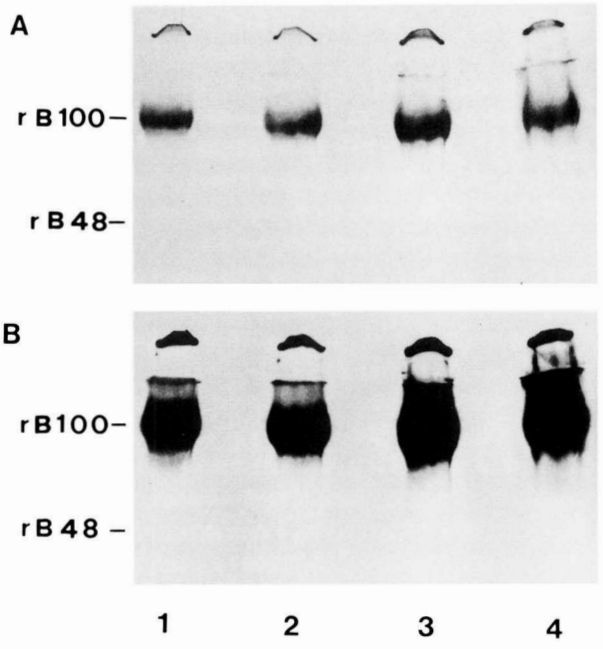

Fig. 4. In vivo labeling of plasma apolipoprotein B in cholesterol-fed 21-day-old chicks. A: Fluorogram of an SDS-polyacrylamide gradient (5–10%) gel electrophoresis of d < 1.019 g/ml plasma lipoproteins isolated from the systemic (lanes 1 and 3) and the porto-mesenteric (lanes 2 and 4) circulation. Lanes 1 and 2: 100 μg of lipoprotein-protein corresponding to 1 × 10^4 cpm; lanes 3 and 4: 200 μg of lipoprotein-protein corresponding to 2 × 10^4 cpm. B: The same fluorogram shown in Fig. 4A after a prolonged exposure of the gel to the X-ray film. The figure is representative of two experiments.

immunoprecipitated from the 12,000 *g* supernatant of intestinal slice homogenates) had the same mobility in SDS-PAGE as apoB-100 secreted by liver slices of the same embryos (**Fig. 6**). In early post-natal life (2 and 7 days after hatching) the major form of apoB secreted by both liver and intestine co-migrated with chick plasma apoB-100. No radioactive bands co-migrating with rat or human apoB-48 were detected (**Fig. 7**). We consistently found that intestinal slices from these chicks secreted an additional form of apoB that migrated in a position similar to that of rat plasma apoB-95 (Fig. 7). This apoB form has been arbitrarily designated as "apoB-95-like." ^{35}S-Labeled apoB immunoprecipitated from the 12,000 *g* supernatant of tissue homogenates confirmed the data shown in Fig. 7 (data not shown).

Liver slices isolated at a later stage of post-natal development (21 days after hatching) secreted exclusively apoB-100 (**Fig. 8A**, lanes 1 and 2). Intestinal slices isolated from the same animals secreted, in addition to apoB-100 and "apoB-95-like," other minor forms migrating as a doublet in the 350,000 molecular weight region (Fig. 8B). ^{35}S-Labeled apoB immunoprecipitated from the 12,000 *g* supernatant of liver slice homogenates consisted of a doublet (apoB-100 and "apoB-95-like") and some minor bands migrating just above rat apoB-48 (**Fig. 9A**). ^{35}S-Labeled apoB immunoprecipitated from the 12,000 *g* supernatant of intestinal slice homogenates consisted of a doublet (apoB-100 and "apoB-95-like") (Fig. 9B). The analysis of apoB forms secreted in vitro by liver and intestinal slices was also conducted in animals that had been fed a cholesterol-rich diet for 2 weeks. As shown in Fig. 8A, B and in Fig. 9A, B, the pattern of apoB forms synthesized and secreted in vitro by liver and intestine of cholesterol-fed chicks was identical to that found in animals fed a standard diet.

In order to ascertain which forms of apoB were present in lipoproteins of intestinal origin, ^{35}S-labeled d < 1.019 g/ml lipoproteins were isolated from the incubation medium of intestinal slices. **Fig. 10A, B** shows that intestinal slices of normally fed chicks (2 and 7 days old) secreted ^{35}S-labeled d < 1.019 g/ml lipoprotein containing exclusively apoB-100.

DISCUSSION

The present study was designed to clarify two points: *a*) whether multiple forms of apoB were present in chick plasma as found in mammalian species; and *b*) which forms of apoB were synthesized and secreted by chick liver and intestine, respectively. In order to investigate whether development and dietary manipulations had any effect on apoB forms, the study was performed in animals that were at various stages of pre- and post-natal develop-

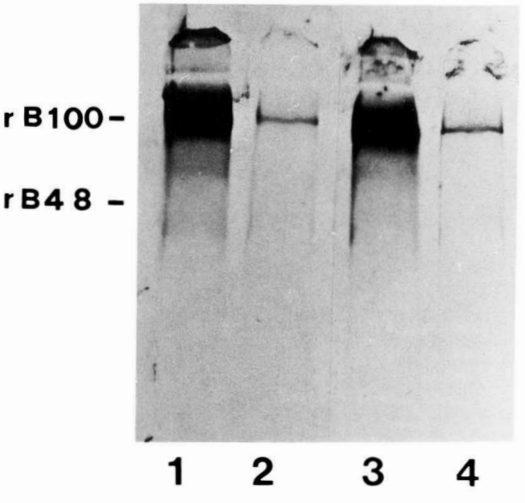

Fig. 5. In vitro secretion of ^{35}S-labeled apolipoprotein B synthesized by liver slices of chick embryo. Fluorogram of an SDS-polyacrylamide gradient (5–10%) gel electrophoresis of ^{35}S-labeled apoB secreted in vitro by liver slices isolated from chick embryos at 8 and 5 days before hatching and incubated with [^{35}S]methionine. Lanes 1 and 3: immunoprecipitated ^{35}S-labeled apoB secreted by liver slices at 8 and 5 days before hatching, respectively; lanes 2 and 4: nonspecific immunoprecipitation. The results shown in this figure are representative of three experiments.

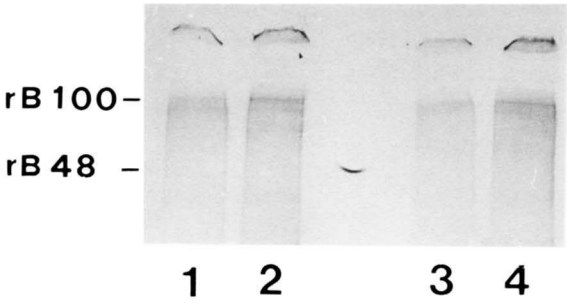

Fig. 6. In vitro synthesis of ^{35}S-labeled apolipoprotein B by intestinal slices of chick embryo. Fluorogram of an SDS-polyacrylamide gradient (5–10%) gel electrophoresis of ^{35}S-labeled apoB synthesized in vitro by intestinal slices isolated from chick embryos at 8 and 5 days before hatching and incubated with [^{35}S]methionine. Lanes 1 and 2: ^{35}S-labeled apoB immunoprecipitated from the 12,000 *g* supernatant of intestinal slice homogenates at 8 and 5 days before hatching, respectively; lanes 3 and 4: nonspecific immunoprecipitation. The results shown in this figure are representative of three experiments.

ment and had been fed a normal or a cholesterol-rich diet.

In a first set of experiments apoB forms were investigated in d < 1.019 g/ml plasma lipoproteins isolated from the systemic circulation of fasted and nonfasted animals. Regardless of the feeding conditions, the d < 1.019 g/ml plasma lipoproteins isolated from the systemic circulation were found to contain exclusively the high molecular weight form of apoB (apoB-100). Since the absence of apoB-48 in the d < 1.019 g/ml plasma lipoproteins of the systemic circulation could reflect a very rapid removal of intestinal lipoproteins from the plasma compartment, the analysis of apoB forms was conducted by using plasma enriched in lipoproteins of intestinal origin. It is generally accepted that in the chick the intestinal lymphatic system is poorly developed (27, 38) and lipoproteins synthesized by the intestinal mucosa are directly secreted into the porto-mesenteric system (27, 33). For this reason Bensadoun and Rothfeld (33) adopted the term of "portomicrons" to indicate the triglyceride-rich lipoproteins secreted by chick intestine. We consistently found that the d < 1.019 g/ml plasma lipoproteins isolated from the porto-mesenteric circulation of nonfasted chicks contained only apoB-100 as the corresponding lipoproteins isolated from the systemic circulation. The analysis of plasma apoB forms was also conducted after the injection of [^{35}S]methionine into the duodenum, on the assumption that in in vivo labeling could allow the detection in plasma of trace amounts of apoB-48 produced by the intestine. ^{35}S-Labeled d < 1.019 g/ml lipoproteins isolated from the systemic and porto-mesenteric circulation of normally fed chicks did not contain radioactive apoB-48. Similar experiments were also performed in cholesterol-fed chicks, on the assumption that cholesterol feeding induced an accumulation in plasma of intestinal lipoproteins (i.e., portomicrons and/or portomicron remnants potentially containing apoB-48). Despite a striking accumulation of cholesterol-rich VLDL and IDL in plasma, no ^{35}S-labeled apoB-48 was detected in the d < 1.019 g/ml plasma lipoproteins isolated from the systemic and the porto-mesenteric circulation of cholesterol-fed chicks.

The study of plasma apoB forms was also conducted by using immunoblotting analysis. This study was designed to ascertain whether our anti-chick apoB IgG could cross-react with the portion of chick apoB-100 that is likely to contain the amino-terminal end of apoB-100 (known to correspond to apoB-48 in mammalian species) (15–17). Our anti-chick apoB-100 IgG cross-reacted with all fragments generated by proteolytic digestion of chick apoB-100, thus indicating that our antibody would have been capable of recognizing chick apoB-48 had the latter been present, even in minute amounts, in d < 1.019 g/ml

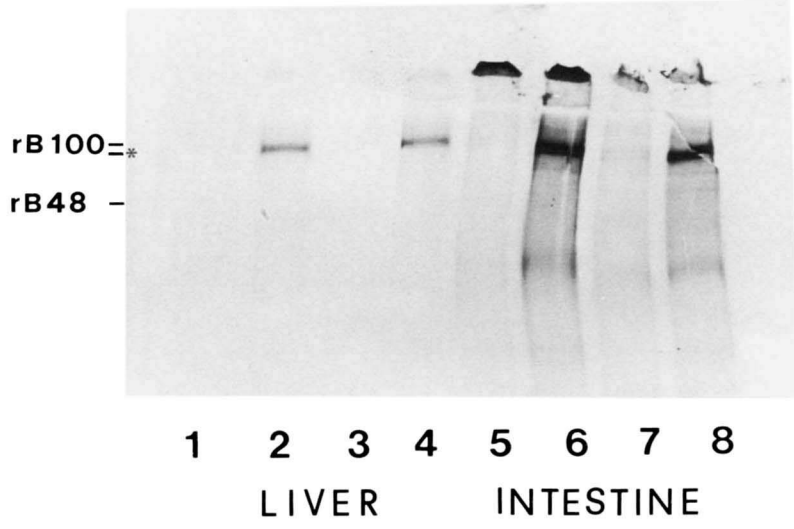

Fig. 7. In vitro secretion of ^{35}S-labeled apolipoprotein B synthesized by liver and intestinal slices of newly hatched chicks. Fluorogram of an SDS-polyacrylamide gradient (5–10%) gel electrophoresis of ^{35}S-labeled apoB secreted in vitro by liver and intestinal slices isolated from 2- and 7-day-old chicks and incubated with [^{35}S]methionine. Lanes 2 and 6: ^{35}S-labeled apoB immunoprecipitated from the incubation medium of tissue slices of 2-day-old chicks; lanes 4 and 8: ^{35}S-labeled apoB immunoprecipitated from the incubation medium of tissue slices of 7-day-old chicks; lanes 1, 3, 5, and 7: nonspecific immunoprecipitation. The star indicates the mobility of rat apoB-95. The results shown in this figure are representative of three experiments.

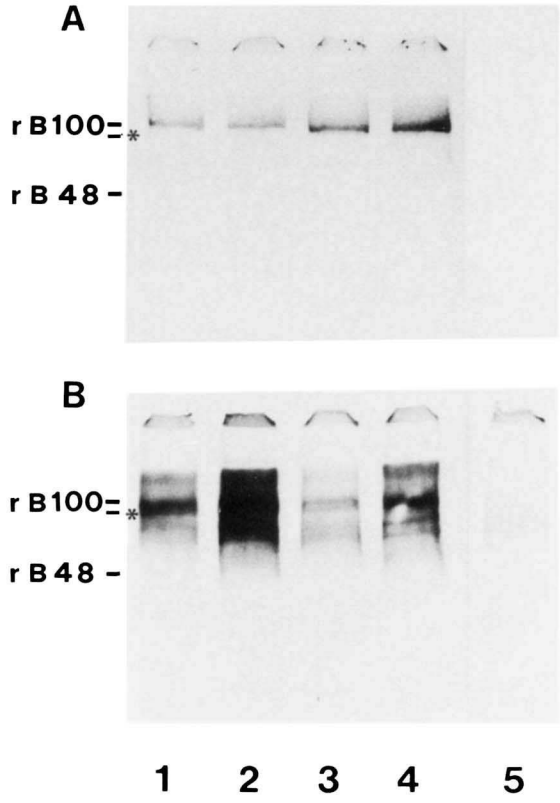

Fig. 8. In vitro secretion of ^{35}S-labeled apolipoprotein B synthesized by liver and intestinal slices of 21-day-old chicks. Fluorograms of SDS-polyacrylamide gradient (5–10%) gel electrophoresis of ^{35}S-labeled apoB secreted in vitro by liver (A) and intestinal (B) slices isolated from control and cholesterol-fed chicks and incubated with [^{35}S]methionine. Lanes 1 and 2: immunoprecipitated ^{35}S-labeled apoB secreted by tissue slices of normally fed chicks; lanes 3 and 4: immunoprecipitated ^{35}S-labeled apoB secreted by tissue slices of cholesterol-fed chicks; lane 5: nonspecific immunoprecipitation. The star indicates the mobility of rat apoB-95. The figure is representative of three experiments.

plasma lipoproteins. Taken together these results, by extending previous observations (18, 19) lead to the conclusion that apoB-48 is absent in chick plasma.

In a second set of experiments we analyzed the apoB forms synthesized in vitro by liver and intestinal slices isolated from animals at various stages of development. At each stage of development liver slices secreted predominantly a single form of apoB that co-migrated with plasma apoB-100 on SDS-PAGE gels. Additional minor bands were occasionally and inconsistently observed. It is likely that they represented proteolytic degradation products of apoB-100 (see below).

The pattern of labeled apoB forms secreted by intestinal slices was more complicated. In the embryos we consistently found that several radioactive peptides were immunoprecipitated by our anti-chick apoB IgG. The most likely explanation for this finding is that in the embryo the material secreted by or associated with intestinal mucosa contains proteases whose activity is poorly controlled by the anti-proteolytic compounds we added to the incubation medium. It should be stressed, however, that apoB found intracellularly consisted predominantly of a single form, similar to liver apoB-100. In post-natal life, however, apoB secreted by the intestine consisted predominantly of two high molecular weight forms: apoB-100 and a slightly lower molecular weight peptide designated "apoB-95-like," simply to indicate that it has an electrophoretic mobility similar to that of rat apoB-95 (3). While it is conceivable that "apoB-95-like" in the chick reflects a minute proteolytic cleavage of apoB-100 (39), we are aware that the presence of this peptide may be due to experimental conditions only. ^{35}S-Labeled apoB secreted by intestine of 21-day-old chicks also contained another doublet with a molecular weight of approximately 350,000; the origin of these bands is unknown. We cannot rule out the possibility that during prolonged incubation there was a leakage of proteins from some damaged tissue slices, leading to the accumulation of incomplete apoB chains in the medium. Of course, proteolytic fragmentation of ^{35}S-labeled apoB-100 may also account for these minor bands. Whichever is the mechanism underlying the origin of these minor bands, we found that the ^{35}S-labeled d < 1.019 g/ml lipoproteins

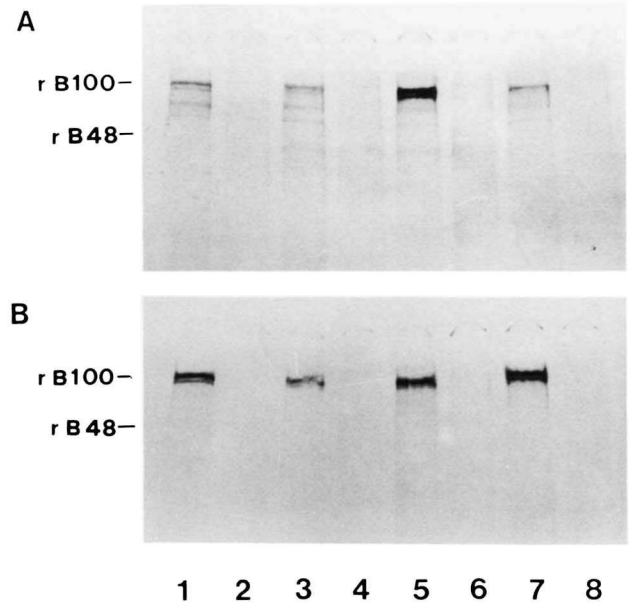

Fig. 9. In vitro synthesis of ^{35}S-labeled apolipoprotein B by liver and intestinal slices of 21-day-old chicks. Fluorograms of SDS-polyacrylamide gradient (5–10%) gel electrophoresis of ^{35}S-labeled apoB synthesized in vitro by liver (A) and intestinal (B) slices isolated from control and cholesterol-fed chicks and incubated with [^{35}S]methionine. Lanes 1 and 3: ^{35}S-labeled apoB immunoprecipitated from 12,000 *g* supernatants of tissue slice homogenates of control chicks; lanes 5 and 7: ^{35}S-labeled apoB immunoprecipitated from the 12,000 *g* supernatants of tissue slice homogenates of cholesterol-fed chicks; lanes 2, 4, 6, and 8: nonspecific immunoprecipitation. The results shown in this figure are representative of three experiments.

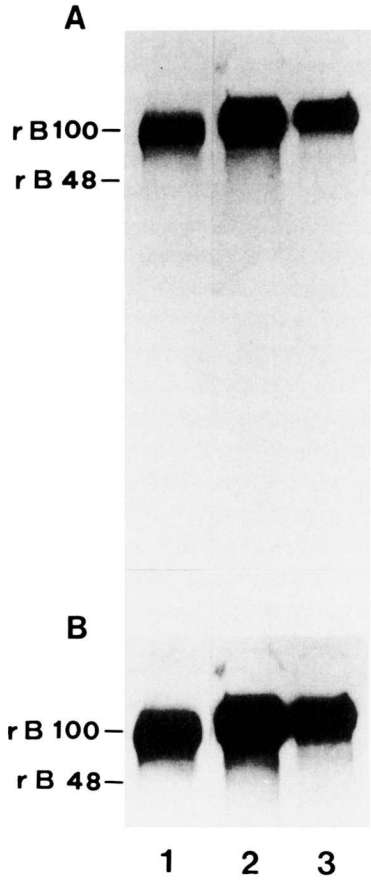

Fig. 10. In vitro secretion of ^{35}S-labeled d < 1.019 g/ml lipoproteins by intestinal slices of normally fed, newly hatched chicks. A: Fluorogram of an SDS-polyacrylamide gradient (5–10%) gel electrophoresis of ^{35}S-labeled d < 1.019 g/ml lipoproteins secreted by intestinal and liver slices incubated in the presence of [^{35}S]methionine. Lanes 1 and 2: ^{35}S-labeled d < 1.019 g/ml lipoproteins isolated from the incubation medium of intestinal slices taken from 2- and 7-day-old chicks; lane 3: ^{35}S-labeled d < 1.019 g/ml lipoproteins isolated from the incubation medium of liver slices from 2-day-old chicks. B: The same fluorogram shown in A after a prolonged exposure of the gel to X-ray film. The figure is representative of two experiments.

isolated from the incubation medium of intestinal and liver slices contained a single, labeled high molecular weight apoB (apoB-100). These in vitro observations are consistent with the idea that no apoB-48 was synthesized and/or secreted by either liver or intestine of the chick at the various stages of postnatal development we have investigated. Furthermore, no synthesis of apoB-48 was induced in either tissues by cholesterol feeding. In conclusion, the absence of apoB-48 in chick plasma reflects the lack of synthesis of this peptide in the intestine.

A major question raised by these observations concerns the evolutionary aspects of apoB-48 formation in the intestine. In the mammalian species studied so far, apoB-100 and apoB-48 are encoded by a single copy gene (10–17). This gene encodes a primary transcript that is modified to produce an mRNA encoding apoB-48 differing from the apoB-100 mRNA by a single base. The difference is a C to U transition resulting in an in-frame stop codon at amino acid 2153 in the apoB-100 sequence (15–17). This mRNA modification appears to be tissue-specific in that the majority, if not all, of apoB mRNA from human and rabbit liver encodes apoB-100 while that from the small intestine encodes apoB-48. While the present study was in progress it was reported that apoB mRNA found in chick liver and intestine is the same size as mammalian apoB mRNA (14 kb) (40) and can encode a protein of ~500,000 daltons. In that study, however, the question of the presence of apoB mRNA modification (i.e., the insertion of an in-frame stop codon) in the intestine leading to the production of apoB-48 was not specifically addressed. Our findings would suggest that in the chick the 14-kb mRNA present in the intestine encodes only apoB-100 and the mechanism for the insertion of a stop-codon at position 2153 of the apoB-100 mRNA is absent or defective in chick intestine. One can speculate, therefore, that the machinery for apoB mRNA modification in the intestine appeared late in the evolution, after the divergence of avian and mammalian species.

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