GROWTH CONTROL OF DIFFERENTIATED FETAL RAT HEPATOCYTES IN PRIMARY MONOLAYER CULTURE

IX. Specific Inhibition of DNA Synthesis Initiation by Very Low Density Lipoprotein and Possible Significance to the Problem of Liver Regeneration

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ABSTRACT

Rat serum very low density lipoprotein (VLDL) inhibits initiation of DNA synthesis in fetal rat hepatocyte cultures; cells engaged in synthesizing DNA resist inhibition. VLDL action is specific and apparently blocks prereplicative protein synthesis. These and other results, from studies of altered blood VLDL levels and [^{3}H]thymidine incorporation into isolated liver nuclei in 70% hepatectomized normal and mutant hyperlipoproteinemic rats, as well as from infusion studies with a "mitogenic" hormone solution, suggest that hepatic VLDL metabolism is linked to the suppression of hepatocyte proliferation.

Mammalian cells in culture usually display a requirement of homologous sera for optimal proliferation (17, 37). However, primary cultures of differentiated fetal rat hepatocytes grow poorly in media containing adult sera obtained from normal or partially hepatectomized rats (H. Leffert, unpublished results), thus preventing further comparative studies of serum growth-promoting effects (22, 33). While testing serum lipoprotein fractions for DNA synthesis initiation activity, based upon previous studies implicating lipids among the set of factors initiating cultured hepatocyte DNA synthesis (19, 27), we observed that rat serum appreciably stimulated DNA synthesis only after removal of bulk lipoproteins. This report describes in more detail the basis of this observation which apparently results from the inhibitory action of one or more components of very low density lipoprotein (VLDL)[1] particles.

MATERIALS AND METHODS

Reagents

Materials required for primary fetal rat hepatocyte monolayer cultures have been described (22–27). Falcon

[1] *Abbreviations used in this paper:* A_{280}, UV absorbance at 280 nm; BSA, bovine serum albumin; dFBS, dialyzed fetal bovine serum; [^{3}H]dT, C[^{3}H]$_3$-thymidine, EDTA, sodium salt of ethylenediamine tetra-acetic acid; ff, homozygous "Fatty" mutant Zucker rats; FF, homozygous wild-type Zucker rats; HDL, high density lipoprotein; LDL, low density lipoprotein; T_3, 3,5,3'-triiodo-L-thyronine; TAGH, isotonic saline solution of T_3, amino acids, glucagon, and heparin; TCA, trichloracetic acid; VLDL, very low density lipoprotein.

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dishes (35-mm plastic style, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) were used to obviate toxicity problems encountered with contemporary NUNC dishes. Sera were purchased from Grand Island Biological Co., Santa Clara, Calif. Isotopes from New England Nuclear Corp. (Gardena, Calif.) included: D-[3-O-C-$^3H]_3$-glucose, sp act 4-5 Ci/mmol; L-[3-^{3}H]arginine, sp act 20-40 Ci/mmol; and C[$^3H]_3$-thymidine ([^{3}H]dT), sp act 18-20 or 40-50 Ci/mmol. T_3 and bovine serum albumin (BSA) and heparin (100 U per mg) were purchased from Sigma Chemical Co., (St. Louis, Mo.) and Nutritional Biochemical (Cleveland, Ohio), respectively. Porcine glucagon was a gift from Dr. W. Bromer, Lilly Research Laboratories, Indianapolis, Ind. AR10 stripping film was obtained from Eastman Kodak Co., Rochester, N. Y.; Sephadex G-100 (40-120 μm) and G-25 (fine) were obtained from Pharmacia Chemical Co., Piscataway, N. J. Bio-Solv and Liquifluor were purchased from Beckman Instruments (Fullerton, Calif.) and New England Nuclear, respectively.

Animals

Adult Fisher/344 male (180-200 g) and 19- to 21-day pregnant female rats were purchased from Simonsen Labs, Gilroy, Calif. Male rats of the strain *Rattus norvegicus* carrying the "Fatty" gene (55) were obtained from the Harriet G. Bird Memorial Laboratory, Stowe, Mass. Homozygous wild-type (FF) and homozygous "Fatty" mutant (ff) Zucker rats weighed 206 ± 57 (SD) g and 302 ± 46 (SD) g, respectively. All animals were housed at 21°C, three to four per cage, fed standard Purina lab chow and H_2O *ad lib,* and accommodated to light between 0800 and 2000 h.

Preparation of Sera

Left-ventricular blood was obtained under ether anesthesia, clotted at 4°C, and centrifuged at 6,000 *g* for 30 min at 4°C. Unless otherwise noted, blood samples from 70% hepatectomized or laparotomized rats were collected 6 h after surgery, and from infused rats as noted (Table II). Fetal bovine sera (FBS) were heat-inactivated, dialyzed, and stored as previously described (23). Protein concentrations were obtained by A_{280} measurements or with the Folin reagent (31).

Preparation of Serum Lipoprotein Fractions and Lipoprotein-Deficient Serum

Rat lipoprotein fractions were isolated by sequential density ultracentrifugation with an SW-41 rotor (Beckman) at 180,000 *g* according to standard procedures (14). Pooled VLDL (free of chylomicra, $d < 1.006$ g/ml) was washed by two additional centrifugation cycles at $d = 1.006$ in 0.15 M NaCl-0.01% (wt/vol) ethylenediamine tetra-acetic acid (EDTA). LDL and HDL were isolated after adjustment of the $d = 1.006$ infranatant to appropriate densities with solid NaBr: fractions $1.006 > d < 1.050$ and $1.050 > d < 1.21$ were designated as LDL and HDL, respectively. Lipoprotein-deficient sera, prepared as described elsewhere (53), contained <0.5% of the initial serum cholesterol concentration as determined by gas-chromatographic analysis.

Resulting fractions and sera were dialyzed exhaustively at 4°C against 0.15 M NaCl with or without 0.01% EDTA. Fractions were sterilized by passage through 0.4-μm filters (Millipore Corp., Bedford, Mass.) and stored at 4°C. Dialysates also were collected and bioassayed (see Fig. 3).

VLDL Protein-Phospholipid Particle Isolation

Water-soluble neutral lipid-free rat VLDL particles were prepared by a partial delipidation method (13). After 6 *n*-heptane extractions at −12°C, VLDL protein recovery was 50-70%. Lipid analyses (51) showed that <1% of VLDL triglyceride and cholesterol and 25-35% of VLDL phospholipid remained associated with the extracted "apoprotein" particles.

VLDL-Lipid Isolation

All operations were performed at 4°C with glass-distilled solvents and acid-washed glassware. 2 ml of VLDL (3.3 mg protein/ml) were extracted four times with 6 vol of chloroform-methanol (1:1), and the resulting lipid extracts were washed as previously described (51, 52). Extracts were partitioned into an "aqueous wash" fraction, shown to contain water-soluble non-lipids, traces of acidic phospholipid and ganglioside (if present in the sample), and into a lipid-soluble fraction containing all neutral and polar lipid. This fraction was purified further by Sephadex G-25 chromatography before separation into neutral lipid, glycolipid, and phospholipid classes with a silicic acid column (52).

Mild alkaline hydrolysis of dried lipid fractions was performed at 37°C for 3 h in 0.1 N KOH in 95% ethanol. Saponified samples were acidified (0.2 ml of 12 N HCl) and extracted with hexane to remove free fatty acids; water-soluble products were neutralized and subsequently tested for biological activity.

A volume of phosphate-buffered saline equivalent to the volume of VLDL extracted was added to each dried lipid fraction and sonicated for 30 s at maximum intensity (Branson Microtip Sonicator, model 140D, Heat Systems-Ultrasonics, Inc., Plainview, N.Y.). Lipid emulsions were stored at 4°C and bioassayed within 12 h of preparation. Parallel mock 0.15 M NaCl extractions were bioassayed to control for residual contamination of fractions by solvents or by trace impurities ("diluent", see Fig. 6).

Ammonium Sulfate Fractionation of Lipoprotein-Deficient Rat Serum

90 ml of sera (A_{280} U/ml = 64; ca. 54 mg protein/ml) from normal or 70% hepatectomized rats were made lipoprotein-deficient (53) and adjusted to initial volumes with 0.15 M NaCl (A_{280} U/ml = 52). Solid $(NH_4)_2SO_4$ was added to 80 ml of this material ($\leq$4 μg cholesterol/ml) at 4°C with constant stirring to obtain these fractions (percentage of saturation): 0–30; 30–50; 50–70; 70–80; and $\geq$80. Fractions were dialyzed exhaustively against 0.15 M NaCl; total protein recovery was 85–90%. Bioassay revealed that >80% of in vitro hepatocyte DNA synthesis initiation-activity (19) was present throughout fractions $\geq$50% saturation (27). Two of these were boiled (50–70%, 5 min; 70–80% 1 min), clarified by centrifugation at 15,000 *g* for 30 min at 4°C, and both supernates were combined with the $\geq$80% fraction (whose activity was heat labile). Precipitated material which formed after boiling was biologically inactive. Pooled active material was dialyzed exhaustively against H_2O at 4°C, lyophilized, and resuspended in 10 ml 0.15 M NaCl. This fraction represented 8–10% of the starting protein (A_{280} U/ml = 35–40) and was used for gel filtration.

Gel Filtration

All operations were performed at 4°C. A glass column (1.5 × 90 cm) was packed with Sephadex G-100 equilibrated in 0.05 M phosphate buffer, pH 7.4. The ammonium sulfate fraction for chromatography (A_{280} U/ml ~ 36, 4.8 ml) was dialyzed against and eluted with similar buffer. Calibration, collection of column fractions, and protein recoveries were carried out as previously described (25).

DNA Synthesis Bioassays

12-day old $G_{0,1}$-arrested hepatocyte cultures were prepared and initiation assays performed as previously described (19), except that 2.3 × 10^5 cells/dish were seeded. DNA synthesis rates at 22–24 h and the percentage of cells entering S phase (i.e., "recruited") were determined by standard [^{3}H]dT (sp act 18–20 Ci/mmol) pulse-labeling and radioautographic techniques, respectively (19), unless otherwise noted. Quiescent 3T3 and fetal rat fibroblast cultures were established, and DNA synthesis initiation assays were performed as described elsewhere (17, 48); methods for culturing SV40-virus transformed 3T3 cells have been described (17).

DNA synthesis was stimulated in intact rats by a 30-min tail vein infusion of 5 ml of TAGH solution (42) containing 3,5,3′-triiodo-L-thyronine (T_3) (100 μg), glucagon (1 mg) and heparin (100 U), pH 7.4. Amino acids used were a 50× concentrate (in 0.15 M NaCl) of the complete mixture present in cell culture medium (22, 23). The infusate was delivered to animals in Bowman cages from a 10-ml syringe fixed to a constant flow infusion pump (Sage Instruments Div., Orion Research Inc., Cambridge, Mass.). Control rats received a 5-ml saline infusion.

DNA synthesis rates were determined in normal and mutant rats (after 70% or sham hepatectomy) or in the infused animals 22–24 h after surgery. Operations were performed under ether anesthesia between 0900 and 1200 h as previously described (29). [^{3}H]dT (sp act 40–50 Ci/mmol; 50 μCi/kg body weight) was administered intrajugularly; nuclei were prepared from caudate and accessory lobes (2). Nuclear aliquots (500 μl) were precipitated by cold 5% vol/vol trichloracetic acid (TCA) onto Whatman GF/C filters, and radioactivity was determined by scintillation counting (19). DNA was quantitated by the diphenylamine reaction (7).

In Vitro Cell Counts

Attached cells were trypsinized and counted with a Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.) (22–27).

In Vitro Precursor Uptake Studies

Labeled precursors were added to cultures ca. 24 h after initiating DNA synthesis. Media were aspired after 30 min, and the monolayers were washed three times with 2 ml of ice-cold Tris-saline, pH 7.4, and extracted with ice-cold TCA (19). For [^{3}H]dT uptake experiments (2.5 μCi/culture; sp act 18–20 Ci/mmol), acid-soluble extracts were pooled from three replicate cultures; the proportion of [^{3}H]dT incorporated into dTTP was quantitated by PEI-cellulose chromatography (34). For arginine and 3-*O*-methyl-D-glucose uptake studies (2.0 and 1.0 μCi/culture, respectively), cultures were washed two times with 2 ml of prewarmed precursor-free and serum-free medium after which 2 ml of similar isotope-containing media were added with or without the initial dFBS and/or VLDL supplements. Acid-soluble radioactivity in 0.5 ml of extract was determined by scintillation counting of samples dissolved in 10 ml of Bio-Solv: Toluene: Liquifluor (100 ml: 1,000 ml: 42 ml) cocktail. Under these conditions, tritium is counted with ca. 50% efficiency. In these experiments, each datum is the mean value of duplicate pools (Fig. 3) or single extracts from triplicate cultures (Fig. 5). Protein synthesis rates were a measure of heat-stable acid-insoluble radioactivity which remained in TCA-extracted monolayers (19).

Data Analysis

For in vitro experiments, unless otherwise noted, each datum is the mean ± 10% of triplicate cultures. Statistical significance (Mann-Whitney U Test; Multiple Range Tests of Dunnett or Duncan) was determined with the UCLA IBM-360 Computer (model 95) and computer program EXBIOL.

RESULTS

Initiation of In Vitro Hepatocyte DNA Synthesis by Native and by Fractionated Rat Sera

Homologous adult rat serum poorly initiated DNA synthesis in $G_{0,1}$-arrested hepatocyte cul-

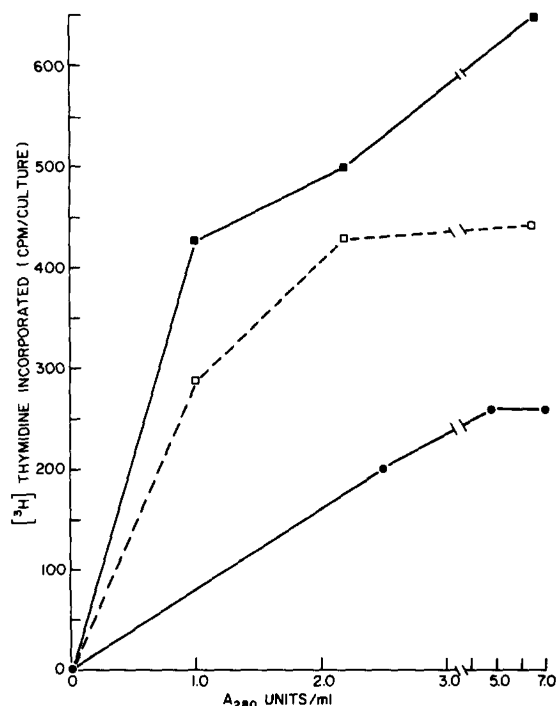

FIGURE 1 Initiation of in vitro hepatocyte DNA synthesis by native and by lipoprotein-deficient rat sera. DNA initiation bioassays were performed and DNA synthesis rates determined as described in Materials and Methods. Basal incorporation rates (serum-free media additions = 200 cpm/culture) were subtracted from plotted data; 15% vol/vol dFBS additions (positive controls) gave 1,600 cpm/culture. Sera tested (A_{280} ~ 60–65 U/ml) included: native rat serum (●—●); lipoprotein-deficient sera obtained from normal or 70% hepatectomized animals (■—■); and lipoprotein-deficient normal serum repleted with 40 μg VLDL protein/ml (□—□). Ordinate: [^{3}H]dT incorporated (cpm/culture). Abscissa: serum A_{280} U added/ml medium.

tures (Fig. 1). [^{3}H]dT incorporation under these conditions, a measure of cellular recruitment into S phase (19), was about doubled.[2] Serum obtained 6 h after 70% hepatectomy enhanced DNA synthesis (33) but, compared to sera from normal or sham-hepatectomized rats, only by 40–50% (data not shown). Similar results were obtained with dialyzed rat sera. By contrast, dFBS was >16 times more active than native rat sera, as determined from slopes of log-dose response curves.

While attempts were being made to fractionate rat sera, lipids were observed to promote initiation of DNA synthesis in this system (19, 27). Therefore, the bulk serum lipoprotein fraction, which carries most serum lipids, was isolated and tested for biological activity. This fraction was nonstimulatory over a broad concentration range (10–400 μg protein/ml medium) and actually inhibited initiation of DNA synthesis ca. 25% at concentrations >200 μg protein/ml medium (not shown). However, the lipoprotein-deficient fraction showed three- to fourfold increased stimulatory activity (Fig. 1). This figure also shows that lipoprotein-deficient sera from 70% hepatectomized rats gave results identical to those obtained with lipoprotein-deficient normal sera. These observations suggest that enhanced DNA synthesis initiation by partially hepatectomized rat sera resulted from differences in the level of inhibitory material present in the lipoprotein fraction.

This hypothesis is supported by the apparent identity between gel filtration profiles of DNA synthesis initiating activity recovered from pooled ammonium sulfate fractions of lipoprotein-deficient sera obtained from either 70% hepatectomized or normal rats (Fig. 2).[3] Two peaks of stimulatory activity eluted: the first appeared in the void volume; the second spread over many fractions which eluted after BSA and contained little A_{280} absorbing material.

Specific Inhibition of Initiation of In Vitro Hepatocyte DNA Synthesis by Rat Serum VLDL

Fig. 3 shows that cellular recruitment into S phase stimulated by lipoprotein-deficient dFBS was inhibited specifically by VLDL at microgram concentrations. Only slight inhibition was caused by LDL or by HDL, or by lipoprotein fraction "dialysates" (0.3 ml/culture). The extent of inhibition could be accounted for neither by cellular detachment (≤10% in all cases) nor by inhibition of [^{3}H]dT uptake into total acid-soluble or dTTP

[2] Earlier studies were conducted under nonarrested culture conditions so that increased incorporation rates probably reflected accelerated rates of ongoing DNA synthesis (33).

[3] Chromatograms of native rat sera, obtained from either 70% hepatectomized or normal rats, produced similar qualitative results compared to lipoprotein-deficient sera, with these exceptions: *(a)* stimulatory activity present in peak I material was generally 65–75% reduced, presumably due to the presence of high molecular weight inhibitory material (see Fig. 3); and *(b)* the presence of stimulatory activity in the region of peak II was variable, presumably due to trailing of inhibitory activity throughout the column.

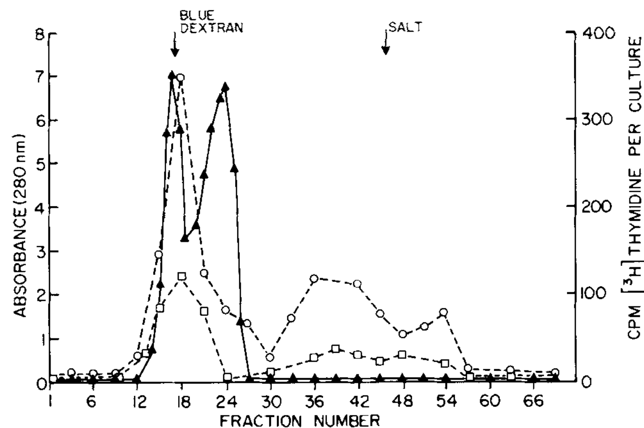

FIGURE 2 Initiation of in vitro hepatocyte DNA synthesis by lipoprotein-deficient rat sera fractionated by ammonium sulfate and gel filtration. Bioassays were performed and data plotted as described in Fig. 1 (basal incorporation rates = 200 cpm/culture; 15% vol/vol dFBS = 1,600 cpm/culture; 15% vol/vol lipoprotein-deficient normal rat serum = 840 cpm/culture). Column fractions added to the cultures (□---□, 0.05 ml; ○---○, 0.20 ml) were derived from sera obtained from normal or 70% hepatectomized rats[3] and were prepared as described in Materials and Methods; protein concentrations were determined spectrophotometrically (▲—▲). BSA standards eluted in fractions no. 18-27. Left ordinate: A_{280} U. Right ordinate: [^{3}H]dT incorporation (cpm/culture). Abscissa: column fraction no.

pools. For example, at VLDL concentrations of 6-30 μg protein/ml, [^{3}H]dT uptake into bulk acid-soluble material was inhibited 40-50% whereas recruitment was inhibited 50-99.9% (Fig. 3); under similar conditions, VLDL did not alter distribution of [^{3}H]dT uptake into dTTP (ca. 60% of the total counts). LDL and HDL fractions also inhibited [^{3}H]dT uptake into the acid-soluble fraction by 30-40% but not recruitment (Fig. 3); apparently, material common to each lipoprotein fraction inhibits [^{3}H]dT uptake. Similar results were obtained using native dFBS to initiate DNA synthesis. Additional controls showed that 85-90% of the attached cells were viable under all conditions (23) and that VLDL lipoprotein neither absorbed nor degraded [^{3}H]dT (25).

Three other systems were used to determine cellular specificity, including SV3T3, quiescent 3T3, and primary fetal rat fibroblasts. VLDL-supplementation at saturating levels (40 μg protein/ml medium) promoted SV3T3 proliferation, determined by cell counts (H. Leffert and D. Young, unpublished results) but neither stimulated nor inhibited serum-initiated DNA synthesis in the other tested cultures.

Additional experiments indicated that VLDL acted similarly when added at saturating levels to lipoprotein-deficient rat serum-induced cultures (Fig. 1). However, the observed inhibition by VLDL (50-60%) was less than that obtained with FBS-induced cultures. Native (normal) rat serum-induced DNA synthesis (Fig. 1) was not further diminished in similar repletion studies.

Temporal Sensitivity to VLDL-Inhibition of Initiation of In Vitro Hepatocyte DNA Synthesis

The quantity of DNA synthesized by $G_{0,1}$-arrested cultures 22-24 h after induction by 10% vol/vol dFBS is proportional to serum-exposure time: 0-12 h produced synthesis rates 90-95% of maximal whereas shorter times (0-4 and 0-8 h) produced proportionately lower rates (36% and 70%, respectively [26]). Because these "commitment" experiments reflected proportions of cells recruited to synthesize DNA (26), it was of interest to determine whether VLDL affected prereplicative events involved with "commitment" processes.

Fig. 4 shows that VLDL inhibited initiation of DNA synthesis to quantitative extents predicted from 10% vol/vol dFBS commitment experiments. For example, the presence of VLDL during the prereplicative phase from 0 to 4, 0 to 8, or 0 to 12 h permitted ca. 64, 30, or 5-10% of the maximal response, respectively. The results (Fig. 4) also suggest that VLDL action was independent

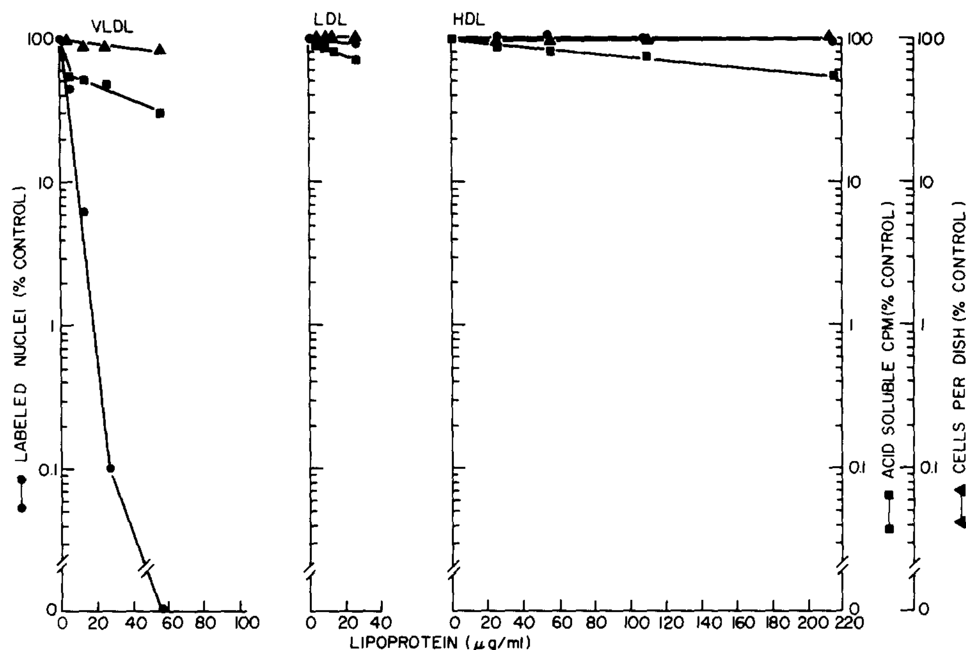

FIGURE 3 Specific inhibition of initiation of in vitro hepatocyte DNA synthesis by rat serum VLDL. Three sets of quiescent cultures received fresh medium changes together with 10% vol/vol lipoprotein-deficient dFBS and varying concentrations (μg lipoprotein-protein/ml medium, abscissae) of VLDL (left panel), or LDL (middle panel), or HDL (right panel). Each culture set then was subdivided into three bioassay groups. The fraction of DNA-synthesizing cells 23.5–24 h later was determined by autoradiography. Each point (●—●, [left ordinate], labeled nuclei) is the average of duplicate cultures, 1,000 cells/dish scored. Control values were: minus serum = 3%; plus serum ± 0.3 ml lipoprotein fraction "dialysates" = 23% ("100%" points). The rate of [^{3}H]dT uptake into TCA-soluble material (group two) was determined between 23.5 and 24 h (■—■, [right ordinate], acid-soluble cpm). Control values were: minus serum = 2,500 cpm; plus serum ± 0.3 ml "dialysates" = 8,800 cpm (100% points). Attached numbers of cells were determined (third group) by trypsinization (▲—▲, [extreme right ordinate]). Control values were: minus serum = 30,000 cells/dish; plus serum ± 0.3 ml "dialysates" = 31,000 cells/dish (100% points).

of initial serum concentrations. DNA synthesis rates observed at 22–24 h were consistent with above predictions when VLDL was added at 0, 4, 8, and 12 h (not shown). VLDL did not block ongoing DNA synthesis because it failed to inhibit [^{3}H]dT incorporation into DNA when added during the labeling interval (Fig. 4). Therefore, VLDL appeared to act only upon cells initiating DNA synthesis.

In Vitro Inhibition of Prereplicative Protein Synthesis by VLDL

Previous studies suggested that membrane events associated with arginine incorporation into protein beyond those involved with arginine uptake into an acid-soluble pool were important in growth regulation (19, 26, 27). Therefore, it was of interest to determine VLDL effects on arginine utilization after dFBS-induced DNA synthesis. 3-*O*-Methyl-D-glucose uptake also was measured because this precursor is not significantly metabolized and therefore serves as a probe for measuring transport (50).

Fig. 5 shows that VLDL had no detectable effect on uptake of either precursor into the acid-soluble fraction; nevertheless, significant inhibition (60–70%) of serum-induced protein synthesis was observed. Therefore, the effects of VLDL were qualitatively similar to those observed with glucagon[4] but considerably more efficient (27). Additional parallel studies, in which comparison was made between the quantity of [^{3}H]arginine remaining precipitable after cold and hot TCA

[4] We have been unable to detect immunoreactive glucagon as a contaminant of our VLDL preparation.

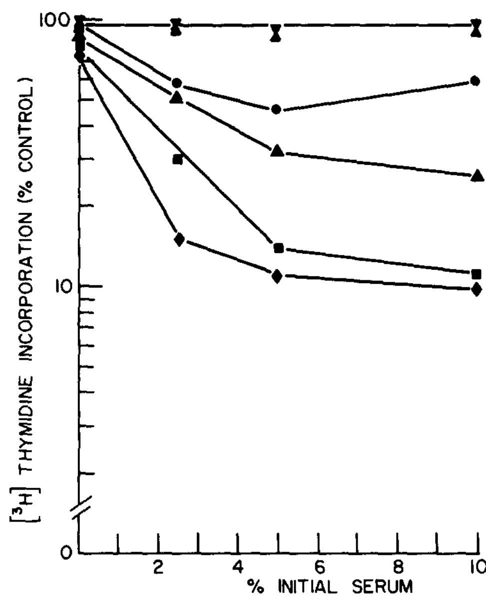

FIGURE 4 Inhibition of initiation of in vitro hepatocyte DNA synthesis as a function of VLDL exposure-time. $G_{0,1}$-arrested cultures were changed to fresh medium supplemented with dFBS (0, or 2.5, or 5.0 or 10.0% vol/vol) and VLDL (40 μg protein/ml). The cultures were subdivided into groups and, at the indicated times (4, ●–●; 8, ▲–▲; or 12 h, ■–■ later), media were aspired, washed twice with 2 ml serum-free medium, and re-incubated with fresh (37°C) media supplemented with the respective initial serum concentrations. Additional cultures received 40 μg VLDL protein/ml continuously (◆–◆) or at 22 h (⧗–⧗). DNA synthesis rates were determined as described in Materials and Methods. "100% serum controls" were: minus serum (140 cpm/culture); 2.5% (600 cpm/culture); 5.0% (800 cpm/culture) and 10.0% (1,100 cpm/culture). Ordinate: [^{3}H]dT incorporation (% control). Abscissa: % initial dFBS concentration.

washing, argue against VLDL-inhibition of tRNA charging because the proportion of tritium in protein (55–60%) and in tRNA (40–45%) was independent of the presence or absence of VLDL.

Partial Purification of Initiation Inhibitors from VLDL Particles

Fig. 6 shows that the total VLDL-lipid fraction was approximately as potent as native VLDL and ca. 10 times more active than VLDL "apoprotein". Mild saponification reduced activity of the lipid fraction ≥80%, suggesting involvement of esterified lipid(s). Both phospholipid and aqueous wash fractions showed considerable inhibitory activity whereas neutral lipids, which comprise the bulk VLDL lipid (20), were inactive. A neutral glycolipid fraction (containing some acidic phospholipids) showed some inhibitory activity, but its potency was considerably less than those of aqueous wash and total phospholipid extracts. Bulk LDL and HDL lipid fractions were inactive (not shown).

Serum Lipoprotein Changes in Adult Rats after 70% and Sham Hepatectomy

Fig. 7 shows that 70% hepatectomy induced specific prereplicative alterations in serum lipoprotein levels. Within 3 h, VLDL levels fell

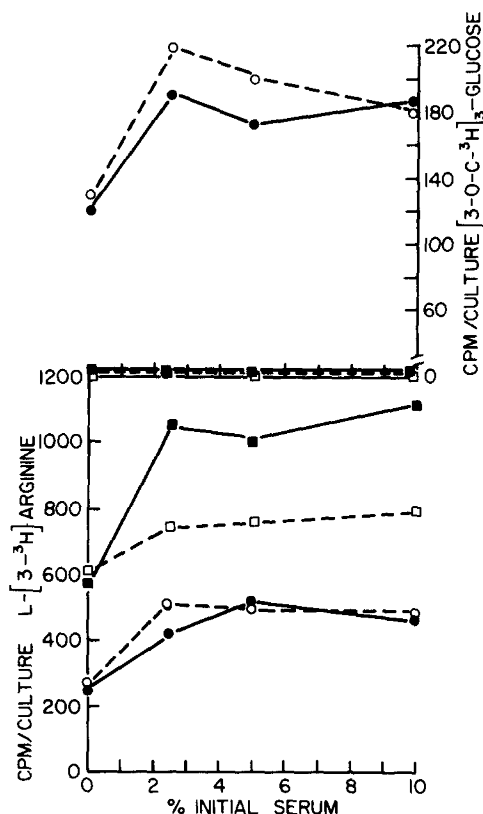

FIGURE 5 VLDL inhibition of serum-stimulated prereplicative protein synthesis in hepatocyte cultures. Arrested cultures were changed to fresh medium containing dFBS (0, or 2.5, or 5.0 or 10% vol/vol, abscissae) with (dashed lines) or without (solid lines) 40 μg VLDL protein/ml medium. Precursor uptake and protein synthesis rates were measured 24.0–24.5 h later as described in Materials and Methods. TCA-soluble (circles) and hot-TCA-precipitable (squares) radioactivity are shown on the ordinates: [3-O-C-^{3}H]$_3$-glucose (top right) and L-[3-^{3}H]arginine (bottom left).

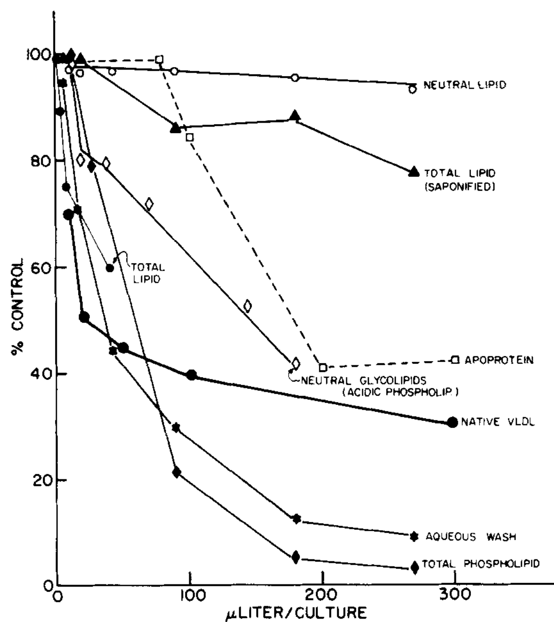

FIGURE 6 Partial purification of VLDL components inhibiting initiation of in vitro hepatocyte DNA synthesis. $G_{0,1}$-arrested cultures were changed to fresh medium containing 10% vol/vol dFBS together with various VLDL particle fractions (0-300 μl, abscissa): native VLDL (●—●); total lipid (●—●); saponified total lipid (▲—▲); neutral lipid (○—○); neutral glycolipid plus acidic phospholipid (◇—◇); aqueous wash (★—★); total phospholipid (◆—◆); and VLDL "apoprotein" (□—□). DNA synthesis rates were determined as described in Materials and Methods. Control values were: minus serum = 240 cpm/culture; plus serum ± 0.3 ml "diluent"/culture = 1,100 cpm/culture (100% points). Ordinate: [^{3}H]dT incorporation (% of control).

>80%, significantly less ($P < 0.05$) than VLDL levels in ether anesthetized or sham-hepatectomized animals (VLDL levels in these latter two groups were statistically similar). VLDL levels in 70% hepatectomized rats remained significantly lowered but, in the control groups, the serum levels rose four- to fivefold within 6 h ($P < 0.01$). Although HDL appeared to vary diurnally, no other differences among the three groups were observed from 0 to 24 h. Fig. 7 also shows that, 12 h after surgery, LDL levels in both laparotomized and 70% hepatectomized groups were higher than those of anesthetized animals ($P < 0.05$).[5] These observations are similar to those made 12 h after surgery with similar groups of rats in a separate study (27). 4-5 days after surgery, VLDL levels in the 70% hepatectomized group were 55-65% of those initially present.

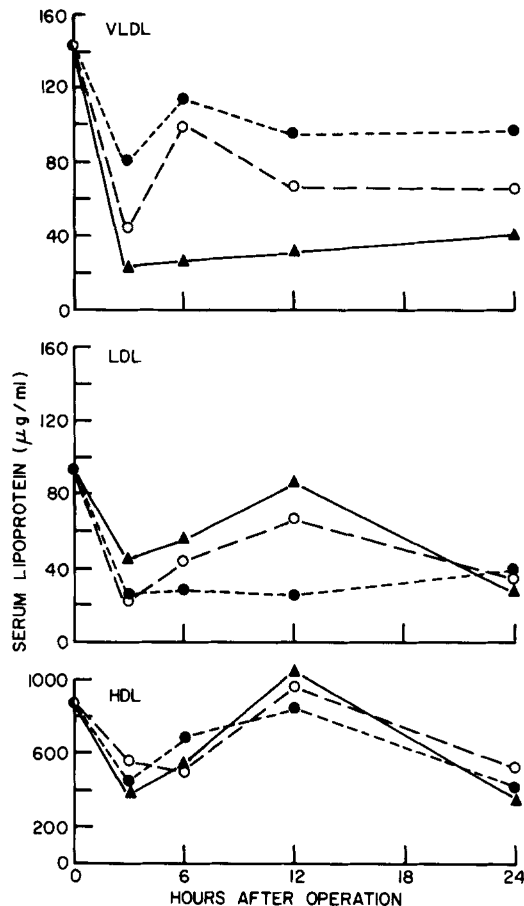

FIGURE 7 Rat serum lipoprotein levels after 70% or sham hepatectomy and after ether anesthesia. Rats were 70% (▲—▲) and sham hepatectomized (○—○), or briefly etherized (●—●), and at varying times (abscissae), three to four animals per group were anesthetized and serum samples prepared for lipoprotein determinations (ordinates) as described in Materials and Methods (VLDL [top panel]; LDL [middle panel]; and HDL [bottom panel]). Food, but not water, was removed from the cages; "zero-time" (0900 h) determinations were made from unstarved animals. Statistical analyses showed significant differences for the following data: VLDL, 70% hepatectomized vs. both sham hepatectomized and etherized rats at 3, 12, and 24 h, $P < 0.05$ and at 6 h, $P < 0.01$; LDL, both 70% heptatectomized and sham hepatectomized vs. etherized at 12 h, $P < 0.05$. At 12 h, all groups of animals had significantly higher serum levels of HDL compared to 3-, 6- or 24-h levels ($P < 0.01$).

[5] Reciprocal changes between VLDL and LDL also have been noted under different conditions (54).

Serum VLDL Levels and DNA Synthesis Rates in Fatty Rats after 70% Hepatectomy

A mutant rat strain (Fatty) which overproduces VLDL and, consequently, is hyperlipoproteinemic has been developed and characterized (38, 39, 55, 56). Table I shows that, after 70% hepatectomy, hepatic DNA synthesis rates in these animals were fourfold less than those in wild-type. This difference correlated inversely with initial serum VLDL levels which were three- to fourfold higher in the mutants; however, both groups had significantly lower VLDL levels 24 h postoperatively, as is shown in Table I.

Serum VLDL Levels and DNA Synthesis Rates in Rats Infused with a TAGH Solution

Table II shows that, under our infusion conditions, the TAGH solution stimulated hepatic DNA synthesis rates fivefold and markedly decreased serum VLDL levels within 30 min, in comparison to saline-infused groups ($P < 0.01$). By 6 h, serum VLDL levels returned to control values, and further decreases (through 24 h) were not observed. Repeated or more prolonged infusions produced both delayed and depressed stimulation of hepatic DNA synthesis; VLDL was not measured under these conditions.

DISCUSSION

A controversial literature surrounds the concept that blood-borne factors of hepatic origin specifically suppress normal hepatocyte proliferation (5, 12, 35). Evidence for their existence in both blood and hepatocytes, however, is lacking (47), and their physiological changes, if any, during in vivo hepatoproliferative transitions are unknown (43). We describe here direct evidence that purified rat VLDL – a circulating lipoprotein produced mainly by adult liver (18, 46) under hormonal control (8,

TABLE I

DNA Synthesis Rates and Serum VLDL Levels in Genetically Obese Fatty Rats after 70% Hepatectomy

Manipulation	Genotype	Serum VLDL* 0 h	Serum VLDL* 24 h	DNA synthesis‡
70% Hepatectomy	FF	52 (3)	31 (4)	11,597 (9)
70% Hepatectomy	ff	167 (5)	42 (5)	2,643 (9)

Mutant "Fatty" rats and wild-type littermates were 70% hepatectomized immediately after sampling 1 ml blood. Food, but not water, was removed from the cages, and hepatic DNA synthesis rates and serum VLDL levels were determined as described in Materials and Methods.

* μg protein/ml. Data show the mean values (number of rats shown in parentheses) and were analyzed by Multiple Range Tests of Dunnett or Duncan. $P < 0.05$ (FF at 0 h vs. FF at 24 h) and $P < 0.01$ (ff at 0 h vs. FF at 0 h or ff at 24 h).

‡ cpm/mg DNA/2 h pulse, 22–24 h postsurgery. Data were analyzed by the Mann-Whitney U Test, $P < 0.02$ (FF vs. ff).

TABLE II

Serum VLDL Levels and DNA Synthesis Rates in Rats Infused with a TAGH Solution

Infusate	Serum VLDL* 0 h	0.5 h	1 h	3 h	6 h	DNA synthesis‡
TAGH	96 ± 12	7 ± 2	11 ± 4	79 ± 13	61 ± 10	2,299 ± 370
Saline	–	86 ± 21	154 ± 18	105 ± 8	69 ± 14	430 ± 116

Intact rats received infusions as described in Materials and Methods. Serum samples were obtained at various times after starting ("0 h"), and VLDL and hepatic DNA synthesis rates were determined as described in Materials and Methods.

* Mean [μg protein/ml] ± SEM, obtained from four rats per group and were analyzed by Multiple Range Tests of Dunnett or Duncan: $P < 0.01$ (TAGH vs. saline at 0.5 and 1 h); $P < 0.05$ (TAGH vs. saline at 3 h); not significant (TAGH vs. saline at 6 h); saline at 1 h was significantly higher than at all other times ($P < 0.01$).

‡ cpm/mg DNA/2 h pulse, 22–24 h postinfusion. The data are mean values ± SEM from five rats per group and were analyzed with the Multiple Range Test of Dunnett ($P < 0.01$).

32, 49) – specifically inhibits initiation of DNA synthesis in differentiated cultures of fetal rat hepatocytes (22–27, 40). Indirect in vivo evidence strongly associating VLDL (and/or its related metabolism) with suppression of hepatic $G_{0,1} \rightarrow S$ transitions also has been obtained. The control of animal cell proliferation and the role that "chalones" may play in these processes have been discussed elsewhere (28).

In vitro findings that VLDL more efficiently inhibits DNA synthesis initiated by FBS (Fig. 3) than by lipoprotein-deficient rat serum (Fig. 1) may involve greater stability of VLDL-particles in fetal serum, and/or "blocking" effects of additional serum lipoproteins (36) or other serum factors. Similar explanations hold for the findings that excess VLDL fails to further diminish low levels of DNA synthesis induced by normal rat serum. Further observations (Fig. 2) that lipoprotein-deficient rat serum fractions – derived from normal or 70% hepatectomized rats – produce identical biological activity profiles suggest that DNA synthesis initiation factors normally are present in blood. This statement is consistent with previous in vitro and in vivo observations (6, 26–30, 42, 45), but proof of identity requires further chemical characterization. Although identity between rat VLDL and the DNA synthesis-initiation inhibitory factor(s) present in dFBS (25) is unlikely, this conclusion must also remain tentative pending further study.

In vitro experiments suggest that VLDL acts by inhibiting prereplicative protein synthesis required for initiating DNA synthesis (Figs. 4 and 5) and not by inhibiting tRNA charging or the uptake of at least one nutrient – arginine – known to limit growth in this system (Fig. 5 [19]). Partial purification of inhibiting material(s) implicates involvement of polar lipids and, possibly, the VLDL "apoprotein". Thus far, the complexity and rapidity ($t_{1/2} \leq 60$ min) of in vivo VLDL serum turnover (9) has precluded in vivo attempts to inhibit, by exogenous VLDL administration, 70% hepatectomy-induced DNA synthesis.

A simple hypothesis may be that a minor class of VLDL lipid influences membrane events regulating critical protein synthetic steps required for initiating DNA synthesis. Consequently, a "region" of hepatocyte membrane lipid, rather than a defined protein receptor, may confer "specificity" to VLDL action. Alternatively, inhibitory action of VLDL polar lipid(s) may be a consequence of surface active effects resulting from emulsification procedures. However, neutral lipid emulsions, which probably are taken up and metabolized by the cells, had no biological activity under similar conditions (Fig. 6). Furthermore, release of cytotoxic-free fatty acids subsequent to cell-mediated VLDL hydrolysis is unlikely because of the presence of albumin in the culture fluids (22, 40). It has not yet been determined whether the aqueous wash fraction of VLDL contains gangliosides, which also may play growth-controlling roles in other cell culture systems (3, 16). However, the Sephadex column purification-step clearly eliminates other low molecular weight water-soluble factors. Because LDL and HDL emulsified lipids are inactive, their major lipid classes, which also are present in VLDL, probably are not involved (again, this assumes intracellular availability). Functions associated with VLDL apoprotein moieties include the capacity for activation of lecithin:cholesterol acyl transferase (10); for activation of lipoprotein lipase (15); and, possibly, for cholesterol transport into cells or exchange with plasma membranes (4). Thus, if apoprotein inhibition (Fig. 6) does not result from bound lipid contamination, its action also could be through modification of hepatic membrane structure.

Two predictions follow from the above discussion. First, hepatic VLDL production should occur predominantly during G_0 (and/or early G_1)[6]; therefore, intrahepatic and serum VLDL levels should be inversely related to hepatic growth states. This prediction is supported by the observation that rapid, specific, and persistent decreases in serum VLDL levels occur after 70% hepatectomy in adult rats (Fig. 7). Moreover, these kinetic changes could have been predicted partly from the rapid elevation of serum glucagon occurring after partial hepatectomy (27, 29) because hyperglucagonemic states suppress hepatic VLDL production (8). However, growth regulation by glucagon is probably complex (6, 27), and other factors, including prostaglandin-E_1, heparin production by mast cells, and alpha$_1$-fetoprotein, also may be involved with suppression of hepatic VLDL production (27, 28). In addition, fetal rat serum VLDL levels are significantly less (by 70–

[6] As yet, we have been unable to detect newly synthesized VLDL in "quiescent" fetal hepatocyte culture fluids, although active synthesis and secretion of other liver-specific proteins occurs (22, 24, 40). By contrast, adult hepatocyte cultures produce VLDL (D. Weinstein, unpublished observations) but do not grow (1).

80%) than those of adults, but increase postnatally as hepatic DNA synthesis declines (11, 41).[7]

Second, genetic and/or environmental conditions which specifically raise or lower hepatic VLDL production and secretion should retard or promote, respectively, initiation of hepatic DNA synthesis. Results with 70% hepatectomized hyperlipoproteinemic mutant rats (Table I), with adult rats infused with a TAGH solution (Table II), and with choline-deficient animals[7] tend to support this prediction.

Fatty rats are hyperinsulinemic (56), overproduce VLDL (38), and have elevated serum VLDL levels (39). Because peripheral antilipolytic effects of insulin could limit fatty acid supplies to regenerating liver which, in turn, may be growth regulatory (27, 44), it cannot yet be concluded that low DNA synthesis rates observed in 70% hepatectomized mutant rats result solely from VLDL action. However, the ability of these animals to regulate peripheral VLDL and insulin levels seems intact because, as in normal rats (29), lower posthepatectomy insulin (not shown) and VLDL levels (Table I) were observed. Whether or not prolonged onset times and/or decreased recruitment of DNA-synthesizing cells cause decreased DNA synthesis rates is, as yet, unknown.

TAGH infusions stimulate hepatic DNA synthesis fivefold and rapidly lower serum VLDL (Table II) as predicted (27). Although each component of the infusate contributes to stimulating DNA synthesis (42), it is not yet known which single or combined components lower serum VLDL; likely candidates are glucagon (8) and heparin (11). Causal relationships between TAGH-induced depression of serum VLDL levels and induction of DNA synthesis would seem likely, but other direct hepatocellular effects of infusate components unrelated to VLDL metabolism also could be important (e.g. putative template activity of heparin [21]). However, both glucagon and heparin (26) fail to initiate in vitro hepatocyte DNA synthesis under a variety of conditions.

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Paper number VIII in this series of growth control studies with cultured fetal rat hepatocytes is reference 27.

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[7] Studies with developing rats indicate that choline deficiency prolongs and elevates hepatic DNA synthesis. Concomitantly, serum VLDL levels decline in the deficient animals (H. Leffert, D. B. Weinstein, A. Rogers, and P. Newberne, manuscript in preparation).

REFERENCES

1. Bisell, D. M., L. E. Hammaker, and V. A. Meyer. 1973. Parenchymal cells from adult rat liver in nonproliferating monolayer culture. *J. Cell Biol.* **59:**722–734.
2. Blobel, G., and V. R. Potter. 1966. Nuclei from rat liver: isolation method that combines purity with high yield. *Science (Wash. D.C.).* **154:**1662–1665.
3. Brady, R. O., C. Borek, and R. M. Bradley. 1969. Composition and synthesis of gangliosides in rat hepatocyte and hepatoma cell lines. *J. Biol. Chem.* **244:**6552–6554.
4. Bruckdorfer, K. R., J. M. Graham, and C. Green. 1968. The incorporation of steroid molecules into lecithin sols, β-lipoproteins and cellular membranes. *Eur. J. Biochem.* **4:**512–518.
5. Bucher, N. L. R., J. F. Scott, and J. C. Aub. 1951. Regeneration of the liver in parabiotic rats. *Cancer Res.* **11:**457–465.
6. Bucher, N. L. R., and M. N. Swaffield. 1975. Regulation of hepatic regeneration in rats by synergistic action of insulin and glucagon. *Proc. Natl. Acad. Sci. U.S.A.* **72:**1157–1160.
7. Burton, K. 1956. A study of conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62:**315–322.
8. Eaton, R. P. 1973. Hypolipemic action of glucagon in experimental endogenous lipemia in the rat. *J. Lipid Res.* **14:**312–318.
9. Fidge, N. H., and P. Poulis. 1974. Studies on the radioiodination of very low density lipoprotein obtained from different mammalian species. *Clin. Chim. Acta.* **52:**15–26.
10. Fielding, C. J., V. G. Shore, and P. E. Fielding. 1972. A protein cofactor of lecithin: cholesterol acyltransferase. *Biochem. Biophys. Res. Commun.* **46:**1493–1498.
11. Fredrickson, D. 1974. Plasma lipoproteins and apolipoproteins. *In* The Harvey Lectures. Aca-

demic Press, Inc., New York. **68:**185–237.

12. GLINOS, A. D. 1960. Environmental feedback control of cell division. *Ann. N.Y. Acad. Sci.* **90:**592–602.
13. GUSTAFSON, A. 1965. New method for partial delipidization of serum lipoproteins. *J. Lipid Res.* **6:**512–517.
14. HAVEL, R. J., H. A. EDER, and J. H. BRAGDON. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:**1345–1353.
15. HAVEL, R. J., C. J. FIELDING, T. OLIVECRONA, V. G. SHORE, P. E. FIELDING, and T. EGELRUD. 1973. Cofactor activity of protein components of human very low density lipoproteins in the hydrolysis of triglycerides by lipoprotein lipase from different sources. *Biochemistry.* **12:**1828–1833.
16. HOLLENBERG, M. D., and P. CUATRECASAS. 1974. Cholera toxin and cell growth: role of membrane gangliosides. *Proc. Natl. Acad. Sci. U.S.A.* **71:**4224–4228.
17. HOLLEY, R. W., and J. A. KIERNAN. 1968. "Contact inhibition" of cell division in 3T3 cells. *Proc. Natl. Acad. Sci. U.S.A.* **60:**300–304.
18. JONES, A. L., and R. K. OCKNER. 1971. An electron microscopic study of endogenous very low density lipoprotein production in the intestine of rat and man. *J. Lipid Res.* **12:**580–589.
19. KOCH, K., and H. L. LEFFERT. 1974. Growth control of differentiated fetal rat hepatocytes in primary monolayer culture. VI. Studies with conditioned medium and its functional interactions with serum factors. *J. Cell Biol.* **62:**780–791.
20. KOGA, S., L. HORWITZ, and A. M. SCANU. 1969. Isolation and properties of lipoproteins from normal rat serum. *J. Lipid Res.* **10:**577–588.
21. KRAEMER, R. J., and D. S. COFFEY. 1970. The interaction of natural and synthetic polyanions with mammalian nuclei. I. DNA synthesis. *Biochim. Biophys. Acta.* **224:**553–567.
22. LEFFERT, H. L., and D. PAUL. 1972. Studies on primary cultures of differentiated fetal liver cells. *J. Cell Biol.* **52:**559–568.
23. LEFFERT, H. L., and D. PAUL. 1973. Serum dependent growth of primary cultured differentiated fetal rat hepatocytes in arginine-deficient medium. *J. Cell. Physiol.* **81:**113–124.
24. LEFFERT, H. L., and S. SELL. 1974. Alpha$_1$-fetoprotein biosynthesis during the growth cycle of differentiated fetal rat hepatocytes in primary monolayer culture. *J. Cell Biol.* **61:**823–829.
25. LEFFERT, H. L. 1974. Growth control of differentiated fetal rat hepatocytes in primary monolayer culture. V. Occurrence in dialyzed fetal bovine serum of macromolecules having both positive and negative growth regulatory function. *J. Cell Biol.* **62:**767–779.
26. LEFFERT, H. L. 1974. Growth control of differentiated fetal rat hepatocytes in primary monolayer culture. VII. Hormonal control of DNA synthesis and its possible significance to the problem of liver regeneration. *J. Cell Biol.* **62:**792–801.
27. LEFFERT, H. L. 1974. Hepatocellular growth control. *In* Workshop on Rat Liver Pathology. P. Newberne and W. Butler, editors. Elsevier Publishing Co., North Holland, Amsterdam. In press.
28. LEFFERT, H., and K. KOCH. 1976. Control of animal cell proliferation. *In* Growth, Nutrition and Metabolism of Cells in Culture, Vol. III. G. H. Rothblat and V. J. Cristofalo, editors. Academic Press, Inc., New York. In press.
29. LEFFERT, H., N. M. ALEXANDER, G. FALOONA, B. RUBALCAVA, and R. UNGER. 1975. Specific endocrine and hormonal receptor changes associated with liver regeneration in adult rats. *Proc. Natl. Acad. Sci. U.S.A.* **72:**4033–4036.
30. LEFFERT, H. L., and N. M. ALEXANDER. 1976. Thyroid hormone metabolism during liver regeneration in rats. *Endocrinology.* **98:**1205–1211.
31. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–275.
32. LUSKEY, K. L., M. S. BROWN, and J. L. GOLDSTEIN. 1974. Stimulation of the synthesis of very low density lipoproteins in rooster liver by estradiol. *J. Biol. Chem.* **249:**5939–5947.
33. PAUL, D., H. LEFFERT, G. SATO, and R. W. HOLLEY. 1972. Stimulation of DNA and protein synthesis in fetal-rat liver cells by serum from partially hepatectomized rats. *Proc. Natl. Acad. Sci. U.S.A.* **69:**374–377.
34. RANDERATH, K. 1966. Thin-Layer Chromatography. Academic Press, Inc., New York. 285.
35. ROGERS, A. E., J. A. SHAKA, G. PECHET, and R. A. MACDONALD. 1961. Regeneration of the liver. *Am. J. Pathol.* **39:**561–578.
36. ROSS, R., and J. A. GLOMSET. 1973. Atherosclerosis and the arterial smooth muscle cell. *Science (Wash. D.C.).* **180:**1332–1339.
37. RUBIN, H. 1971. Growth regulation in cultures of chick embryo fibroblasts. *In* Growth Control in Cell Cultures: A CIBA Foundation Symposium. G. E. W. Wolstenholme and J. Knight, editors. Churchill Livingstone, London. 127–145.
38. SCHONFELD, G., and B. PFLEGER. 1971. Overproduction of very low-density lipoproteins of genetically obese rats. *Am. J. Physiol.* **220:**1178–1181.
39. SCHONFELD, G., C. FELSKI, and M. A. HOWALD. 1974. Characterization of the plasma lipoproteins of the genetically obese hyperlipoproteinemic Zucker Fatty rat. *J. Lip. Res.* **15:**457–464.
40. SELL, S., H. LEFFERT, U. MUELLER-EBERHARD, S. KIDA, and H. SKELLY. 1975. Relationship of the biosynthesis of alpha$_1$-fetoprotein, albumin, hemopexin and haptoglobin to the growth state of fetal

rat hepatocyte cultures. *Ann. N.Y. Acad. Sci.* **259**:45–58.

41. SELL, S., M. NICHOLS, F. F. BECKER, and H. L. LEFFERT. 1974. Hepatocyte proliferation and α_1-fetoprotein in pregnant, neonatal, and partially hepatectomized rats. *Cancer Res.* **34**:865–871.
42. SHORT, J., R. F. BROWN, A. HUSAKOVA, J. R. GILBERTSON, R. ZEMEL, and I. LIEBERMAN. 1972. Induction of DNA synthesis in the liver of the intact animal. *J. Biol. Chem.* **247**:1757–1766.
43. SIMARD, A., L. CORNEILLE, Y. DECHAMPS, and G. W. VERLY. 1974. Inhibition of cell proliferation in the livers of hepatectomized rats by a rabbit hepatic chalone. *Proc. Natl. Acad. Sci. U.S.A.* **71**:1763–1766.
44. ŠIMEK, J., VL. CHMELAŘ, J. MĚLKA, J. PAZDERKA, and A. CHARVÁT. 1967. Influence of protracted infusion of glucose and insulin on the composition and regeneration activity of liver after partial hepatectomy in rats. *Nature (Lond.).* **213**:910–911.
45. STARZL, T. E., A. FRANCAVILLA, C. G. HALGRIMSON, F. R. FRANCAVILLA, K. A. PORTER, T. H. BROWN, and C. W. PUTNAM. 1973. The origin, hormonal nature, and action of hepatotrophic substances in portal venous blood. *Surg. Gynecol. Obst.* **137**:179–199.
46. STEIN, O., and Y. STEIN. 1967. Lipid synthesis, intracellular transport, storage and secretion. I. Electron microscope radioautographic study of liver after injection of tritiated palmitate or glycerol in fasted and ethanol-treated rats. *J. Cell Biol.* **33**:319–339.
47. VERLY, W. G., Y. DECHAMPS, J. PUSHPATHADAM, and M. DESROSIERS. 1971. The hepatic chalone. I. Assay method for the hormone and purification of the rabbit liver chalone. *Can. J. Biochem.* **49**:1376–1383.
48. VESER, J., and W. FRANK. 1972. Über die bedentung von kälberserum fur kulturen embryonaler rattenzellen. *Z. Naturforsch. Teil. B. Anorg. Chem. Org. Chem. Biochem. Biophys. Biol.* **27b**:1573–1574.
49. WATKINS, M. L., N. FIZETTE, and M. HEIMBERG. 1972. Sexual influences on hepatic secretion of triglyceride. *Biochim. Biophys. Acta.* **280**:82–85.
50. WEBER, M. J. 1973. Hexose transport in normal and Rous sarcoma virus transformed cells. *J. Biol. Chem.* **248**:2978–2983.
51. WEINSTEIN, D. B., J. B. MARSH, M. C. GLICK, and L. WARREN. 1969. Membranes of animal cells. IV. The lipids of the L cell and its surface membrane. *J. Biol. Chem.* **244**:4103–4111.
52. WEINSTEIN, D. B., J. B. MARSH, M. C. GLICK, and L. WARREN. 1970. Membranes of animal cells. VI. The glycolipids of the L cell and its surface membrane. *J. Biol. Chem.* **245**:3928–3937.
53. WEINSTEIN, D. B., T. E. CAREW, and D. STEINBERG. 1975. Uptake and degradation of low density lipoprotein by swine arterial smooth muscle cells with inhibition of cholesterol biosynthesis. *Biochim. Biophys. Acta.* In press.
54. WILSON, D. W., and R. S. LEES. 1972. Metabolic relationships among the plasma proteins. *J. Clin. Invest.* **51**:1051–1057.
55. ZUCKER, L. M. 1965. Hereditary obesity in the rat associated with hyperlipidemia. *Ann. N.Y. Acad. Sci.* **131**:447–458.
56. ZUCKER, L. M., and H. N. ANTONIADES. 1972. Insulin and obesity in the Zucker genetically obese rat "Fatty". *Endocrinology.* **90**:1320–1330.