LIPOGENESIS AND THE SYNTHESIS AND SECRETION OF VERY LOW DENSITY LIPOPROTEIN BY AVIAN LIVER CELLS IN NONPROLIFERATING MONOLAYER CULTURE

Hormonal Effects

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ABSTRACT

The nonproliferating chicken liver cell culture system described yields cell monolayers with morphological and lipogenic properties characteristic of the physiological-nutritional state of donor animals. Synthesis and secretion of fatty acid, cholesterol, and very low density lipoprotein (VLDL) occur at in vivo rates and respond to hormones and agents which affect these processes in vivo. Cells derived from fed chickens maintain high rates of synthesis of fatty acid and cholesterol for several days if insulin is present in the medium. High rates of fatty acid synthesis are correlated with the appearance of membrane-enclosed triglyceride-rich vesicles in the cytoplasm; deletion of insulin causes a decrease ($T_{1/2}$ = 22 h) in fatty acid synthetic activity. Addition of glucagon or cyclic AMP (cAMP) causes an immediate cessation of fatty acid synthesis and blocks the appearance of the triglyceride-rich vesicles. Fatty acid synthesis in liver cells prepared from fasted chickens is <5% that of cells from fed animals. After 2–3 days in culture with serum-free medium containing insulin ± triiodothyronine, fatty acid synthesis is restored to normal; glucagon or dibutyryl cAMP blocks this recovery. Liver cells derived from estradiol-treated chickens synthesize and secrete VLDL for at least 48 h in culture. Electron micrographs of these cells reveal more extensive development of the rough endoplasmic reticulum and Golgi complex compared to cells from untreated chickens. Whereas [^{3}H]leucine incorporation into total protein is unaffected by estrogen treatment, [^{3}H]leucine incorporation into cellular and secreted immunoprecipitable VLDL is markedly increased indicating specific activation of VLDL apopeptide synthesis; 8–10% of the labeled protein synthesized and secreted is VLDL. Dodecyl sulfate-acrylamide gel electrophoresis of immunoprecipitated ^{3}H-VLDL reveals three major apopeptides of 300,000, 11,000, and 8,000 daltons corresponding to those of purified chicken VLDL.

Among the major functions of the liver cell are lipogenesis and the synthesis and secretion of very low density lipoprotein (VLDL) for use by extrahepatic cells. These processes have been exten-

sively investigated with perfused livers, liver slices, and, more recently, with isolated hepatocytes (8–10, 21, 26, 35, 40, 48). Studies with these liver preparations are limited in that only acute effects on lipogenesis and VLDL synthesis can be explored, since the preparations are viable for only a few hours. On the other hand, cells in primary culture retain differentiated function for several days. During this time, factors affecting lipogenesis and VLDL synthesis at the level of transcription, translation, and enzyme turnover can be investigated.

We have developed an avian liver cell primary culture system which meets these objectives. Chicken liver cells are particularly well-adapted for studies on lipogenesis and VLDL synthesis and secretion. The liver accounts for nearly all *de novo* fatty acid synthesis in the chicken (41), and provides a substantial fraction of the fatty acid utilized by other tissues including adipose tissue. Moreover, in avian species the liver has the unique role of synthesizing and secreting lipid and protein precursors, notably VLDL, for egg yolk formation in the ovary (36). Since these processes are under the control of estrogen in avian species (22, 30), it is possible to increase hepatic VLDL output 10- to 50-fold by administering this hormone (29). This can be accomplished in the livers of both male and sexually-immature chickens. Within a few days after the injection of estrogen, there is an extraordinary rise in the level of VLDL in the blood to 10–15 g/100 ml of serum (22, 30). The source of the increased plasma levels of VLDL induced by estrogen has been conclusively shown to be the liver (44). Thus, cells in primary culture from the livers of estrogen-treated chickens offer advantages for the study of lipogenesis and lipoprotein assembly and secretion.

Various modifications of the method of Berry and Friend (4), involving perfusion of the liver with collagenase, have been widely used for the preparation of hepatocytes from the rat (9, 10, 11, 12, 25, 40, 54) and the chicken (9). Anatomical considerations make this procedure cumbersome for 2- to 3-wk-old chicks, and maintenance of sterile conditions during cell preparation is difficult. A technique for the preparation of viable liver cells from the young chicken which avoids hepatic perfusion and which can be performed under sterile conditions has been developed in our laboratory. In monolayer culture, these liver cells have the capacity to carry out lipogenesis and VLDL assembly and secretion at rates which approximate those of liver tissue from animals in different physiological states, i.e., fasted, fed, and estrogenized. Such cells exhibit morphological characteristics comparable to those of intact liver and, in addition, are responsive to hormones and agents known to affect these processes in vivo. A preliminary report of these findings has been made (49).

MATERIALS AND METHODS

Materials and Miscellaneous Methods

White Leghorn chickens (10–15 days old) of mixed sex were maintained on a low fat, high carbohydrate diet for at least 48 h before sacrifice unless otherwise indicated. In some experiments, chickens were injected daily for 3 days before sacrifice, with 17-β-estradiol (20 mg/day per kg body weight, intraperitoneally) in propylene glycol. DNase I and selected lots of type III collagenase were obtained from Worthington Biochemical Corp. (Freehold, N. J.). Collagenase was dissolved in phosphate-buffered saline (PBS) to give a concentration of 150 U/ml and was stored at −20°C until used. DNase was dissolved in 1 mM HCl to a concentration of 2.5 mg/ml. Serum for the culture medium was obtained from 12- to 18-mo-old white Leghorn roosters. After allowing 90 min for clotting to occur, clots were cut into small pieces and the blood was centrifuged at 10,000 *g* for 20 min. The serum was filtered twice under vacuum through Schleicher and Schuell no. 589 filters (Schleicher & Schuell, Keene, N. H.), after which it was sterilized by passage through a 0.45 μm Millipore filter (Millipore Corp., Bedford, Mass.) with a prefilter and stored at −20°C. Basal Medium (Eagle) (10×) with Earle's salts, without L-glutamine and $NaHCO_3$ (BME), was obtained from Grand Island Biological Co. (Grand Island, N. Y.).

PBS, pH 7.3, contained (grams per liter): KCl, 0.2; KH_2PO_4, 0.2; NaCl, 8; and $Na_2HPO_4 \cdot 7\ H_2O$, 1.48. Phosphate buffer (10×) contained (grams per liter): KH_2PO_4, 2; $Na_2HPO_4 \cdot 7\ H_2O$, 14.8, and was adjusted to pH 7.6 with NaOH. Eagle's medium phosphate buffered (EPB) contained BME supplemented with phosphate buffer, 20 mM D-glucose, streptomycin (0.1 mg/ml), penicillin (100 U/ml), 2% rooster serum, and amino acids as follows (milligrams per liter): L-alanine, 50; L-arginine hydrochloride, 150; L-asparagine, 57; L-aspartate, 120; L-cysteine, 90; L-cystine, 15; L-glutamate, 150; L-glutamine, 600; glycine, 100; L-histidine hydrochloride, 150; L-isoleucine, 100; L-leucine, 25; L-lysine hydrochloride, 205; L-methionine, 100; L-phenylalanine, 100; L-proline, 100; L-serine, 100; L-threonine, 150; L-tryptophan, 50; L-tyrosine, 100; L-valine, 150. Amino acids were adjusted to pH 7.3. Bovine insulin and porcine glucagon, provided by Dr. Walter N. Shaw (Eli Lilly and Co., Indianapolis, Ind.), were dissolved in water (1 mg/ml), adjusted to pH 2.8 and stored at 4°C. Cholera enterotoxin and sodium heparin were obtained

from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, N. Y.). Amino acids, streptomycin sulfate, penicillin G, 17-β-estradiol, and triiodothyronine were from Sigma Chemical Co. (St. Louis, Mo.). All hormone, enzyme, and amino acid solutions used for cell culture were sterilized by passage through 0.22-μm Millipore filters. Falcon (VWR Scientific Div., San Francisco, Calif.) and Nunclon plastic tissue culture dishes (Vangard Systems Inc., Dobbs Ferry, N. Y.) were used interchangeably except where indicated. Reeve-Angel ultra-fine glass fiber filter discs (2.5 cm) were obtained from Arthur H. Thomas Co. (Philadelphia, Pa.). All reagents for electron microscopy were obtained from Electron Microscopy Sciences (Fort Washington, Pa.). NCS tissue solubilizer was obtained from Amersham/Searle Corp. (Arlington Heights, Ill.), and Triton X-100 from Packard Instrument Co. (Downer's Grove, Ill.). Sodium [1-^{14}C]acetate, L-[4,5-^{3}H(N)]leucine, and [5,6-^{3}H]uridine were obtained from New England Nuclear (Boston, Mass.). Lactic dehydrogenase was measured by a modification of the method of Stolzenbach (47). The assay mixture contained, in a volume of 1 ml, 100 mM potassium phosphate, pH 7.0, 0.67 mM sodium pyruvate, 0.2 mM NADH, 0.2% Triton X-100, and sample.

Preparation of Chicken Liver Parenchymal Cells

All procedures were carried out at room temperature unless otherwise indicated. Five 13- to 17-day-old chickens (about 100 g each) were decapitated, feathers and skin were removed, the carcasses were rinsed with 70% ethanol, and the livers were removed aseptically in a laminar-flow hood. Livers were rinsed with PBS and minced finely, after which the tissue mince was rinsed with PBS and transferred to a 250-ml Erlenmeyer flask. 20 ml of collagenase solution, 0.15 ml of DNase, and 2.5 ml of 400 mM glucose were added to the minced tissue (about 30 ml). The tissue suspension was incubated for 20 min at 37°C in a gyratory shaker set at 130 oscillations/min. After the tissue had settled, the supernate (~20 ml) containing dissociated cells was transferred to two sterile plastic tubes with a blunt large-bore pipette and was centrifuged at 190 *g* for 1 min. The cell pellet was subjected to a procedure for lysing erythrocytes (5, 52), while the remaining tissue mince was undergoing further collagenase treatment as described below. The cell pellet was resuspended in 5 ml of a 0.14 M NH_4Cl-0.017 M Tris (Cl^-) solution at pH 7.2. After 5 min, 5 ml of EPB containing 2% rooster serum was added and the suspension was centrifuged at 190 *g* for 1 min. The cell pellet was washed two more times with EPB (containing 2% rooster serum), finally resuspended in 0.5 ml of this medium, and set aside (at 25°C, exposed to air) to be pooled with cells obtained in subsequent processing.

To the remaining tissue mince were added 20 ml of collagenase and 1 ml of glucose. The mince was dispersed by repeatedly drawing it in and out of a blunt large-bore pipette, after which the suspension was incubated and processed as before. This procedure was repeated one additional time. Pieces of tissue remaining after the three collagenase treatments were further dissociated by vigorous in-and-out pipetting action as above. This suspension was filtered through a 100-mesh silk screen and the filtered cells were subjected to the erythrocyte lysis procedure. The washed cells from each stage of the procedure were combined, filtered through 100-mesh silk screen to remove aggregated Kupffer cells and any clumped cells, and the filtrate was brought to a final volume of 20 ml with EPB (containing 2% rooster serum). Cell number was determined and the viability estimated by trypan blue exclusion. Cell suspensions usually contained 40–50 × 10^6 cells/ml having a viability of 85–95%. The entire hepatocyte isolation procedure required about 2 h.

Preparation and Maintenance of Liver Cell Monolayers

Aliquots of cell suspensions (within 10–15 min of isolation) were pipetted into cell culture dishes containing medium previously equilibrated in 10% CO_2-90% air at 37°C. Except where indicated, the standard culture medium consisted of BME supplemented with 5% rooster serum, 0.22% $NaHCO_3$, 2 mM L-glutamine, 20 mM glucose (total concentration, 25 mM) streptomycin (0.1 mg/ml), penicillin (100 U/ml), and amino acids (see Materials and Miscellaneous Methods). In all experiments, except those on VLDL synthesis and secretion, plating densities were 3 × 10^6 cells/35-mm dish (2 ml of medium) and 10 × 10^6 cells/60-mm dish (4 ml of medium). When VLDL synthesis and secretion were investigated, 18 × 10^6 cells/60-mm dish (4 ml of medium) were plated. The plates were swirled gently to disperse the cells and then were incubated in a humidified forced-draft incubator under 10% CO_2-90% air at 37°C; at equilibrium, the pH of the medium was 7.25–7.35. Medium changes were made every 24 h unless otherwise specified. Insulin, glucagon, and triiodothyronine were added at levels of 5, 5, and 1 μg/ml of medium, respectively, where indicated.

Isolation and Purification of Labeled and Unlabeled Serum VLDL

Blood from laying hens was collected and held at room temperature for 30 min. Clots were rimmed, and after standing for an additional 30 min, the serum was separated by centrifugation at 12,000 *g* for 15 min. VLDL was separated from the serum by centrifugation at serum density ($d \cong 1.006$ g/cm^3) at 150,000 *g* for 48 h in a preparative ultracentrifuge at 15°C. The upper VLDL layer was collected and resuspended in 0.15 M NaCl containing 10 mM sodium phosphate, pH 7.5, and

0.5 mM EDTA. The resuspended VLDL (21 mg of protein in 3 ml) was applied to a 2.2 × 40 cm column of Sepharose 6B (Pharmacia Fine Chemicals, Piscataway, N. J.) previously equilibrated with the elution buffer at 2°C. The column was eluted with 0.15 M NaCl containing 10 mM sodium phosphate, pH 7.5, and 0.5 mM EDTA, and the eluate was collected fractionally. Fractions comprising the majority (~75%) of the void volume protein peak were pooled and dialyzed against two changes of 100 vol of glass-distilled water. Protein in VLDL samples was measured by the method of Lowry et al. (34) modified such that reagent A contained 0.4% sodium cholate.

To prepare ^{3}H-VLDL labeled primarily in the apoprotein portion of the molecular aggregate, 4-day-old chickens were injected intraperitoneally with a priming dose of 0.5 mg of 17-β-estradiol in propylene glycol. After 3 wk each chick received a second injection of the hormone (20 mg/kg of body weight). [^{3}H]Leucine (1.0 mCi) was injected 18 h later, and after an additional 4 h, blood was collected and ^{3}H-VLDL isolated as described above. The labeled VLDL was then dialyzed against 10 mM sodium phosphate, pH 7.4, 0.15 M NaCl, and 0.5 mM unlabeled leucine. The final VLDL product had a protein concentration of 2.1 mg/ml and a specific activity of 130,000 cpm/mg of protein.

Preparation of Anti-VLDL Antibody and the Immunochemical Measurement of VLDL Apoprotein Synthesis and Secretion

Antibody against chicken serum VLDL apoproteins was generated in male, white New Zealand rabbits by injecting 0.25 mg of purified VLDL protein (1.0 mg/rabbit) mixed with Freund's complete adjuvant into each foot pad. At 2 and 4 wk, the procedure was repeated. Blood was collected 2 wk after the last injection, and booster injections were given every 2 mo thereafter. The Ouchterlony double-diffusion technique (42) was used to verify the specificity of the antiserum. Immunotitration equivalence-point titers were in the range of 50 μl antiserum/35 μg VLDL protein.

To follow the kinetics of [^{3}H]leucine incorporation into total protein and into immunoprecipitable VLDL in cells and medium, liver cell monolayers (18×10^6 cells/60 mm dish) were incubated in 2 ml of medium containing [^{3}H]leucine for up to 6 h. The medium contained all amino acids as described in Materials and Methods, with the omission of unlabeled L-leucine. After incubation, the medium was removed and aliquots were taken for TCA precipitation of total protein and immunoprecipitation of VLDL as described below. PBS (2 ml) was added to each dish and cells were scraped and transferred to a Dounce homogenizer. The suspension was homogenized (50 strokes) with a tight-fitting pestle at 37°C, and an aliquot (0.1 ml) was added to a tube containing 0.1 ml of cold 20% TCA. Precipitated protein was collected on glass fiber filters which were washed with 100 ml of cold 5% TCA and then with 100 ml of cold 95% ethanol. Filters were placed in counting vials, dried at 100°C, and counted in toluene liquid scintillator. The remaining cell homogenate was transferred to a 37°C water bath; sodium deoxycholate was added to give a final concentration of 1%, and the suspension was homogenized. After centrifugation at 30,000 *g* for 15 min at 25°C, an aliquot (0.2 ml) of the supernate was mixed in a Beckman microfuge tube (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) with 10 μl of carrier chicken serum VLDL (35 μg of protein), VLDL antiserum (50–100 μl), and sodium deoxycholate to give a final concentration of 1%. Tubes were shaken, incubated for 2 h at 37°C, then for 24 h at 4°C, after which they were centrifuged for 2 min in a Beckman microfuge and the pellets were washed 4 times with 0.15 M saline. The tip of the tube, containing the immunoprecipitate, was sliced off and transferred to a counting vial containing 0.32 ml of 1% sodium dodecyl sulfate (SDS). After 2 h at 50°C, ^{3}H-activity was determined in 3 ml of Triton-containing liquid scintillator. Immunotitration curves were run under these conditions with each batch of antiserum to establish the level needed for maximum immunoprecipitation of VLDL protein.

Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis of VLDL Apoproteins

Unlabeled and [^{3}H]leucine-labeled VLDL or immunoprecipitates were dissolved in 100 μl of 2% SDS at 55°C, 2-mercaptoethanol was added to a final concentration of 1%, and the solution was heated for 2 min at 100°C. Samples were applied in 20% glycerol with bromophenol blue tracking dye to 3.3 or 10% acrylamide gels containing 0.1% SDS prepared as described by Weber et al. (53). After electrophoresis at 2 mA/gel for 20 min and 8 mA for 1½–2 h, the gels were exposed to 10% TCA overnight and then either were stained with Coomassie blue or were frozen and sliced for ^{3}H-analysis. Gel slices were incubated overnight at 37°C in 0.3 ml of 90% NCS solubilizer and then counted in 3 ml of toluene scintillator.

Incorporation of [^{14}C]Acetate into Fatty Acids and Cholesterol

Cell monolayers in 35-mm dishes were incubated for 1 h with 1 ml of medium containing 5 mM sodium [1-^{14}C]acetate (300–800 cpm/nmol). The cells were then scraped from culture dishes, transferred to tubes containing 0.9 g of KOH, and the plates were rinsed with 1 ml of PBS which was then added to the tubes. An equal volume of absolute ethanol was added (final concentration of KOH, ca. 4 N) and the tubes were placed in a boiling water bath for 2 h. Nonsaponifiable lipids were

extracted into petroleum ether and used for cholesterol isolation as described below. The aqueous phase was then acidified to a pH <1.0 with concentrated HCl. Fatty acids were extracted into petroleum ether; the extract was washed with 5% acetic acid and taken to dryness under N_2. The samples were counted in toluene scintillator.

The extract containing nonsaponifiable lipids was washed twice with 0.1 N NaOH and twice with 5% acetic acid. The washed extract was dried under N_2 and cholesterol was isolated as the digitonide by a modification of the method of Sperry and Webb (46) and Crawford (13). The residue was dissolved in 2 ml of acetone-absolute ethanol (1:1). Two drops of glacial acetic acid and 0.2 ml of 1% carrier cholesterol in absolute ethanol was added, and cholesterol was precipitated by the addition of 5 ml of 0.5% digitonin in 70% ethanol. After standing at room temperature for 24 h, the tube was centrifuged at 27,000 *g* for 10 min. The pellet was washed once with 4 ml of acetone-diethyl ether (1:1) and once with 4 ml of diethyl ether, after which it was dried at room temperature, dissolved in 1 ml of methanol, and counted in toluene scintillator.

Isolation of Cytoplasmic Triglyceride-Rich Vesicles from Chicken Liver Cells

The medium was removed from cells cultured in the presence of insulin for 24–48 h. After washing the monolayer twice with PBS, cells were lysed by adding glass-distilled water and aspirating the cells with a Pasteur pipette. The mixture was gently homogenized (30 strokes) at 2°C in a Dounce homogenizer with a loose-fitting pestle, after which the homogenate was transferred to a centrifuge tube. Centrifugation at 900 *g* for 5 min caused the lipid vesicles to float, giving rise to a layer which was carefully removed with a Pasteur pipette. The vesicles were resuspended in water and centrifuged again as described above; this procedure was repeated one additional time. Examination of the vesicle preparation by phase contrast microscopy revealed a uniform population of vesicles closely resembling that observed *in situ*.

Characterization of Labeled Lipids in Cells and in Triglyceride-Rich Vesicles

After incubating the cell monolayer with [1-^{14}C]acetate, cells were scraped from the culture dish and the dish was washed with PBS. Cells and washings were homogenized at 2°C in a Dounce homogenizer with a tight-fitting pestle. One volume of cell homogenate was extracted with 20 vol of chloroform:methanol (2:1, vol/vol) containing 10 mM acetic acid. After washing with 20 vol of 0.15 M NaCl followed by 12 vol of Folch upper phase solution (43), the extracts were evaporated to dryness under N_2 and resuspended in a small volume of chloroform. This concentrated extract was applied to silica gel thin-layer plates and chromatographed using a 1,2-dichloroethane:glacial acetic acid (100:1) solvent system; authentic palmitic acid, tripalmitin, phosphatidyl choline, cholesterol, and cholesteryl esters were cochromatographed as standards. Spots were visualized with 2,7-dichlorofluorescein, cut out, and counted in toluene scintillator. To characterize labeled lipids in triglyceride-rich vesicles, an aliquot was subjected to chloroform-methanol extraction and thin-layer chromatography as described above for cell homogenates.

Electron Microscopy

Medium was removed from the cell monolayer and cells were fixed in the 35-mm Falcon culture dish for 1 h at 2°C with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. The monolayer was then fixed with osmium tetroxide, stained with uranyl acetate, embedded in Epon and prepared for electron microscopy as described by Huang and Pagano (24). Serial sections were cut with a diamond knife on an LKB ultramicrotome (LKB Instruments, Inc., Rockville, Md.), mounted on copper grids (200 mesh), and stained with lead citrate. Electron micrographs were obtained at 60 kV with a Siemens Elmiskop I double condenser electron microscope.

RESULTS

Characteristics and Morphology of Chicken Liver Cells in Monolayer Culture

Unlike most procedures for the isolation of biochemically competent nonproliferating liver cells from the rat (4, 9–12, 25, 40, 54), perfusion of the liver is not required to obtain viable liver cells for primary monolayer culture from the 2- to 3-wk-old chicken. By avoiding the cumbersome perfusion technique, sterility is more readily maintained during the preparation of hepatocytes. The collagenase tissue dissociation procedure described in Materials and Methods produces a good yield of liver cells with high viability and plating efficiency. When freshly prepared, 85–95% of the hepatocytes in the cell suspension exclude trypan blue dye, while 95–98% of cells in the monolayer exclude the dye after 24–48 h in culture. The cell suspension is briefly exposed to dilute NH_4Cl which preferentially lyses erythrocytes (5, 52) which would otherwise contaminate the preparation. The combination of this procedure with filtration of the cell suspension through a 100-mesh silk screen to remove Kupffer cells gives rise to cell monolayers that are >95% parenchymal cells by

microscope examination. The cell yield for a typical preparation in which 5 livers (~15 g wet weight of tissue) are used is 10^9 cells or 7×10^7 cells/g of tissue. Neither cell yield nor viability is significantly affected by fasting the donor animal for 48 h or by prior estrogenization, experimental treatments the biochemical effects of which will be described subsequently.

Within 2 h after plating, more than 95% of the cells have attached firmly to the surface of the culture dish. It was observed during initial studies that the presence of homologous serum in the medium was necessary to maximize the rate and extent of cell attachment to the dish; calf serum, fetal calf serum, and horse serum were ineffective in replacing chicken serum for optimal cell attachment. Rooster serum, rather than hen serum or growing chick serum, was used in the present studies on *de novo* lipogenesis because of its lower lipid content. Upon attachment, cells begin to lose their refractile appearance, flatten, and spread out on the dish (Fig. 1*A*). Within 4–5 h after plating, nearly half of the cells have flattened (Fig. 1*B*), markedly increasing their area of contact with the dish and apparent diameter (average diam., 20–30 μm). When contact with neighboring cells is made, spreading ceases and polygonal cell boundaries are established. At appropriate plating densities, a confluent monolayer is achieved with 24–48 h. Illustrated in Fig. 1*C* is a confluent monolayer of avian liver cells in culture with insulin for 45 h. The polygonal character of the cells in the monolayer is reminiscent of that in thin sections of avian liver (23). The presence of insulin in the medium is required for optimal cell flattening and spreading; in its absence cells attach normally, but remain spherical and do not produce confluent monolayers.

It has been established (results not shown) that avian liver cells cultured under the conditions described do not proliferate. There is neither an increase in cell number nor significant incorporation of [*methyl*-^{3}H]thymidine into TCA-precipitable nucleic acid from the time of plating. It will be demonstrated subsequently, however, that avian liver cells are responsive to hormones and actively carry out the synthesis of fatty acids, cholesterol, protein, and RNA for up to 4–5 days in culture. Fibroblast contamination and proliferation are minimal in the liver cell cultures; confluent monolayers usually contain <3% fibroblasts even after 4–5 days in culture.

When liver cells from normally fed chickens are maintained in monolayer culture in the presence of insulin for 24–48 h, substantial amounts of lipid, primarily triglyceride, are synthesized *de novo* (Table I). Although an appreciable amount of this lipid is secreted into the medium (20–30% of the [^{14}C]acetate incorporated into long chain fatty acyl groups), a substantial quantity of lipid accumulates in refractile triglyceride-rich vesicles in the cytoplasm (Fig. 2*B*, cells in culture for 48 h). The lipid cores of the vesicles stain intensely with the lipid stain, Oil Red EGN. Illustrated in Fig. 3*A* is an electron micrograph of a cell containing the cytoplasmic vesicles which vary in diameter from 0.1 to 2 μm. Electron micrographs at higher magnification (Fig. 3*B*) reveal that the lipid-containing vesicles are bounded by a membrane bilayer. Also visible in Fig. 3*A* and *B* are vesicles containing osmiophilic structures that are similar to VLDL particles described by others (1, 20); some of the smaller triglyceride-rich vesicles have the appearance of multivesicular bodies or secondary lysosomes. Detailed examination of the electron micrographs suggests that vesicle expansion occurs by membrane fusion with both smaller lipoprotein-containing vesicles and smaller triglyceride-rich vesicles (see unbroken arrows, Fig. 3*B*). The triglyceride-rich vesicles can be isolated by flotation at low centrifugal force after cell lysis by gentle homogenization in hypotonic medium. On the basis of experiments in which cells were labeled with a lipogenic presursor, [^{14}C]acetate, it is evident (Table I) that the newly synthesized lipid in the isolated vesicles is primarily triglyceride with only small amounts of phospholipid, free fatty acid, cholesterol, and cholesterol ester. Similar experiments with [^{3}H]leucine indicate a low content of newly synthesized protein in the vesicles.

The addition of glucagon (or dibutyryl cAMP) to the insulin-containing medium totally prevents the accumulation of triglyceride-rich vesicles (Fig. 2*A*). Moreover, it will be shown later that this inhibitory effect is the result of the specific suppression of *de novo* lipogenesis by these agents. Electron micrographs (Fig. 4*A*) of cells in monolayer culture for 48 h in a glucagon-containing medium reveal a cytoplasm devoid of triglyceride-rich vesicles. Dibutyryl cAMP has the same effect (result not illustrated). Exposure to glucagon for 48 h produces an alteration in the structure of the mitochondrial membranes of avian liver cells (Fig. 4*B*). Many mitochondria exhibit focal thickening along the external membrane sur-

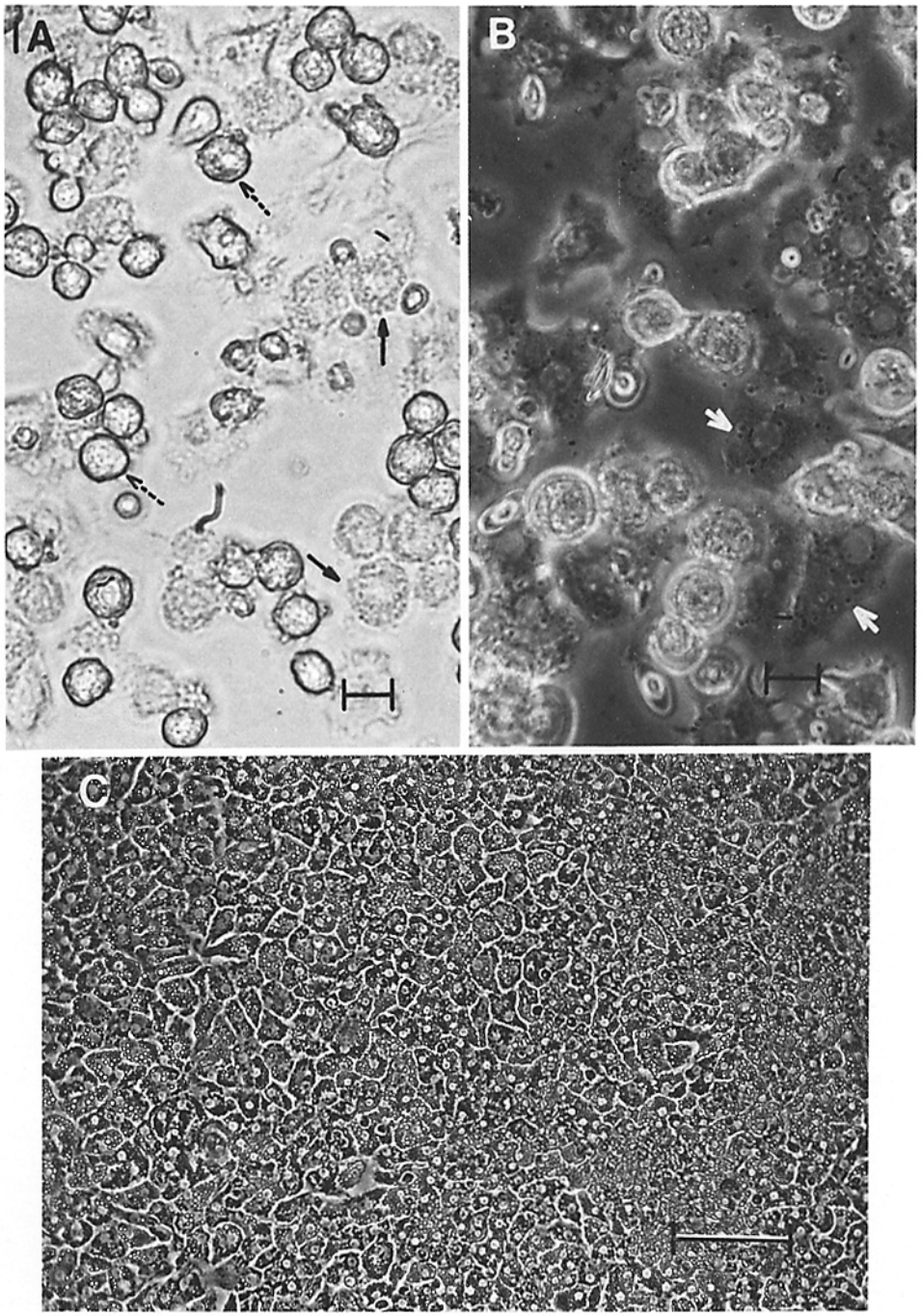

FIGURE 1 Light micrographs of chicken liver cells in monolayer culture. Normal chicken liver cells at (*A*) 2 h, (*B*) 4 h, and (*C*) 48 h after plating in standard culture medium containing 5% rooster serum and insulin. Liver cells from fed 15-day-old chicks were isolated and plated as described in Materials and Methods. Medium was aspirated just before photography to remove unattached cells. Upon attachment, cells appear spherical and refractile, as in (*A*) (broken arrows); after flattening and spreading on the dish, their refractile character (*A* and *B*, unbroken arrows) is lost. Confluent monolayers of polygonal cells as in (*C*) are obtained between 24 and 48 h after plating. Scale bars in (*A*) and (*B*), 10 μm; in (*C*), 100 μm. (*A* and *B*) × 620; (*C*) × 155.

TABLE I

*Composition of Lipids in Cells and Cytoplasmic Vesicles Synthesized from [^{14}C]Acetate**

Lipid	Distribution of ^{14}C-activity in lipid from:	
	Cells	Cytoplasmic vesicles
	%	
Triglyceride	80.9	94.4
Phospholipid	8.0	2.4
Cholesterol		
Free	4.2	0.7
Esterified	2.0	1.2
Fatty acid	4.9	1.3

* Liver cells from fed chickens were maintained in culture for 24 h in the standard medium containing 5% rooster serum and 5 μg of insulin/ml. After a 1-h incubation with [1-^{14}C]acetate, cellular lipids were extracted with a chloroform-methanol mixture and the extract was subjected to thin-layer chromatography (see Materials and Methods for details). To label the cytoplasmic vesicles, cells in culture for 24 h were labeled with 5 mM [1-^{14}C]acetate for 20 h and then chased with 5 mM unlabeled acetate for 5.5 h. Vesicles were isolated as described in Materials and Methods and their labeled lipid composition was determined as described above. Each data point represents the mean of duplicate dishes.

face. In these regions, membrane whorls appear to occupy the intercristal space between the inner and outer mitochondrial membranes. Despite this apparent alteration in mitochondrial structure, many major energy-requiring biochemical functions are not impaired. For example, the rates of protein and RNA synthesis of cells exposed to both glucagon and insulin for periods up to 4 days are comparable to those of cells exposed to insulin alone. During this period the rates of protein and RNA synthesis increased 2- to 2.5-fold.

In the intact animal, *de novo* fatty acid synthesis by the liver is markedly curtailed by fasting (31, 32). Similarly, liver cell monolayers derived from fasted chickens have <5% of the fatty acid synthetic capacity of those prepared with cells from fed chickens. However, when carried for 2–3 days under cell culture conditions in serum-free medium containing insulin (± triiodothyronine), this capacity is restored to normal levels; when carried in culture for 4–5 days, supranormal levels of fatty acid synthesis are achieved. Shown in Fig. 2 *C* and *D* are cell monolayers prepared from fasted chickens (40 h) and then maintained in culture medium containing insulin for 4 and 5 days, respectively. It is evident that the cytoplasmic cell compartment has become engorged with the triglyceride-rich vesicles. This process is accelerated by triiodothyronine and can be completely prevented by the presence of glucagon or dibutyryl cAMP in the medium (not illustrated). Importantly, these morphological findings correlate well with biochemical parameters of lipogenesis to be presented in a later section. It should be noted that all of the lipid accumulated in the cytoplasmic triglyceride-rich vesicles pictured in Fig. 2 *C* and *D* arose via *de novo* synthesis, since the medium was lipid-free i.e., serum-free.

Cells derived from the livers of chickens treated with 17-β-estradiol (20 mg/kg of body weight per day for 3 days) and carried in culture for 24 h with insulin present differ morphologically and functionally from those prepared from normal or fasted animals. The major morphological difference is the more extensive development of the rough endoplasmic reticulum (Fig. 5 *A* and *B*) and Golgi complex (Fig. 6 *A*) in cells from estrogenized animals. This is correlated with the observation that such cells have a greatly increased capacity to synthesize and secrete VLDL (results to be described in a later section). It is particularly significant that the structural integrity of organelles critical to lipoprotein synthesis and secretion, i.e., the rough endoplasmic reticulum (Fig. 5 *B*), Golgi complex (Fig. 6 *A*), lipoprotein vesicles (Fig. 6 *B*), and microvilli (Fig. 6 *B*), remains intact under primary cell culture conditions for 24–48 h.

Effect of Hormones on Fatty Acid and Cholesterol Synthesis by Monolayer Cultures of Liver Cells from Fed and Fasted Chickens

The liver is the major site of *de novo* fatty acid synthesis in the chicken, providing adipose tissue with a large fraction of its fatty acid precursors for lipogenesis (39, 41). Monolayer cultures of liver cells from fed chickens synthesize fatty acids from acetate at substantial rates, 1.5–3.0 μmol of acetate incorporated into fatty acyl groups per minute per gram dry weight of liver when measured 4–48 h after plating. The composition of lipids synthesized from [^{14}C]acetate by chicken liver cells in monolayer culture for 24 h is illustrated in Table I, triglyceride being the major lipid formed. The pattern of lipids synthesized approximates that of serum VLDL (2), the principal lipid-containing secretory product of avian liver.

The high initial rate of fatty acid synthesis by liver cells in monolayer culture can be maintained

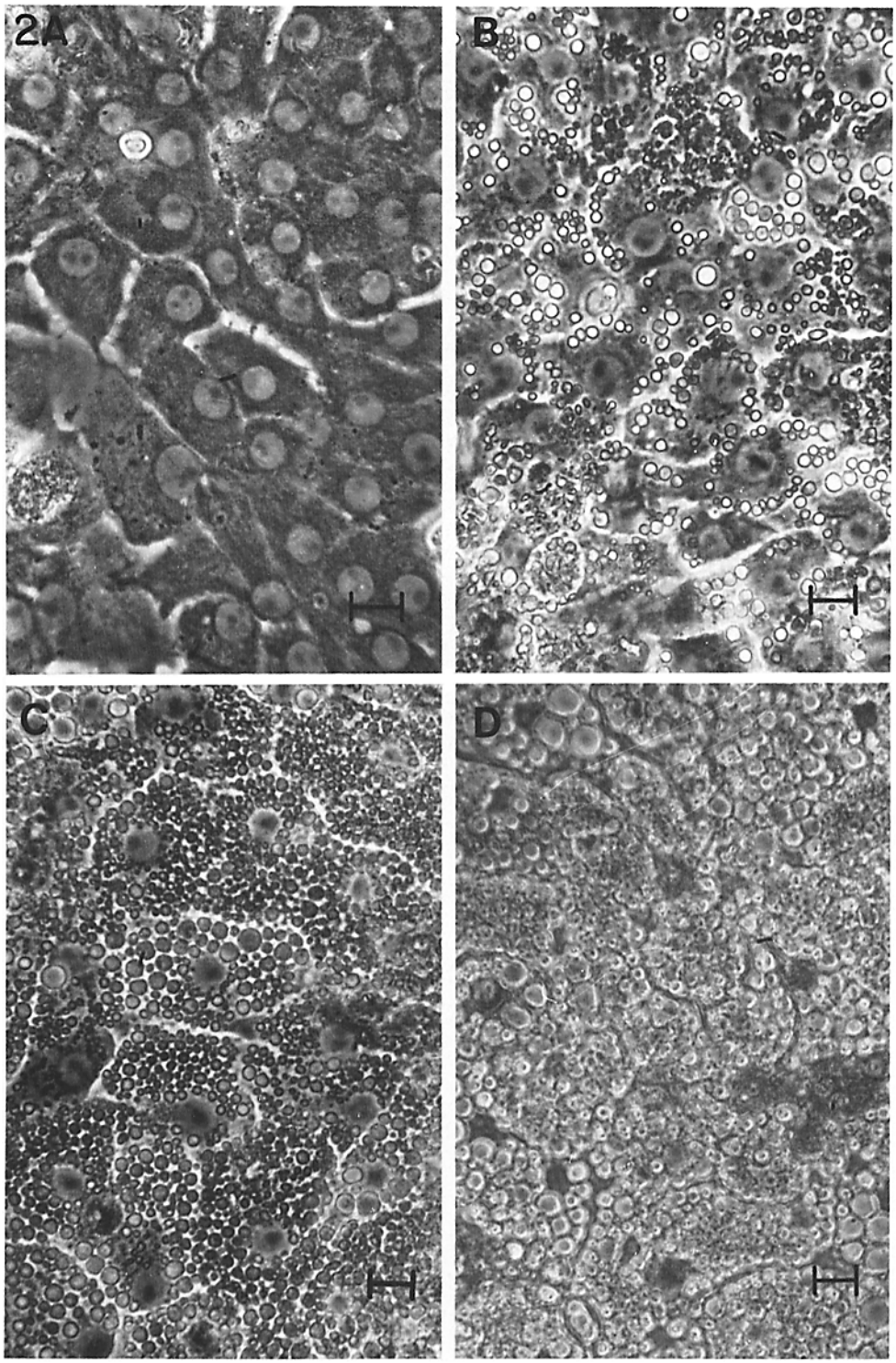

FIGURE 2 Light micrographs of chicken liver cells in monolayer culture. Liver cells from fed (*A* and *B*) and fasted (*C* and *D*) chickens. Cells from fed chickens were maintained in monolayer culture for 48 h with standard medium containing 5% rooster serum, and in (*A*), insulin plus glucagon, or in (*B*), insulin. Note the presence of cytoplasmic triglyceride-rich vesicles in (*B*) and their absence in (*A*). In (*C*) and (*D*), cells from chickens fasted for 36 h were plated in standard culture medium containing 1% rooster serum and insulin. At 4 h, medium was removed and replaced with the same medium minus serum after which cells were maintained in culture with insulin in (*C*) for 96 h and in (*D*) for 120 h. With cells from fasted chickens, the accumulation of triglyceride-rich vesicles was delayed by 24–36 h relative to that of cells from fed chickens. Bars, 1 μm. (*A*) × 800; (*B-D*) × 621.

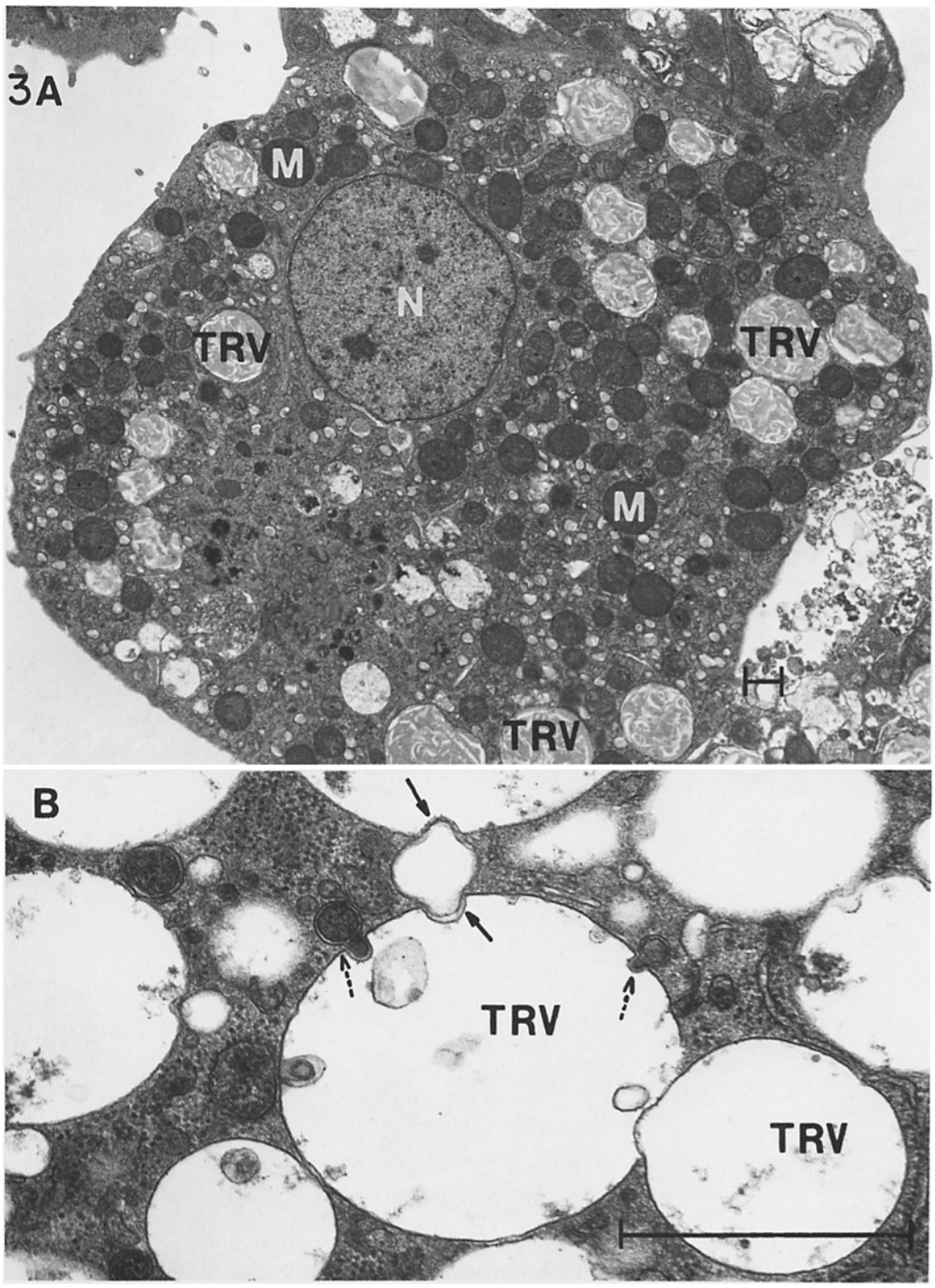

FIGURE 3 Electron micrographs of liver cells in monolayer culture. (*A*) Liver cells from fed chickens in monolayer culture for 24 h in standard medium containing 5% rooster serum and insulin, illustrating cytoplasmic triglyceride-rich vesicles. M, mitochondria; N, nucleus; TRV, triglyceride-rich vesicles. (*B*) Membrane-enclosed, triglyceride-rich vesicles at higher magnification. Large triglyceride-rich vesicles appear to be undergoing fusion with smaller triglyceride-rich vesicles (unbroken arrows) and lipoprotein-containing vesicles (broken arrows); the latter contain osmiophilic particles having the properties of VLDL. The triglyceride-rich vesicles are bounded by a membrane bilayer. Bars, 1 μm. (*A*) × 5,200; (*B*) × 42,000.

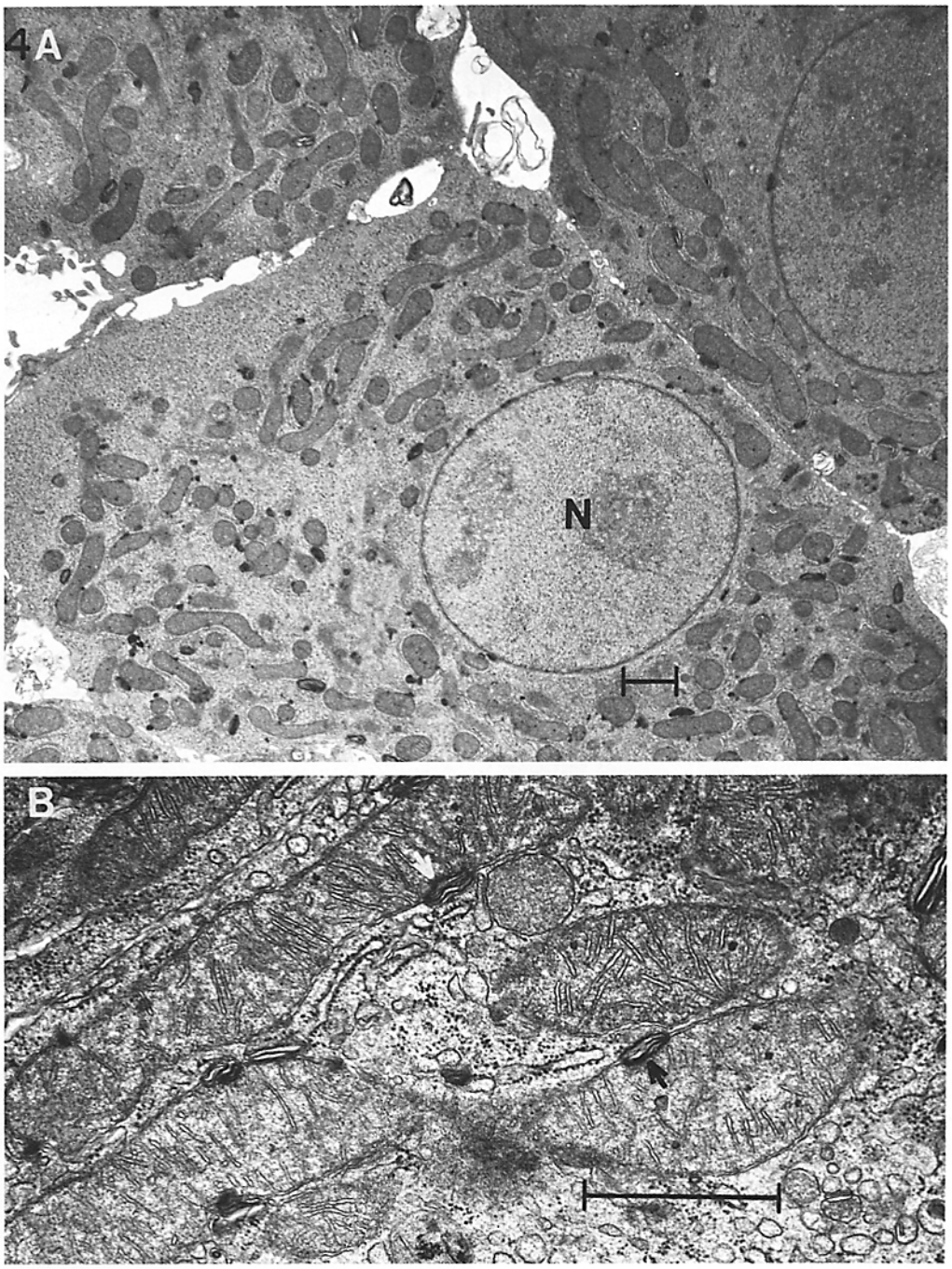

FIGURE 4 Electron micrographs of liver cells in monolayer culture. (*A*) Liver cells in monolayer culture for 24 h in standard medium containing 5% rooster serum and both insulin and glucagon. Glucagon prevents the formation of the cytoplasmic triglyceride-rich vesicles shown in Fig. 3. N, nucleus. (*B*) Mitochondria of the same cells at higher magnification, illustrating altered membrane structure. The focal thickenings observed on the mitochondrial surface in (*A*) are shown at higher magnification in (*B*) to be due to membrane whorls (arrows) in the intercristal space between the inner and outer mitochondrial membranes. Bars, 1 μm. (*A*) × 7,000; (*B*) × 28,000.

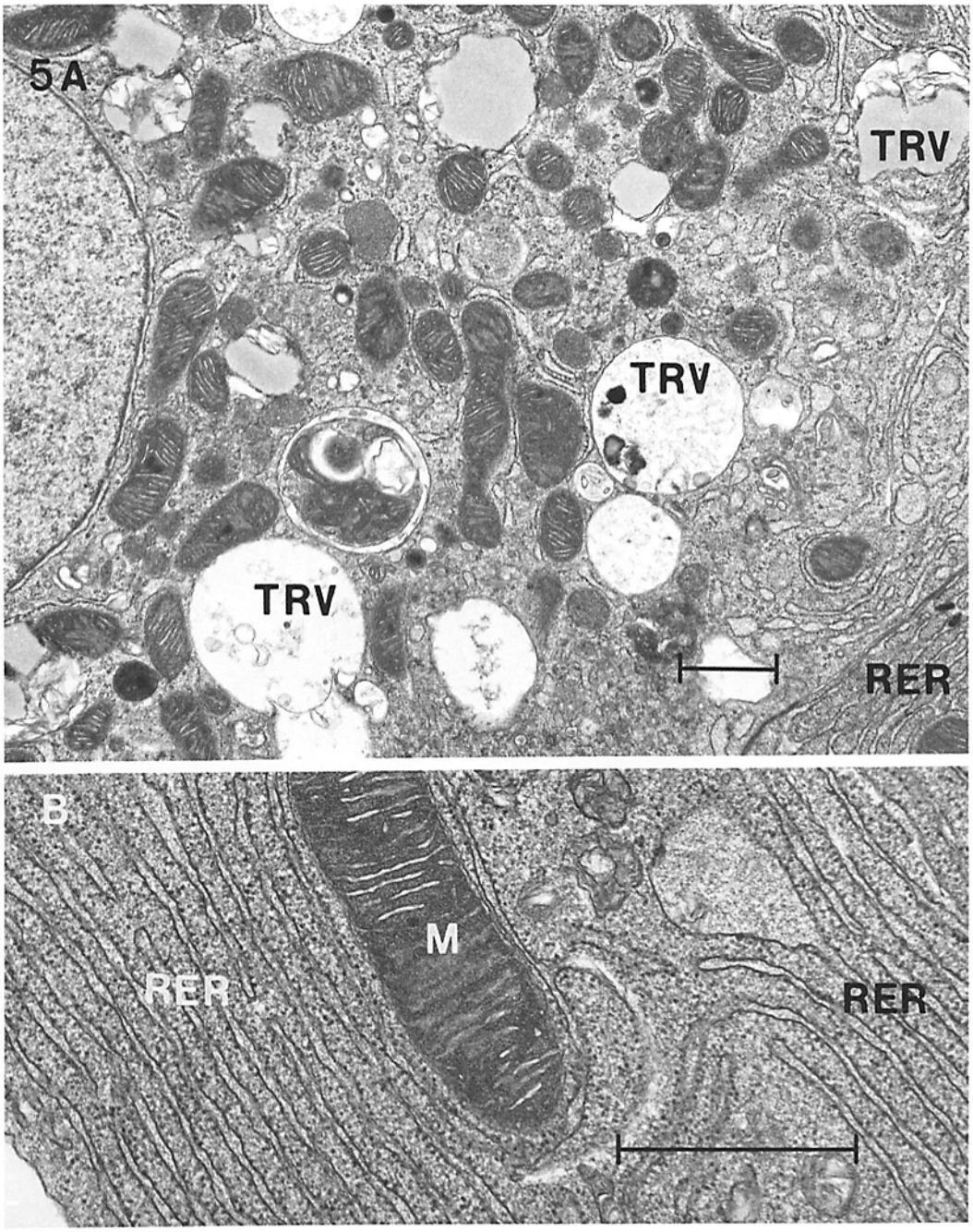

FIGURE 5 Electron micrographs of liver cells in monolayer culture. Regions of liver cells from estradiol-treated chickens in monolayer culture for 24 h in standard medium containing 5% rooster serum and insulin. Illustrated in (*A*) and (*B*) is the extensive development of the rough endoplasmic reticulum (*RER*) and in (*A*) the presence of triglyceride-rich vesicles (*TRV*). M, mitochondria. Bars, 1 μm. (*A*) × 14,000; (*B*) × 35,000.

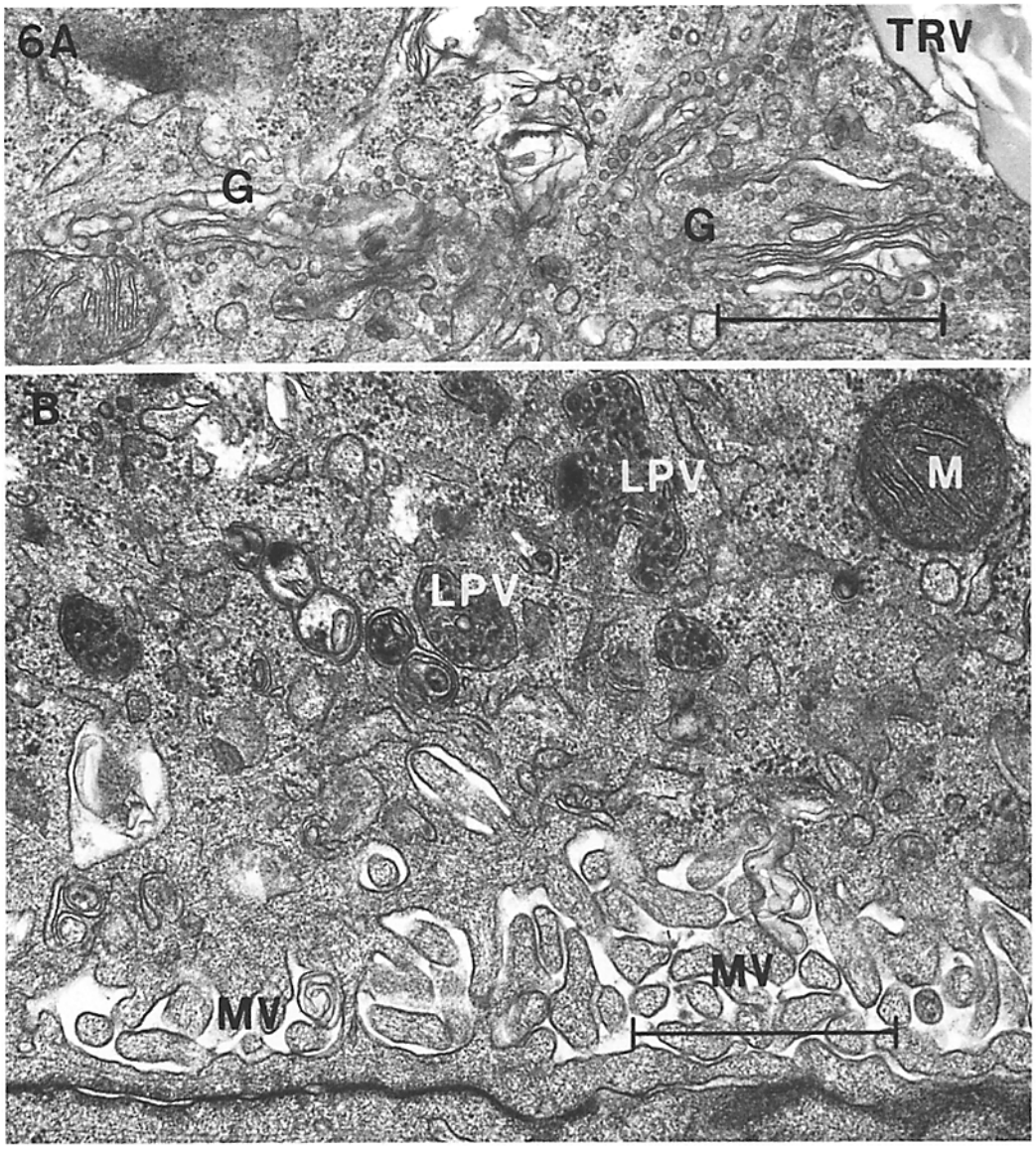

FIGURE 6 Electron micrographs of liver cells in monolayer culture. (*A*) Golgi-rich region and (*B*) microvilli of liver cells from estradiol-treated chickens in monolayer culture for 24 h in standard medium containing 5% rooster serum and insulin. Shown in (*B*) near the microvilli are numerous lipoprotein vesicles; contained within these vesicles are osmiophilic electron-dense structures which closely resemble VLDL particles. TRV, triglyceride-rich vesicles; G, Golgi complex; M, mitochondria; LPV, lipoprotein vesicle; MV, microvilli. Bars, 1 μm. (*A*) × 28,000; (*B*) × 35,000.

for up to 48 h, provided a rich medium containing insulin is used (Fig. 7*A*). If insulin is omitted, the fatty acid synthetic capacity of the cells declines slowly with a half-life of about 22 h (Fig. 7 A); 5×10^{-2} μg of insulin/ml of medium is sufficient to maintain a maximal rate of fatty acid synthesis for 24–48 h. Preliminary results[1] indicate that this decline is associated with a corresponding decrease in the cellular level of acetyl-CoA carboxyl-

[1] Watkins, P. A., and D. M. Tarlow. Manuscript in preparation.

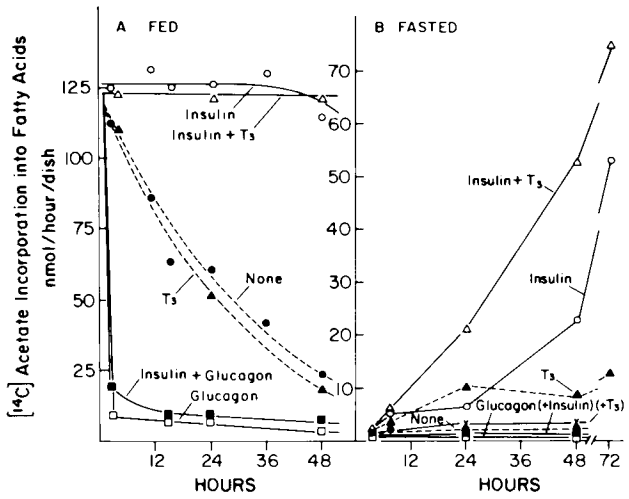

FIGURE 7 The effect of insulin, triiodothyronine, and glucagon on fatty acid synthesis by liver cells in monolayer culture derived from fed and fasted chickens. Liver cells (2.8×10^6 cells/35-mm dish) from (A) fed or (B) 36-h-fasted chickens were maintained in monolayer culture in the presence of the hormones indicated. Cells from fed chickens were provided with the standard medium containing 5% rooster serum; cells from fasted chickens were carried on the same medium containing 1% rooster serum for 4 h after plating, after which the medium was removed and replaced with serum-free medium for the duration of the experiment. At the times indicated, fresh medium containing 5 mM [1-^{14}C]acetate was added and the cells were incubated for 1 h. Incorporation of ^{14}C-activity into total fatty acyls of cells plus medium was determined as described in Materials and Methods. ●, no hormone; ○, insulin; ▲, triiodothyronine (T_3); □, glucagon; △, insulin + T_3; ■, glucagon + insulin; ×, glucagon + insulin + T_3.

ase, a key regulatory enzyme in fatty acid biosynthesis (18, 19, 31, 32, 38). The addition of glucagon either in the presence or in the absence of insulin causes an immediate (within 15 min) and almost complete inhibition of [^{14}C]acetate incorporation into fatty acids (Fig. 7 A). Glucagon inhibits the rate of incorporation of ^{3}H from ^3H_2O into fatty acids to the same extent (results not shown), which indicates that *de novo* fatty acid synthesis per se is affected; 5×10^{-3} μg of glucagon/ml of medium is required for half-maximal inhibition of fatty acid synthesis. These biochemical changes correlate well with the morphological alterations caused by the same hormones. It should be recalled that insulin induces the accumulation of triglyceride-rich cytoplasmic vesicles and that this effect is completely blocked by glucagon (Fig. 2 *B* and *A*, respectively).

Earlier investigations (31, 32) have established that fasting or rendering an animal diabetic drastically curtails hepatic lipogenesis. Recovery from these states by feeding carbohydrate or by administering insulin requires at least 24 h in vivo (31). These effects can be simulated under cell culture conditions with liver cells obtained from fasted chickens. The rate of fatty acid synthesis in "fasted" hepatocytes is initially <5% that of "fed" cells and remains constant without hormone addition (Fig. 7 B). Addition of insulin to the culture medium causes a gradual restoration of synthetic capacity of fasted cells to normal over the course of several days. This process is accelerated when triiodothyronine is present in addition to insulin (Fig. 7 B); 5×10^{-5} μg of triiodothyronine/ml of medium is required to elicit a half-maximal increase of fatty acid synthesis in the presence of insulin. Concomitant with the restoration of the lipogenic rate is a corresponding return to normal of the activity of acetyl-CoA carboxylase[1]. Glucagon blocks the increase in the rate of [^{14}C]acetate incorporation in the presence of insulin or insulin plus triiodothyronine (Fig. 7 B). In contrast to fasted liver cells, fed cells are unresponsive to triiodothyronine (Fig. 7 A).

The magnitude of the glucagon-induced inhibition of fatty acid synthesis in fed hepatocytes and the rapidity with which it occurs prompted a further study of this phenomenon. The mode of action of glucagon is thought to be via the second messenger, cAMP. Therefore, cAMP, its dibutyryl derivative (N^6,O^2-dibutyryl cAMP), and cholera toxin, a potent activator of adenyl cyclase

(3), were tested as potential inhibitors. Like glucagon, both dibutyryl cAMP and cholera toxin markedly inhibit fatty acid synthesis (Table II). Inhibition by cAMP is not as great, presumably because of its slower rate of entry into cells as compared with dibutyryl cAMP (45). The presence of insulin in the culture medium does not prevent the inhibitory action of either glucagon or dibutyryl cAMP (Table II).

Like lipogenesis, cholesterol synthesis by the liver provides another basic precursor for the assembly of VLDL. This normal hepatic function is also retained by chicken liver cells in primary culture. Moreover, physiological agents known to affect cholesterogenesis in vivo have comparable effects. As illustrated in Table III, insulin is required to maintain the rate of cholesterol synthesis at its high initial level, a rate equivalent to that observed in vivo (8). Both glucagon and dibutyryl cAMP, which inhibit fatty acid synthesis almost completely, even in presence of insulin (Fig. 7 and Table II), have a less drastic short-term effect on cholesterogenesis under similar conditions. However, after exposure to these agents for 24 h (in the presence of insulin), cholesterogenesis is decreased by 60–70%. Certain oxygenated derivatives of cholesterol, i.e., 7-ketocholesterol and 25-hydroxycholesterol, are potent inhibitors of cholesterogenesis by other cell types in culture (6, 28). These agents appear to act by blocking the synthesis of HMG-CoA reductase (6, 28) and by another mechanism not yet elucidated (6). When monolayer cultures of chicken liver cells are incubated for 4 h with 7-ketocholesterol or 25-hydroxycholesterol in the presence of insulin, there is little effect on cholesterogenesis; a much greater inhibition occurs after 24 h, particularly with 25-hydroxycholesterol (Table III). Inhibition by these agents is specific in that neither protein nor RNA synthesis is affected.

TABLE II

*Hormonal Effectors of Fatty Acid Synthesis by Chicken Liver Cells in Primary Culture**

Additions	Rate of [^{14}C]acetate incorporation into fatty acids	
	nmol/h/dish	*% of control*
Exp I		
None	59	100
Glucagon	1.2	2
diBu-cAMP	6.5	11
cAMP	16	28
Cholera toxin	2.9	5
Exp II		
None	67	100
Glucagon	3.4	5
diBu-cAMP	6.7	10
Insulin	72	107
Insulin + glucagon	6.6	10
Insulin + diBu-cAMP	3.3	5

* Liver cells isolated from normal fed chicks were plated (2.8×10^6 cells/35-mm dish) and incubated for 4 h. Medium was then removed and fresh medium containing the indicated additions and 5 mM sodium [1-^{14}C]acetate (except when cholera toxin was tested) was added. After a 1-h incubation the amount of [^{14}C]acetate incorporated into total fatty acids (free plus saponifiable) of cells and medium was determined as described in Materials and Methods. When cholera toxin was added, cells were incubated for an hour with the toxin before the addition of [^{14}C]acetate. Insulin and glucagon were added at a level of 5 μg/ml, cholera toxin at 1 μg/ml, cAMP and dibutyryl cAMP (diBu-cAMP) (N^6,O^2-dibutyryl adenosine 3′,5′-cyclic phosphate) at 0.1 mM. Each data point represents the mean of duplicate dishes.

TABLE III

*Factors Affecting Cholesterogenesis in Chicken Liver Cells in Primary Culture**

Additions	Rate of [^{14}C]acetate incorporation into cholesterol at:	
	4 h	24 h
	nmol/h/dish	
None	3.0	0.7
Glucagon	2.1	0.4
diBu-cAMP	1.6	0.3
Insulin	3.1	3.4
Insulin + glucagon	2.8	1.2
Insulin + diBu-cAMP	2.1	1.0
Insulin + 7-ketocholesterol	2.6	1.9
Insulin + 25-hydroxycholesterol	2.5	0.4

* Liver cell monolayers (2.8×10^6 cells/35-mm dish) prepared using cells from fed chicks were incubated for 4 or 24 h with the additions. Fresh medium containing 5 mM sodium [1-^{14}C]acetate was then added and incubation was continued for 1 h. The incorporation of [^{14}C]acetate into cholesterol of cells and medium was determined as described in Materials and Methods. Insulin, glucagon, and 7-ketocholesterol were added at a level of 5 μg/ml, 25-hydroxycholesterol at 10 μg/ml, and dibutyryl cAMP (diBu-cAMP) (N^6,O^2-dibutyryl adenosine 3′,5′-cyclic phosphate) at 0.1 mM. 1% rooster serum was present during the first 4 h of incubation. Each data point represents the mean of duplicate dishes.

Synthesis and Secretion of VLDL by Monolayer Cultures of Liver Cells from Estradiol-Treated and Untreated Chickens

To measure the synthesis and secretion of VLDL by immunoprecipitation, it was necessary to purify, characterize, and obtain antibody against this lipoprotein. Purification of VLDL from laying hen serum was accomplished by flotation at serum density in the ultracentrifuge followed by gel filtration on Sepharose-6B (see Materials and Methods for details).

The apoprotein subunit composition of chicken VLDL was determined by dodecyl sulfate-acrylamide gel electrophoresis after dissociating the purified lipoprotein with sodium dodecyl sulfate. Under these conditions, chicken VLDL gives rise to 3 major stained apoprotein bands (Fig. 8, gels *A* and *B*). The largest of these, band I on gel *A*, has a molecular weight around 300,000, based on its mobility in 3.3% acrylamide gels, and appears to correspond to the *B* apopeptide of human VLDL (7). This band stains positively with periodic acid-Schiff reagent (15), indicating that it is a glycoprotein. When tested in the dodecyl sulfate-acrylamide gel electrophoresis system, human VLDL gives rise to a major stained apoprotein band (results not shown) with a mobility identical to that of band I of gel *A* (Fig. 8). Chicken VLDL, like human VLDL, gives rise to a diffuse stained apoprotein band (Fig. 8, gel *A*, band II) just beyond the tracking dye on 3.3% acrylamide gels. This band can, however, be resolved into two stained apoprotein bands (Fig. 8, gel *B*, bands II and III) when the pore size of the gel is reduced by raising the acrylamide concentration to 10%. The slower moving of these two bands (band II) has a slightly faster mobility than cytochrome *c*, indicating a molecular weight of 10–11,000. The faster moving band (band III) has a molecular weight of 7–8,000 based on its mobility relative to insulin.

Antiserum for immunoprecipitation was obtained from rabbits immunized with purified VLDL. Antibody generated against VLDL precipitates only the VLDL component of laying hen serum as indicated by the appearance of a single precipitin band upon Ouchterlony double-diffusion analysis (Fig. 9).

The capacity of chicken liver cells in monolayer culture (with insulin present) to synthesize and secrete VLDL was investigated utilizing the anti-

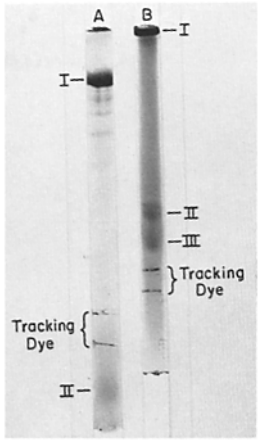

FIGURE 8 Polyacrylamide gel electrophoresis of dodecyl sulfate-dissociated chicken VLDL. VLDL isolated from laying hens' serum was dissociated with SDS, subjected to electrophoresis on (*A*) 3.3% and (*B*) 10% acrylamide gels, and then stained as described in Materials and Methods. To each gel were applied 50 μg of dissociated VLDL protein.

VLDL antibody for immunoprecipitation. To maximize VLDL synthesis, 17-β-estradiol was administered (20 mg/day per kg body weight, intraperitoneal) to the donor animals for 3 days before liver cell isolation. Synthesis and secretion of VLDL protein were measured by following the rate of [^{3}H]leucine incorporation into immunoprecipitable [^{3}H]VLDL appearing in the cells and medium. It is evident (Fig. 10 B) that during a 4-h labeling period substantial ^{3}H-label is incorporated into immunoprecipitable VLDL appearing in the medium. That this immunoprecipitable ^{3}H-activity is VLDL is indicated by its flotation when the medium with unlabeled VLDL added is centrifuged at serum density for 14 h at 300,000 *g*. Less than 1% of the label in the immunoprecipitate is extractable with acidic chloroform-methanol (2:1, vol/vol), demonstrating that ^{3}H is not incorporated to a significant extent into the lipid components of VLDL. When subjected to dodecyl sulfate-acrylamide gel electrophoresis, the ^{3}H-VLDL immunoprecipitate exhibits apoprotein patterns on both 3.3 and 10% acrylamide gels (Fig. 10 B) identical to those of purified VLDL (Fig. 8) and authentic ^{3}H-VLDL isolated from the serum of an estrogen-treated chick injected with [^{3}H]leucine (Fig. 10 A). A similar VLDL-apoprotein labeling pattern is obtained (Fig. 10 C) for the immunoprecipitate of cellular material. ^{3}H-

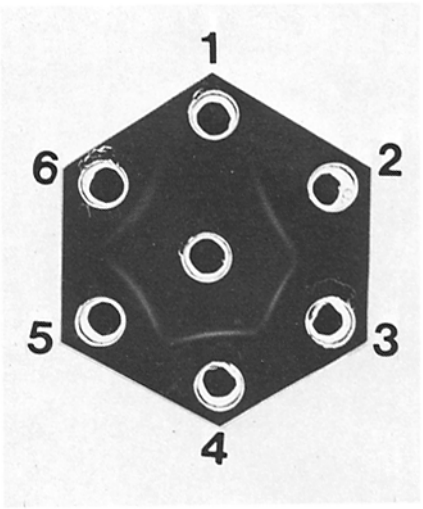

FIGURE 9 Ouchterlony double diffusion of anti-VLDL antiserum against laying hen serum. Agarose gels (0.5%) contained 0.15 M NaCl, 0.02% sodium azide, and 20 mM potassium phosphate, pH 7.0. The center well contained 10 μl of rabbit anti-VLDL antiserum; Wells 1-4 contained 0, 5, 10, and 20 μl, respectively, of laying hen serum diluted 1:4; wells 5 and 6 contained 10 and 20 μl, respectively, of undiluted laying hen serum. Plates were developed at room temperature for 3 days.

VLDL appearing in the medium during the 4-h incubation with [^{3}H]leucine appears to arise via secretion rather than by leakage from damaged cells. This is indicated by the fact that <2% of the total cellular lactate dehydrogenase, a cytoplasmic marker enzyme, appears in the medium during the 4-h labeling period. In contrast, immunoprecipitable ^{3}H-VLDL secreted into the medium accounts for 25–30% of the total ^{3}H-VLDL (cellular plus medium) during this period. Pulse-chase experiments (not shown) reveal that 80% of cellular ^{3}H-VLDL formed during a 1-h pulse with [^{3}H]leucine appears in the medium during a 2-h chase with unlabeled leucine. In addition, dodecyl sulfate-acrylamide gel electrophoretic patterns of labeled VLDL incubated with cells in culture for up to 24 h are identical with those shown in Fig. 10 B. This and the fact that the level of immunoprecipitable ^{3}H-VLDL did not decrease, shows that degradation of VLDL is not significant. Thus, it may be concluded that liver cells from estrogenized chicks in monolayer culture synthesize and secrete VLDL. Moreover, these processes are still active in cells maintained in culture for 48 h.

In avian species the liver has the unique role of synthesizing and secreting lipid and protein components, primarily as VLDL, for egg yolk formation in the ovary (36). Since this process is under the control of estrogen, it was of interest to compare the rates of VLDL synthesis and secretion by liver cell monolayers derived from untreated and estradiol-treated chickens. As illustrated in Fig. 11 B, after a short lag period, liver cells from estrogen-treated chickens secrete ^{3}H-VLDL at a rate five times that of cells from untreated controls. Intracellular levels of newly synthesized ^{3}H-VLDL are increased as well in cells from estrogenized animals. The estrogen effect on VLDL synthesis and secretion is highly specific in that total protein synthesis and secretion is increased by no more than 10% by estradiol treatment (Fig. 11 A). A comparison of the relative rates of [^{3}H]leucine incorporated into immunoprecipitable VLDL (Fig. 11 B) and into TCA-precipitable protein (Fig. 11 A) reveals that 10% of the labeled protein secreted is VLDL. In terms of total protein synthesized, i.e., that in cells plus medium, 8.5% is VLDL apoprotein.

Insulin (0.05–5 μg/ml) promotes a two- to three-fold increase in the rate of VLDL apoprotein synthesis as measured by [^{3}H]leucine incorporation into immunoprecipitable VLDL (results not shown). This effect is not immediate, but is elicited within 24 h and is retained for 72 h. Insulin also causes an increase of similar magnitude in [^{3}H]leucine and [^{3}H]uridine incorporation into cellular protein and RNA, respectively. Hence, the effect of insulin on VLDL apoprotein synthesis is probably not specific. It is also of interest that neither glucagon nor dibutyryl cAMP blocks the insulin-induced increase in protein or RNA synthesis.

DISCUSSION

The investigation of hepatic metabolism has been greatly facilitated by the use of isolated liver cells. Hepatocyte isolation techniques most commonly involve perfusion of the liver via the portal vein with collagenase and hyaluronidase (4, 9–12, 25, 40, 54). Although external digestion of embryonic chick liver with these enzymes produces viable hepatocytes (16), such cells lack significant lipogenic activity (17) since differentiation of the lipogenic enzymes occurs only after hatching. We have found the external digestion procedure to be satisfactory for use with liver tissue from 2- to 3-wk-old chickens. By use of this method large quantities of viable cells having in vivo lipogenic

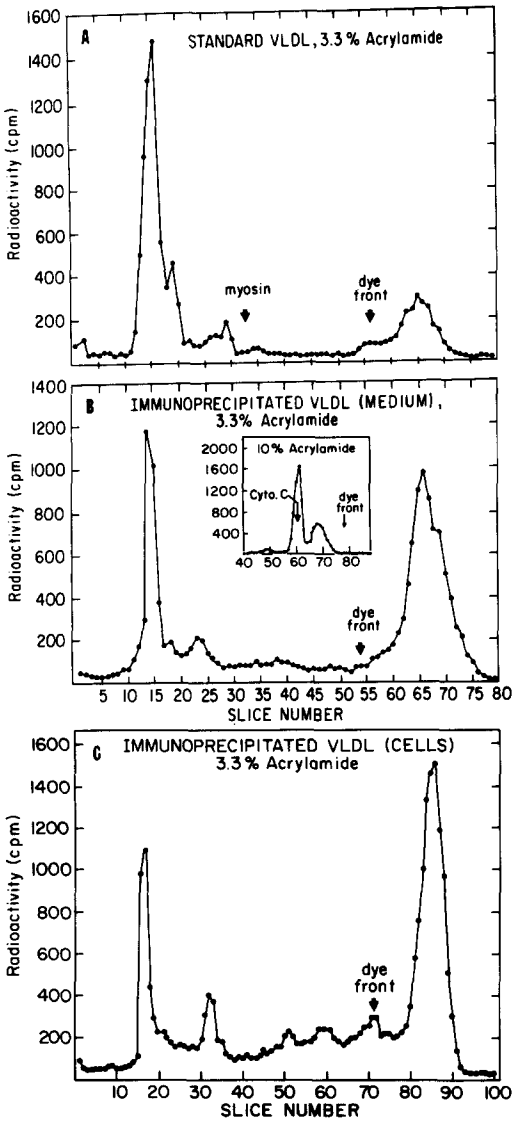

FIGURE 10 Dodecyl sulfate-acrylamide gel electrophoretic patterns of: (A), ^{3}H-VLDL labeled in vivo with [^{3}H]leucine and (B) and (C) cellular and secreted ^{3}H-VLDL immunoprecipitates, respectively, from liver cell monolayers incubated with [^{3}H]leucine. For (A) serum ^{3}H-VLDL was isolated from the serum of an estrogenized chicken after injecting [^{3}H]leucine (see Materials and Methods for details). The ^{3}H-VLDL was dissociated with SDS and then subjected to dodecyl sulfate-acrylamide gel electrophoresis as described in Materials and Methods. For (B) and (C), liver cells from 13-day-old estradiol-treated chickens were plated in standard culture medium containing 5% rooster serum and insulin. After 4 h the medium was removed and replaced with 2 ml of medium containing 100 μCi of [5,6-^{3}H]leucine (5 mCi/μmol) in place of unlabeled leucine. 4 h later, ^{3}H-VLDL was immunoprecipitated from cell extracts and medium, dissociated with SDS, subjected to dodecyl sulfate-acrylamide gel electrophoresis, gel was sliced (80 or 100 slices), and the slices were analyzed for ^{3}H-activity as described in Materials and Methods.

rates can be prepared quickly under aseptic conditions.

One of the principal limitations of isolated hepatocyte systems has been their relatively short duration of viability. Therefore, the development of a cell culture system in which chicken liver hepatocytes can be maintained for several days should prove extremely useful for long-term studies on the regulation of hepatic lipid metabolism. The ability of chicken liver cells to form monolayers greatly increases the utility of this system. The presence of homologous serum was found to be necessary for attachment of chicken liver cells to culture dishes, but not for their subsequent maintenance in monolayer culture. This is evident from the fact that fasted hepatocytes, plated for 4 h in the presence of 1% serum and carried in serum-free medium thereafter, retain their viability and their responsiveness to hormones. The presence of insulin in the medium was found to accelerate cell flattening and spreading as reported for another system (37). Although the monolayers used in the present studies were maintained for only 4–5 days, preliminary studies indicate that liver cells in culture remain viable for 10 days or longer.

Liver cells derived from chickens of differing nutritional-physiological state, e.g. fasted, fed, or estrogen-stimulated, behave in monolayer culture like their counterparts in vivo with respect to lipogenesis and VLDL apoprotein synthesis. Their morphological properties in culture are characteristic of the physiological-nutritional state of the donor animal. Of significance is the fact that changes in apparent physiological state can be brought about in culture by treatment with appropriate hormones. In particular, the hormonal status of liver cells in the fasted or fed states can be simulated with glucagon or insulin and triiodothyronine, respectively. It is established (31, 32) that fasting or rendering an animal diabetic drastically curtails lipogenesis. Like fasting or the diabetic state where the plasma insulin/glucagon ratio is depressed (50), deleting insulin from the culture medium leads to an 80–85% loss of fatty acid synthetic capacity of liver cells in culture within 48 h. Glucagon or dibutyryl cAMP greatly acceler-

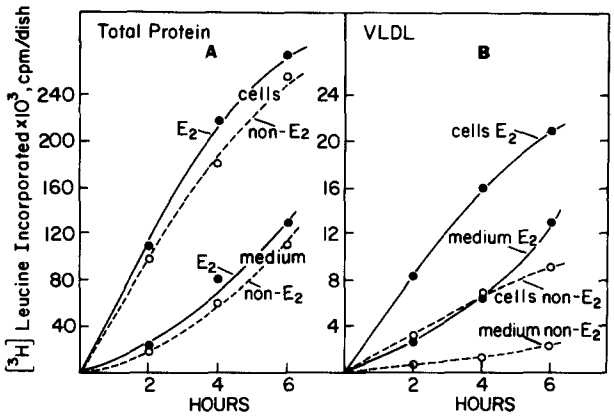

FIGURE 11 The effect of estradiol treatment in vivo on the synthesis and secretion of immunoprecipitable ^{3}H-VLDL by chicken liver cells in monolayer culture. Liver cell monolayers from untreated (non-E_2) and estradiol-treated (E_2) chickens were prepared and incubated with [^{3}H]leucine-containing medium as described in Fig. 10. [^{3}H]leucine incorporated into cellular and secreted TCA-precipitable protein (total protein) and immunoprecipitable VLDL was determined as described in Materials and Methods.

ates the decline in activity. This pattern is consistent with the fact that hepatic cAMP levels rise during fasting or as a result of diabetes (27). Despite a low level of lipogenesis, glucagon- or dibutyryl cAMP-treated cells synthesize RNA and protein at rates comparable to or exceeding initial levels, suggesting that the metabolic integrity of the cells is preserved.

Like the situation in vivo, liver cells in monolayer culture derived from fasted chickens exhibit a greatly reduced fatty acid synthetic capacity, as well as low acetyl CoA carboxylase activity.[1] Both activities can be restored to normal levels in culture by insulin with or without triiodothyronine, although the combination of the two hormones accelerates recovery. It is interesting that embryonic chick liver cells in culture, which are virtually devoid of the enzymes of fatty acid synthesis, require only triiodothyronine for their induction; insulin has little or no effect (17). Thus, the hormonal stimuli for the induction of hepatic fatty acid synthetic capacity during neonatal differentiation appear to differ from those required for recovery from the fasted state.

Of importance is the fact that glucagon (or dibutyryl cAMP) opposes the effect of insulin by totally blocking the recovery of lipogenesis. Recent investigations in our laboratory (51)[1] indicate that cAMP exerts its inhibitory effect on hepatic fatty acid synthesis by two mechanisms, one via short-term control and the other through long-term control. In the first instance, cAMP acts within minutes to drastically reduce the cytoplasmic concentration of citrate, an allosteric activator of acetyl CoA carboxylase (32, 33), allowing the enzyme to revert to its inactive conformation. Since the carboxylase catalyzes the first committed step of fatty acid synthesis, the pathway is blocked at its point of inception. This finding accounts for the rapid decrease in fatty acid synthesis by cAMP observed in Fig. 7 A. In addition, cAMP appears to block the insulin-induced synthesis of acetyl CoA carboxylase[1] per se. Thus, a rise in cAMP concentration upon deleting insulin from the medium would be expected to promote a decay in lipogenic capacity by both the short-term and long-term mechanisms.

The increased hepatic output of VLDL in response to estrogen stimulation (29) is also manifested by liver cells in monolayer culture. Cells isolated from estradiol-treated chickens synthesize and secrete VLDL into the medium at substantially greater rates than cells from untreated controls. There is an excellent correlation between this biochemical function and the morphology of cells in culture from estrogen-treated chicks; such cells exhibit more extensive development of the rough endoplasmic reticulum and Golgi complex, as well as the appearance of greater numbers of lipoprotein vesicles. Compelling evidence has been obtained that liver cells in monolayer culture actively secrete VLDL. This is substantiated by pulse-chase studies (49)[2] in which >80% of cellular VLDL formed during a 1-h [^{3}H]leucine pulse is chased into the medium as immunoprecipitable

[2] Reed, R. E., and M. D. Lane. Manuscript in preparation.

^{3}H-VLDL in 2 h. Since <2% of total cellular lactate dehydrogenase activity appears in the medium during this time, true secretion of VLDL and not leakage from damaged cells must have occurred. Thus, liver cells in culture retain the capacity to carry out all phases of lipoprotein production: synthesis, assembly, packaging, and secretion.

A significant fraction of the triglyceride synthesized by liver cells maintained in culture for several days under lipogenic conditions, i.e., insulin present, is incorporated into membrane-enclosed vesicles which appear in the cytoplasm. Both the number and size of the triglyceride-rich vesicles increase with time in culture, leading to a broad size distribution. Although the vesicles are surrounded by a membrane, electron microscopy reveals no association between mature vesicles and cytoplasmic membrane systems. The vesicles can in fact be isolated intact after cell lysis in hypotonic medium. It is evident that there is an association between vesicles; electron micrographs (Fig. 3*B*) reveal that both small lipoprotein vesicles and small triglyceride-rich vesicles interact with larger triglyceride-rich vesicles. The mechanism by which vesicle growth occurs appears to be fusion of interacting vesicles followed by coalescence of their lipid contents (Fig. 3*B*). While this process appears to occur to a limited extent in rat (14) and avian (23) liver cells in vivo, it becomes a predominant outlet for fatty acid and triglyceride synthesized by chicken liver cells after 48 h in culture.

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