

GROWTH CONTROL OF DIFFERENTIATED FETAL RAT HEPATOCYTES IN PRIMARY MONOLAYER CULTURE

VI. Studies with Conditioned Medium and Its Functional Interactions with Serum Factors

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ABSTRACT

Serum-deficient ($\leq 0.00003\%$ vol/vol) conditioned medium (CM) obtained from primary cultures of fetal rat hepatocytes initiates DNA synthesis and mitosis in homologous quiescent cultures. CM similarly prepared from 3T3 fibroblast cultures is inactive.

At least two conditioning factors are involved in initiating DNA synthesis. The first of these, arginine, is obligatory, synthesized by the cells, and released into the culture medium. The second, a lipid or lipid-containing material, is stable to pH extremes (pH 2, pH 10) and chromatographs with an apparent $R_f \sim 0.5$ on silica gel thin-layer plates using hexane-ether (4:1) as the solvent system.

It is suggested that these cultured hepatocytes enter or leave the G_0 or early G_1 phase of the cell cycle as determined in part by their capacity to use available conditioning factor and nutrient components of the medium, in particular, arginine. Serum factors including serum fraction I (4), insulin, and possibly, lipid-like conditioning material appear to initiate DNA synthesis by controlling cellular processes involved with the enhanced utilization and synthesis of growth-limiting nutrients.

INTRODUCTION

Differentiated fetal rat hepatocytes selected to grow in arginine-free medium as primary monolayer cultures are being used as an in vitro model system to study hepatocellular growth control (1). Apart from their serum factor requirements (2-4), these cultured cells also display an additional but poorly understood obligatory requirement of conditioned medium (1, 3).

In the preceding report (4), it was observed that

washed hepatocytes plated into ornithine-supplemented, serum-free medium and not subjected to medium changes are, nevertheless, capable of survival and DNA synthesis. These observations suggested that ornithine might support the production of conditioning factors in addition to its precursor involvement with intracellular arginine biosynthesis (1, 3), one or more of which might be directly involved with initiating hepatocellular

DNA synthesis. Therefore, the nature of the conditioning effect was studied in more detail using conditions permitting assessment of the individual contributions of serum, conditioned medium, and nutrient components of the medium.

MATERIALS AND METHODS

Reagents

Amino acids (*dl*- or *l*-ornithine, and *l*-arginine-HCl), putrescine dihydrochloride, dT,¹ crystalline bovine insulin, and albumin were obtained from Sigma Chemical Corp., St. Louis, Mo. [³-³H]-*l*-arginine (sp act 20.6 Ci/mmol), [³-³H]-*dl*-ornithine (sp act 2.38 Ci/mmol) and [³H]dT (sp act 20.0 Ci/mmol) were purchased from New England Nuclear, Boston, Mass.

Fetal Rat Hepatocyte Tissue Culture

PLATING: Fetal rat hepatocytes were prepared for plating essentially as previously described (1-5). In the experiments described here, four pregnant rat litters, on the average totaling about 30 fetuses, were used and the dissected livers subjected to four incubations in 40 ml of a 0.3% vol/vol collagenase-physiological salt solution. The pooled, ice-cold cell suspension (160 ml) was mixed with an equal volume of ice-cold medium supplemented with 10% vol/vol dFBS, and distributed as 40-ml aliquots into plastic 50-ml screw-cap tubes (Falcon Plastics, Div. of B.-D. Laboratories, Los Angeles, Calif.). The culture medium consisted of Dulbecco and Vogt's modification of Eagle's medium formulated without arginine (1) and unless otherwise noted, supplemented with *dl*-ornithine (0.4 mM) as previously described (4). This medium will be referred to as "basal medium". After the first centrifugation step (4), the resulting pellets (ca. 0.6 ml packed cells with 0.1 ml residual supernate) were again resuspended with 10 ml basal medium. The contents of the eight tubes were pooled to yield, on the average, 80 ml of a cell suspension (ca. 2.5×10^6 cells/ml) with an average residual concentration of exogenously added serum equivalent to $\leq 0.0005\%$ vol/vol. The use and preparation of other materials required for establishing these cultures is described elsewhere (1-5). Pleuropneumonia-like organism contamination was found to be negative as determined by autoradiography (1).

PREPARATION OF QUIESCENT FETAL RAT HEPATOCYTE CULTURES FOR ASSAYS OF INITIATION OF DNA SYNTHESIS AND MITOSIS: Although previous studies (3) indicated that quiescent cultures could be prepared by plating fetal hepatocytes together

with arginine-free medium supplemented with 1.75% vol/vol dFBS, the use of these cultures would create problems of interpretation owing to the simultaneous presence of serum and conditioning factors. These problems were circumvented by using step-down conditions to generate quiescent cultures (5).

Fetal rat hepatocytes were plated ($2.0-2.5 \times 10^5$ cells/30-mm diameter dish) with 2 ml basal medium supplemented with 10% vol/vol dFBS. 34 h postplating, the medium was aspirated and replaced with 2 ml similar fresh medium. 56 h postplating, the medium on these dishes was aspirated and 2 ml fresh 50% CM² was added. Arginine levels in 50% CM did not exceed ca. $10 \mu\text{M}$ (5). Similar results were obtained whether or not these dishes were washed twice with 2 ml serum-free basal medium.² During the next 7-9 days, cell multiplication occurred as indicated by an 80-100% increase in the number of attached cells (day no. 2: $0.12-0.25 \times 10^5$ cells/30-mm dish; day no. 9: $0.24-0.65 \times 10^5$ cells). Between 9 and 11 days postplating, although the cells were not confluent at this time, cell multiplication ceased, the basal rate of DNA synthesis³ had declined to a constant low value

² If dishes are not washed, about $10 \mu\text{l}$ of old medium are not removed by aspiration and are carried over into the freshly added medium.

³ It has previously been reported that in this system [³H]dT uptake into DNA is linearly proportional to the attached cell density ($1-10 \times 10^4$ cells/dish) (2). Thus, for any given single plating in which the cell culture density is identical throughout, observed differences in DNA-synthesizing capacity are valid measures of differences among different samples of putative growth-promoting materials. In these studies, large numbers of cultures (500-600 dishes) derived from a single plating were always used for any single set of experiments. At the time the assays were carried out, the numbers of attached cells did not vary from dish to dish by more than $\pm 10\%$. But, owing to difficulties in determining exact gestational age, litter size, and liver weight etc., it has not always been possible, from different pools of fetuses, to begin each set of experiments with cultures of uniform cell number; hence, for each experiment, the initial cell density has always been indicated. Although this variability has tended in some experiments ($0.2-0.3 \times 10^6$ cells/dish) to reduce the absolute amount of [³H]dT uptake, it has not altered the qualitative results. Furthermore, the accuracy of these assays was verified by separate control studies which showed that: (a) rates of [³H]dT incorporation into alkali-stable material (2) were linear for the duration of pulse times used; (b) percentages of DNA-synthesizing cells were proportional to the amount of [³H]dT incorporated per culture for pulse times of equal duration and with dishes of equal cell numbers (2); and (c) errors of measurement amongst duplicate or triplicate cultures did not fluctuate by more than $\pm 10\%$.

¹ *Abbreviations used in this paper:* bovine serum albumin, BSA; conditioned medium, CM; dialyzed fetal bovine serum, dFBS; thymidine, dT; methyltriated thymidine, [³H]dT; trichloroacetic acid, TCA.

(20–100 cpm/dish), and the percentage of DNA-synthesizing cells was less than 2% as determined by autoradiography after a [³H]dT pulse of 12–24 h. Upon reinitiation of cell multiplication (216 h postplating, see Results) these cultured cells were found to have retained differentiated function as indicated by their capacity to incorporate 3-[³H]-*dl*-ornithine into protein as L-[³H]arginine (1) and to release into the culture medium with a specific time course-synthesized alpha-fetoprotein (5).

SERUM FRACTION I PREPARATION: This serum fraction was obtained from dFBS by a two-step procedure described in the preceding report (4). The apparent molecular weight of SFI as judged by gel filtration is $\geq 120,000$ daltons, and the purification of SFI relative to whole serum is ca. 12-fold. The material was stored at -20°C for periods up to 4 mo without detectable losses of activity.

CONDITIONED MEDIUM PREPARATION: CM was prepared by plating 4×10^6 freshly isolated hepatocytes into 9-cm diameter plastic tissue culture dishes (NUNC, Roskilde, Denmark) together with 10 ml of arginine-free, ornithine-free medium. For different experiments, various supplements, as indicated, were added at zero time and unless noted, the medium was collected 44 h postplating and centrifuged (International Clinical Desk-Top, 2,000 rpm, 10 min). The supernatant CM was collected, sterilized by filtration (Nalgene 0.45- μm filter, Nalge Co., Nalgene Labware Div., Rochester, N. Y.) to remove debris, and then stored at 4°C for periods up to 6–8 wk without detectable losses of activity.

50% CM was formulated by adding 40 ml of a suspension of 10^8 freshly prepared, twice-washed hepatocytes into 215 ml basal medium, and then plating 4×10^6 cells/10 ml basal medium/9-cm diameter dish. After the sterilization step at 44 h, this medium was diluted 50% with fresh basal medium. The final amount of residual serum which may have been present would be equivalent to $\leq 0.00005\%$ (vol/vol). This CM was used to generate quiescent cultures (see above).

Dialysis of CM was carried out at 4°C for 24 h against 2 vol of fresh basal medium using $\frac{5}{8}$ -inch dialysis tubing (3). Tubing treated in this manner was found to retain $\geq 99\%$ of the radioactivity associated with ¹²⁵I-insulin dissolved in a 1% wt/vol BSA solution. Concentration of high molecular weight material in CM was performed by exhaustive dialysis against isotonic saline followed by ultrafiltration dialysis of the dialysand at 4°C using PM 10 filters (Amicon Corp., Lexington, Mass.); the filtrate of this material remained once concentrated whereas the retentate was 10 times concentrated.

Acidification (pH 2) or alkalization (pH 10) of undilute CM was carried out at 4°C by the addition of 6 N HCl or NaOH, respectively, for 24 h, followed by reneutralization to an initial pH of 7.4.

For the preparation of 3T3 fibroblast CM, 4×10^6 logarithmically growing (10% dialyzed bovine serum) cells were harvested by trypsinization (2), washed twice with basal medium, and plated together with 10 ml basal

medium into a 9-cm diameter dish. All subsequent steps were identical to those used with fetal hepatocyte cultures.

DNA SYNTHESIS ASSAYS: The rate of incorporation of [³H]dT was determined by 2-h pulse labeling of duplicate or triplicate cultures with 0.05 ml of a cocktail so that the final thymidine concentration in the medium was 3×10^{-6} M, 1.25 $\mu\text{Ci/ml}$ (2). The medium was aspirated and the dishes were washed twice with Tris-saline, pH 7.4 (2), 2 ml/wash. Ca^{++} - and Mg^{++} -free trypsin solution (2) was then added (2 ml/dish) and the dishes were incubated at 37°C for 30 min. Vigorous pipetting by hand was required to ensure removal of more than 95% of the attached cells; this material was then either directly filtered (Whatman GF/C 2.4-cm glass fiber filters presoaked in 5% TCA), or added to glass tubes containing 5 ml of ice-cold 7.2% TCA vol/vol which was then filtered. Large quantities of dishes (600) could rapidly be processed by this latter procedure although the recovered acid-precipitable counts were slightly reduced (15–20%). No substantial qualitative differences were observed with either of the two procedures. The filters were washed with ice-cold 5% TCA and ice-cold ethanol, air-dried, and counted in plastic scintillation vials containing 5 ml Liquifluor-toluene scintillant (1:237 vol/vol, New England Nuclear) with a Beckman Counter Model LS-233 (Beckman Instruments, Inc., Fullerton, Calif.). Tritium counting efficiency was about 48%.

The percentage of cells synthesizing DNA was determined by autoradiography using Kodak AR-10 stripping film as previously described (4) (Eastman Kodak Co., Rochester, N. Y.). Cultures were pulsed for 6–8-h periods (less than 25% of the maximal population doubling time [4]) by adding 0.05 ml of [³H]dT in water (1.25 $\mu\text{Ci/ml}$ medium). At least 1,000 cells/dish were scored; a nucleus with ≥ 10 overlying grains was considered to be labeled. Since cultured fetal rat hepatocytes have a tendency to form monolayer aggregates by surface migration after having been plated (1–4), only those cells with well-defined cytoplasmic and nuclear boundaries were scored over many randomly chosen central and increasingly peripheral sites on the dish.

CELL COUNTS: The number of attached cells per dish, which has been shown to be a valid measurement of cell multiplication, was measured with a Coulter Counter (Coulter Electronics, Inc., Fine Particle Group, Hialeah, Fla.) as previously described (3). Errors of measurement among duplicate or triplicate cultures were $\pm 10\%$.

AMINO ACID ANALYSIS: The concentration of basic, acidic, and neutral amino acids released into the culture medium was determined as previously described (3); for arginine determinations, no prior dilution of the sample was made. Ornithine was separated from lysine with a Beckman Model 121 automatic amino acid analyzer, using a column (0.9 \times 24 cm) packed with PA 35 resin. The sample (0.5 ml) was applied to the column and eluted for 210 min (0.25 M sodium citrate, pH 4.25)

at 32.5°C; the elution buffer was then changed (0.38 M sodium citrate, pH 5.66), and 25 min later, the temperature was changed to 62.5°C. The run was then continued for an additional 115 min.

CONDITIONING FACTOR PURIFICATION: 100 ml CM were extracted five times with ethyl ether at 4°C (2:1, vol/vol). The ether layer was brought to dryness over air at 20°C. The residue was redissolved in 0.5 ml ethyl ether, applied to thin-layer plates of Silica Gel-G (5 × 20 cm, Brinkmann Instruments Inc., Westbury, N. Y.), and developed to 15 cm in a solvent system of hexane-ether (4:1). Gel strips 1-cm wide were collected by scraping and eluted for 48 h at 4°C with 1 ml 95% ethanol. Parallel plates were run and developed with iodine vapor to locate migrating components. These plates were not used for bioassays.

Portions of the remaining aqueous layer were redialyzed exhaustively at 4°C against twice-concentrated basal medium to replete the aqueous phase to normal nutrient levels. Fresh basal medium was extracted and worked up similarly to serve as an additional control.

ARGININE-UTILIZATION ASSAYS: Incorporation of [³H]-L-arginine into TCA-soluble and insoluble material was determined by adding to each quiescent culture 2 ml fresh arginine-free and serum-free medium together with the radioactive precursor (0.2 μCi/ml). At varying times (0–180 min) the dishes were removed from the incubator, the medium was aspirated, and the attached cells were rapidly washed three times, for 60 s with ice-cold Tris-saline, pH 7.4 (2) to remove extracellular radioactivity. TCA (5% vol/vol, 2 ml/dish) was then added, and the acid-soluble material extracted (4°C, 24 h). A second similar extraction failed to remove additionally detectable radioactivity. 0.2-ml aliquots of extracted material were mixed with 10 ml Aquasol (New England Nuclear) and counted. The TCA was aspirated and the dishes were washed three times with ice-cold Tris buffer and incubated with twice-concentrated trypsin-TD (2) (37°C, 40 min, 2 ml/dish). After vigorous pipetting by hand, greater than 95% of the fixed cellular material could be removed as determined by standard protein assays (6). At the time and for the duration of the experiment, the number of cells per 30-mm diameter dish was ca. 0.41×10^6 , corresponding to ca. 10 μg protein. The trypsinized material was filtered on Whatman GF/C filters and prepared for scintillation counting by procedures similar to those described above for [³H]dT uptake.

RESULTS

The Conditioning Effect

Cultured fetal rat hepatocytes require CM for cell multiplication, as shown in Fig. 1. Cells plated (4×10^5 cells/dish) into arginine-free, 10% vol/vol dFBS-supplemented medium divided if no further medium changes were made; but if similar fresh

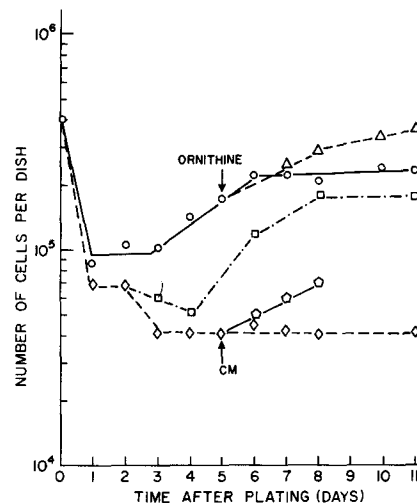


FIGURE 1 Obligatory growth requirement of conditioned medium (CM) by cultured fetal rat hepatocytes. Fetal rat hepatocytes were plated (4×10^5 cells/dish) into 2 ml arginine-free medium containing 10% vol/vol dFBS. No further changes were made on one group of cultures (O—O); a similar group received 0.4 mM *dl*-ornithine 5 days postplating (Δ---Δ). Two other groups of cultures underwent a change of medium at 24 h postplating and received 2 ml identical plating medium. At 48 h, one group (□---□) received 10% vol/vol dFBS-containing CM (see Materials and Methods) while the other (◇---◇) received fresh plating medium. 5 days postplating, this latter group was again subjected to a medium change to similar 10% vol/vol dFBS-CM (pentagons). Cell multiplication was determined by counting attached cells recovered by trypsinization. Abscissa: time after plating (days). Ordinate: number of cells per dish.

medium was added as replacement 24 and 48 h postplating, detectable cell multiplication was arrested. These results were not due to cell settling (3), nor to the mechanical detachment of cells with proliferative capacity arising from the medium changes per se, because cell-free medium collected from parallel cultures 44 h postplating promoted cell multiplication, although with differing kinetics,⁴ when added on day no. 2 or day no. 5 to growth-arrested cultures. Similar qualitative results were obtained when cells were plated under identical conditions with basal medium.

⁴Since evidence has been presented elsewhere that fetal hepatocytes cultured in this manner are a homogeneous cell population (3), the 2-day lag in the proliferative response to CM added on day no. 2, which is not seen when CM is added on day no. 5 (Fig. 1), would imply that the metabolic, or cell cycle states of day 2 cells differ from those of day 5 cells.

Previous observations also indicated that fetal hepatocytes plated without exogenously added serum display increased DNA synthesis rates (4). Together, these results suggested that obligatory growth-promoting material not identical with ornithine or nondialyzable serum factors accumulates in hepatocyte culture medium.

Growth-Promoting Effects of CM Prepared Without Exogenously Added Serum

Various CM preparations were tested on quiescent cultures and found to have growth-promoting activity. The results are shown in Figs. 2 and 3.

Three observations indicated that cells stimulated to initiate DNA synthesis and to divide by

CM in a dose-dependent manner originated from a G_0 (7) and/or early G_1 population: (a) the kinetics of nuclear labeling (Fig. 2) and DNA synthesis rates (5, 8) showed parallel time courses with 8–12-h lags before detectable increases which preceded mitotic figures by 12–14 h; (b) if [^3H]dT was added together with 50% CM, with or without 10% dFBS at zero time, and Colcemid (0.1 $\mu\text{g}/\text{ml}$) 24–36 h later, then >90% of the detectable collected mitotic figures at 36 h were labeled as determined by autoradiography; and (c) the observed increases in the numbers of attached cells per dish at 120 h approximated those expected on the basis of the observed maximal percentages of DNA-synthesizing cells (Fig. 2).

Although maximal proportions of CM-induced DNA-synthesizing cells varied in different experi-

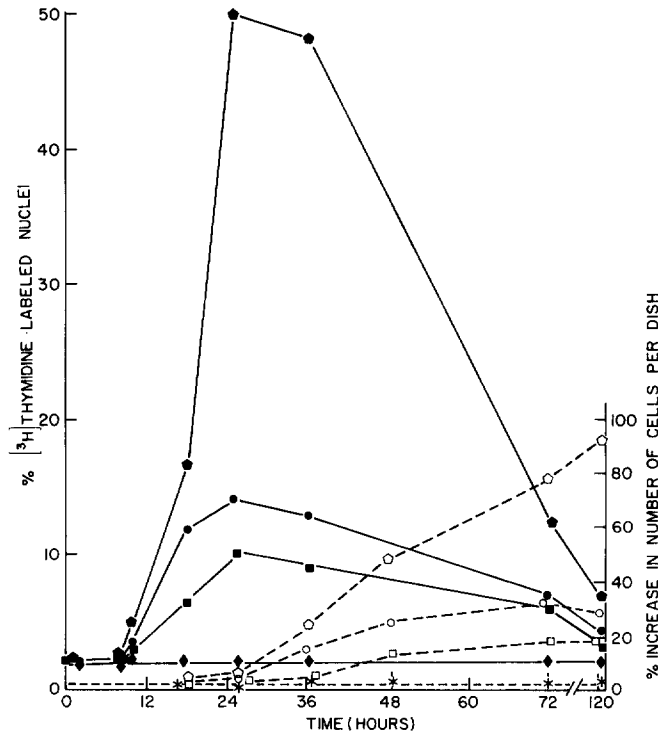


FIGURE 2 Stimulation of the initiation of DNA synthesis and cell division in quiescent fetal rat hepatocyte cultures by serum-free ($\leq 0.00005\%$ vol/vol) CM. Quiescent cultures were prepared by the procedure described in Materials and Methods. At 216 h postplating (time zero), some cultures (0.29×10^5 cells/dish) received no medium change and either no addition or 0.2 ml isotonic saline addition (\blacklozenge or \ast). Other cultures received a medium change to 2 ml fresh basal medium \pm 10% vol/vol dFBS, or to 2 ml CM derived from 3T3 cultures (also \blacklozenge or \ast), or to 2 ml 30% CM (\blacksquare or \square); or to 2 ml 50% CM (\bullet or \circ); or to 2 ml 50% CM supplemented with 10% vol/vol dFBS (closed pentagons or open pentagons). At varying times the percentage of DNA-synthesizing cells (solid lines) was determined by autoradiography while cell multiplication (dashed lines) was determined by counting attached cells recovered by trypsinization. Abscissa: time after additions (h). Left ordinate: percent labeled nuclei. Right ordinate: percent increase in number of cells per dish.

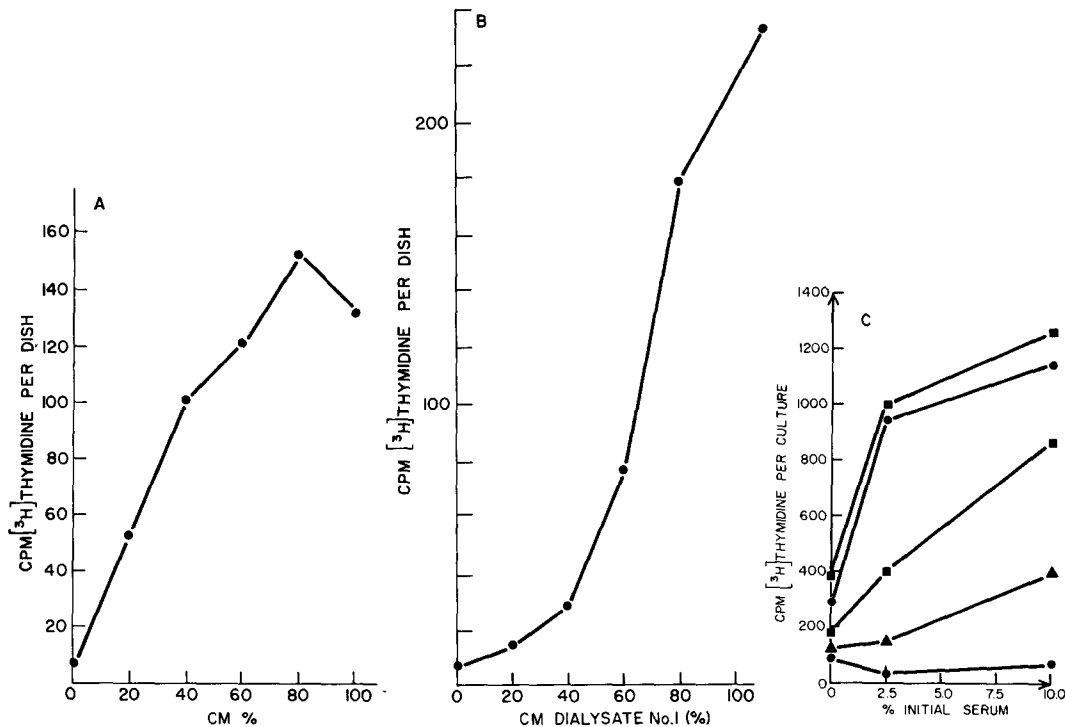


FIGURE 3 Dose response curves to undialyzed CM, CM dialysate, and serum interaction with respect to initiating DNA synthesis in quiescent cultures. Quiescent fetal rat hepatocyte cultures were prepared by the procedure described in Materials and Methods. At 216 h postplating (time zero), the spent media were aspirated and replaced with 2 ml of the media to be tested with or without supplements as indicated below. DNA synthesis was determined by 2-h pulse-labeling the cultures between 22 and 24 h with [³H]dT (1.25 μCi/ml, 3 × 10⁻⁸ M dT) and measuring the incorporation of radioactivity into TCA-insoluble material. (A) Dose-response to undialyzed CM. CM was prepared as described in Materials and Methods and diluted with fresh basal medium. Abscissa: percent CM in fresh basal medium. Ordinate: cpm [³H]dT incorporated per culture. The number of cells per culture at time zero was 0.24 × 10⁶. (B) Dose-response to a dialysate of CM in the presence of excess serum. 50% CM was prepared and dialyzed as described in Materials and Methods (see also Table II, dialysate no. 1). The CM dialysate was diluted with fresh basal medium and then supplemented with 10% vol/vol dFBS. Abscissa: percent CM dialysate in fresh serum-supplemented basal medium. Ordinate: cpm [³H]dT incorporated per culture. The number of cells per culture at time zero was 0.27 × 10⁶. (C) Efficiency of stimulation of the initiation of DNA synthesis: mutual enhancement by both conditioned medium and serum. CM was prepared as described in Materials and Methods and diluted with fresh basal medium which in some cases was supplemented with different concentrations of dFBS (0–10% vol/vol). Abscissa: percent initial serum supplemented to medium. Ordinate: cpm [³H]dT incorporated per culture. The number of cells per culture at time zero was 0.55 × 10⁶. The percent vol/vol CM in basal medium was: 0 (●—●), 25 (▲—▲), 50 (■—■), 100 (●—●), and 75 (■—■).

ments,³ in any single experiment stimulation of DNA synthesis was proportional to the initial CM concentration from 0–80% vol/vol (Fig. 3 A). If CM was supplemented with dFBS, synergistic stimulatory responses were observed (Figs. 2, 3 C) with about 50% of the cells participating under maximal conditions in the first round of DNA synthesis (Fig. 2).

It would appear unlikely that CM was acting to

dilute an inhibitor released into the culture medium by 9-day old quiescent cells because if spent medium from these cultures was diluted 50% vol/vol with CM and put back on to the same cultures, then DNA synthesis was initiated, whereas dilution of the spent culture medium with fresh basal medium was nonstimulatory. The additional possibility that stimulatory conditioning material “neutralized” (9) putative growth-inhibi-

tory material released into the culture medium by quiescent cells also was ruled out by an additional set of control experiments which showed that the quantitative stimulatory capacities of fresh CM diluted 50% with either fresh basal medium or fresh basal medium exposed to quiescent cultures for 24 h were identical. However, higher concentrations (>75–80%) of undialyzed CM (Fig. 3 A, C) but not CM-dialysate (Fig. 3 B) inhibited initiation of DNA synthesis, which suggested that nondialyzable inhibitory material also was present in CM. This was supported by the finding that 10-fold concentration of the dialysate by ultrafiltration with PM10 membranes yielded material which markedly inhibited (80–90%) initiation of DNA synthesis.

Studies on the Nature of Conditioning Material Required to Initiate DNA Synthesis and Mitosis

Although arginine levels in the medium previously were reported to be about 2 μM (3), it appeared likely that at least some of the conditioning material consisted of arginine because of the observations that ornithine, but not putrescine, enhanced cell multiplication in both slowly (3) and rapidly growing cultures (Fig. 1).

Four different lines of evidence would tend to support this: (a) fresh medium supplemented with 5% vol/vol dFBS and low levels of arginine (3–4 μM) stimulated initiation of DNA synthesis in quiescent cultures to near maximal levels, but not when arginine levels were $\leq 1.0 \mu\text{M}$ (Fig. 4 C, D); (b) *l*-enantiomer, but not *d*-enantiomer, precursors of arginine, such as ornithine or citrulline, were effective supplements to arginine-free medium for the preparation of active CM, whereas supplements consisting of no addition, 0.4 mM putrescine, or 0.1 mM uridine were ineffective as determined by autoradiographic measurements of DNA-synthesizing cells in DNA initiation assays (Table I); (c) functionally active 50% CM was found by amino acid analysis to contain arginine ($1.1 \times 10^{-5} \text{ M}$), $\geq 80\%$ of which could be accounted for by synthesis⁵ from $[3\text{-}^3\text{H}]\text{dl}$ -ornithine⁶;

⁵ Serum proteolysis (10), protein degradation, or extracellular enzymatic conversion of arginine precursors was unlikely to have made sufficient amounts of arginine available for growth because (a) 10% vol/vol dFBS-supplemented basal medium incubated 44 h at 37°C failed to stimulate detectable DNA synthesis using initiation

and (d) dialysis of CM removed DNA synthesis stimulatory activity but repletion of exhaustively dialyzed CM with $1.1 \times 10^{-5} \text{ M}$ arginine restored 70–80% of the initial stimulatory activity (Table II).

Two unexpected results were obtained in these experiments. Exogenous arginine, when added either to the spent medium of day no. 9 cultures (Fig. 4 A, B) or to fresh, serum-free medium (Fig. 4 C, D), stimulated DNA synthesis. Furthermore, simultaneous addition of ornithine (Fig. 4 A, C) produced a slightly synergistic effect, whereas exogenously added ornithine with or without dFBS had no detectable stimulatory activity if arginine was not present (Fig. 4 B, D). These observations raised the possibility that arginine and ornithine in the correct proportions would account entirely for the DNA-synthesizing stimulatory activity of CM. Additional results argue against this, however,

assays; and (b) 1.75% vol/vol dFBS-supplemented CM failed to stimulate DNA synthesis (Table I) whether or not ornithine (0.4 mM) was subsequently added after CM preparation. 10% vol/vol dFBS-supplemented CM similarly tested produced slight stimulation (Table I) not enhanced by subsequent ornithine supplementation. Amino acid analysis of 1.75% dFBS-CM revealed the presence of a low level of arginine (ca. 1.2 μM), whereas analysis of 10% dFBS-CM revealed the presence of slightly higher levels (2.0 μM).

⁶ 10 ml of CM (0.4 mM, *dl*-ornithine) were prepared in the presence of 3- $[^3\text{H}]\text{dl}$ -ornithine (5 $\mu\text{Ci/ml}$) as described in Materials and Methods. This CM, containing 9.2×10^6 cpm/ml, was diluted 50% with TCA (10% vol/vol) so that the resulting diluted CM would be expected to contain approximately 0.1 mM *l*-ornithine and 2.3×10^6 cpm/ml. Analysis of the diluted CM after centrifugation with no visible precipitate indicated 10% reduction of ornithine, i.e., the concentration remained about equal to $9 \times 10^{-5} \text{ M}$; the arginine concentration was found to be $11 \times 10^{-6} \text{ M}$. Amino acid analysis of 0.5 ml diluted CM (ca. 1.1×10^6 cpm) revealed that 800,000 and 80,000 cpm were present in the ornithine and arginine peaks, respectively (300,000 counts were found in the acidic and neutral peaks). Thus, the specific activity of *l*-ornithine would be ca. 1,780 cpm/ 10^{-10} mol (800,000 cpm/ 450×10^{-10} mol). Assuming that one molecule of *l*-ornithine is converted to one molecule of *l*-arginine, the quantity of synthesized *l*-arginine would be 9×10^{-9} mol/ml ($80,000 \times 2/1,780$) or $9 \times 10^{-6} \text{ M}$. The ratio 9:11 (0.82) is thus a *lower limit* for the quantity of synthesized *l*-arginine released into CM under the above conditions, owing to the possible loss of labeled arginine into the ornithine peak via extracellular (arginase?) hydrolysis which was not corrected for.

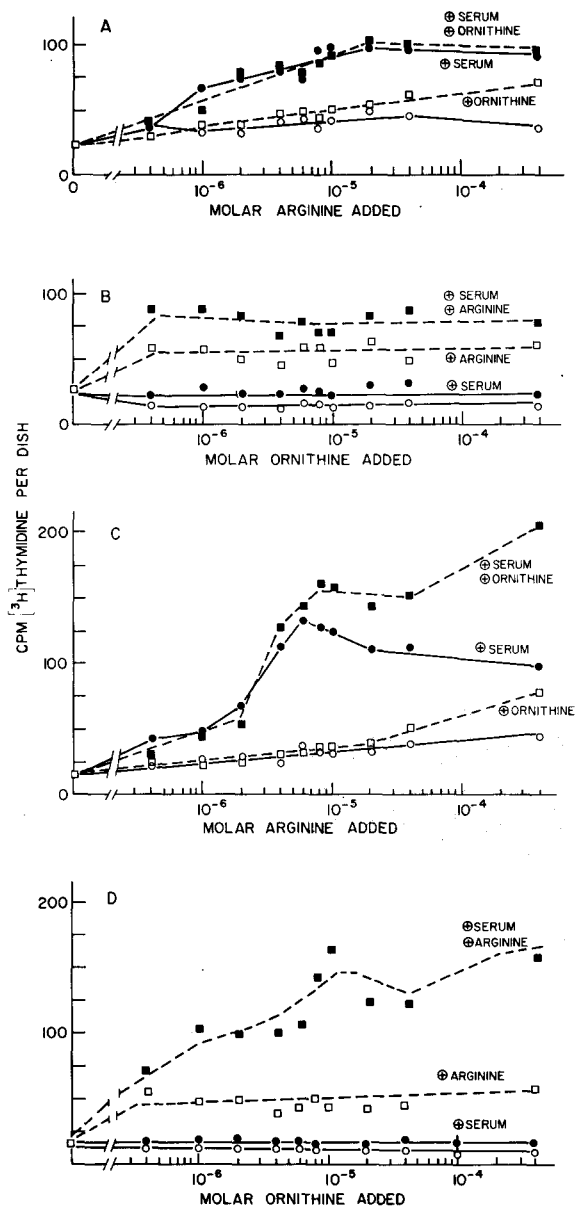


FIGURE 4 Requirement for limiting nutrients with respect to the initiation of DNA synthesis and serum interactions. Quiescent fetal rat hepatocyte cultures (0.22×10^5 cells/dish) were prepared as described in **Materials and Methods**, and divided into two groups. In the first group, additions were made to spent medium at 216 h postplating (panels A, B). In the second group, the media were changed 216 h postplating to 2 ml fresh, arginine-free media which were then supplemented as indicated in panels C and D. DNA synthesis was determined by 2-h pulse-labeling the cultures between 22 and 24 h with $[^3\text{H}]\text{dT}$ ($1.25 \mu\text{Ci/ml}$, 3×10^{-6} M dT) and measuring radioactivity incorporated into TCA-insoluble material. In some cases (dashed lines) either L-arginine or *dl*-ornithine was added in excess (0.4 mM). All concentrations are final concentrations per ml culture medium. Where indicated, dFBS supplementation was 5% vol/vol. Abscissas: A and C, final molar concentration of L-arginine; B and D, final molar concentration of *dl*-ornithine. Ordinates: A–D, cpm $[^3\text{H}]\text{dT}$ incorporated per culture.

TABLE I
Effects of Different Supplements Added to Arginine-Free, Serum-Free Medium on Conditioning Factor Production by Cultured Fetal Rat Hepatocytes

Medium conditioned by fetal rat hepatocytes supplemented with:	[³ H]dT-labeled nuclei			
	h after medium change			
	0	8	16	28
	%	%	%	%
Nothing	2	1	3	1
dFBS (1.75% vol/vol)	—	2	2	3
dFBS (10.0% vol/vol)	—	2	3	7
Putrescine (0.4 mM)	—	2	2	3
Uridine (0.1 mM)	—	1	1	2
l-Citrulline (0.4 mM)	—	2	4	22
l-Ornithine (0.4 mM)	—	2	6	34
d-Ornithine (0.4 mM)	—	2	2	3

Washed suspensions of fetal rat hepatocytes were prepared for DNA initiation assays as described in Materials and Methods. Conditioned media were produced by plating fetal rat hepatocytes (4×10^6 cells/9-cm diameter dish) with 10 ml arginine-free medium supplemented with the materials listed above. The amount of residual serum which may have been present in CM derived from serum-free platings would be $\leq 0.00005\%$ vol/vol. CM were centrifuged, sterilized as described in Materials and Methods, and added (2 ml) to 9-day old quiescent cultures (ca. 0.44×10^6 cells/dish). At various times, the percentage of cells synthesizing DNA was determined by pulse-labeling the cultures for 8 h with [³H]dT (1.25 μ Ci per ml) and preparing the dishes for autoradiography as described in Materials and Methods.

because, as shown in Fig. 4C, fresh serum-free medium containing excess l-ornithine (0.2 mM) and a concentration of arginine equal to that present in 50% CM (10 μ M) failed to stimulate DNA synthesis. Moreover, as shown in Table II, additional DNA synthesis-initiating activity apparently remained in a nondialyzable form because reconstituted dialysand ([arginine] = 1.1×10^{-5} M) was fivefold more stimulatory than basal medium similarly reconstituted with arginine.

These observations indicated that CM contained additional material functionally capable of inducing DNA synthesis. Direct evidence for this is presented in Table II. Here it is shown that extraction of CM with ethyl ether removed most of the stimulatory material without reducing the capacity of appropriately supplemented basal medium to support initiation of DNA synthesis. Material recovered from ether extracts of CM was chromatographed on thin-layer silica-gel plates in a sol-

vent system of hexane-ether (4:1) and was found to yield fractions (R_f 0.4–0.7) which stimulated the initiation of DNA synthesis in a dose-dependent manner. The addition of thin-layer chromatography ethanol eluates $>5 \mu$ l could not be accomplished in these studies due to toxic impurities in the gel preparations. No detectable stimulatory activity could be extracted from fresh l-arginine-supplemented (2×10^{-5} M) basal medium under similar conditions. Parallel chromatography studies with CM ether extracts further indicated a correspondence between a unique spot of iodine vapor-positive material ($R_f = 0.50$) and the peak of DNA-synthesis stimulatory activity ($R_f \sim 0.5$ –0.6). Prior exposure to CM for 24 h at 4°C to pH extremes (pH 2, pH 10) failed to reduce activity, as further shown in Table II.

Regulation of Cultured Fetal Rat Hepatocyte Growth by Arginine, Ornithine, and Serum Factors

Quiescent fetal rat hepatocyte cultures originate from 2-day old cultures (see Materials and Methods) exposed 7–8 days to 50% CM, the initial arginine level of which was found to be ca. 10 μ M. As the cell population grew and came to rest by day no. 9, further daily amino acid analyses of the culture media indicated that arginine was being depleted, resulting in a final level barely detectable ($\leq 1 \mu$ M). Readdition of arginine (0.4 mM) to spent medium in day no. 9 cultures did not produce a significant stimulation of DNA synthesis (Fig. 4 A), but if arginine was added together with fresh serum-free medium as noted above, then DNA synthesis was stimulated to a small but significant amount, as shown in Fig. 4 C. In separate experiments (5), it was observed by autoradiography that these increased DNA synthesis rates were indicative of only a small increase in the percentages of cells synthesizing DNA (4–5%, background less than 2%). These observations suggested that in addition to arginine, other substances may have been depleted from the medium, and this was verified by the finding that as the cell population became quiescent, cysteine levels in spent medium also had undergone $\geq 90\%$ reduction (initial concentration, ca. 0.4 mM).

Readdition of 5% vol/vol dFBS to spent medium failed to promote initiation of DNA synthesis as shown in Fig. 4 A; similar results were observed when serum was added together with

TABLE II
 Partial Physical Characterization of DNA Synthesis-Initiating Material in Fetal Rat Hepatocyte Conditioned Medium

Addition	[³ H]dT cpm/culture	Addition	[³ H]dT cpm/culture
Controls			
Nothing	15	pH stability	
Basal medium + dFBS, 5% vol/vol	20	CM, 50%, pH 2 (24 h)	187
Basal medium + L-arginine, 2 × 10 ⁻⁵ M	35	CM, 50%, pH 10 (24 h)	177
Basal medium + L-arginine, 2 × 10 ⁻⁵ M + dFBS, 5% vol/vol	300	Lipophilic nature	
Basal medium + L-arginine, 4 × 10 ⁻⁴ M	50	CM, 100%, ether-extracted	30
Basal medium + L-arginine, 2 × 10 ⁻⁵ M + dFBS, 5% vol/vol + ethanol, 2 μl	309	Basal medium, ether-extracted	35
Basal medium + ethanol, 2 μl	27	Basal medium, ether-extracted + L-arginine, 2 × 10 ⁻⁵ M + dFBS, 5% vol/vol	325
CM, 20%	58	TLC-CM eluate, R _f = 0.40-0.47, 1 μl	40
CM, 50%	175	TLC-CM eluate, R _f = 0.40-0.47, 2 μl	64
CM, 100%	190	TLC-CM eluate, R _f = 0.47-0.53, 1 μl	85
Dialyzability		TLC-CM eluate, R _f = 0.47-0.53, 2 μl	110
CM, Dialysate no. 1	83	TLC-CM eluate, R _f = 0.73-0.80, 1 μl	39
CM, Dialysate no. 2	46	TLC-CM eluate, R _f = 0.73-0.80, 2 μl	71
CM, Dialysate no. 3	14	TLC-basal medium, R _f = 0.47-0.53, control, 2 μl	28
CM, Dialysand (no. 3)	16	TLC-basal medium, R _f = 0.47-0.53, control, 5 μl	8
CM, Dialysand + L-arginine, 4 × 10 ⁻⁴ M	145		

Washed suspensions of fetal rat hepatocytes were prepared for DNA initiation assays as described in Materials and Methods. The additions (2 ml medium, serum or CM fractions as noted) were made to the 9-day old quiescent cultures (0.30 × 10⁵ cells/dish), and the rate of DNA synthesis was determined by 2-h pulse-labeling the cultures 22-24-h later with [³H]dT (1.25 μCi/ml, 3 × 10⁻⁶ M dT). 40 ml CM prepared as described in Materials and Methods were dialyzed against 80 ml basal medium (4°C, 48 h). The resulting dialysate (no. 1) was sterilized and put aside for testing; similar dialyses were then performed in tandem in order to prepare dialysates nos. 2 and 3, stopping with the final dialysand (no. 3, 40 ml). pH treatments of CM, followed by reneutralization, were carried out as described in Materials and Methods as were the preparations of thin-layer silica gel chromatography fractions derived from ether-extracts of CM (TLC-CM eluates) and basal medium. The addition of 1 μl TLC-eluate was equivalent to 10% CM. Thin-layer chromatography-eluate additions were made to basal medium supplemented with 2 × 10⁻⁵ M L-arginine.

fresh arginine-free medium with or without ornithine (Fig. 4 B). This indicated that exogenous arginine was required for new and continued rounds of DNA synthesis, and receives some experimental support from the observation (5) that under maximal conditions of growth stimulation (fresh basal medium supplemented with 0.4 mM arginine and 5-10% vol/vol dFBS), DNA synthesis rates, which declined after the 32nd h (Fig. 2), began to increase again at about the 40th h (5).

These observations raised the possibility that under these culture conditions one or more serum factors initiate DNA synthesis by controlling cellular processes which stimulate utilization and/or synthesis (3) of growth-limiting nutrients, particularly arginine.

Direct evidence supporting a "utilization" mechanism is shown in Fig. 5. Here it may be seen that two serum components known to be involved with initiating DNA synthesis in this system, immunoreactive insulin-enriched SFI (4) and crystalline insulin (8), both stimulated increased rates of arginine utilization by quiescent cells, as determined by measuring the cumulative incorporation

of [³H]-L-arginine into cold TCA-soluble and insoluble material. Rate changes were not observed until 30-40 min (insoluble counts, Fig. 5 B) and 90-120 min (soluble counts, Fig. 5 A) after the start of the experiment. About 60-70% of the cold acid-precipitable counts at all time points measured remained acid-precipitable after heat treatment (90°C, 20 min). Precursor incorporation into this material and the subsequent stimulation of DNA synthesis both were inhibited >95% by cycloheximide (5.0 μg/ml). No substantial quantitative or qualitative differences were observed when guanido-[¹⁴C]-L-arginine was used as a precursor.

Indirect evidence supporting a "synthesis" mechanism is shown in Fig. 4 C and 4 D. Maximal initiation of DNA synthesis could be achieved only when arginine, serum, fresh medium, and ornithine were combined; neither putrescine (10⁻⁸-10⁻⁴ M) nor uridine (10⁻⁵-10⁻³ M), compounds taken up by the cells (11, 12), were capable of substituting for arginine and/or ornithine. In addition, when arginine and serum were present in excess, then the stimulation of DNA synthesis was proportional to

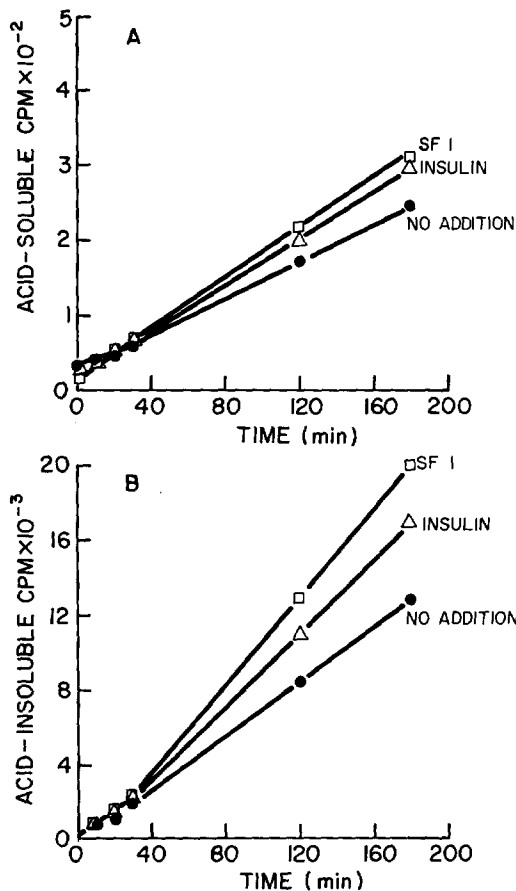


FIGURE 5 Stimulation of arginine-utilization by insulin and serum fraction I (SFI). 2 ml fresh, arginine-free medium was supplemented with [^3H]-L-arginine (0.2 $\mu\text{Ci/ml}$) and either 0.1 ml isotonic NaCl (●—●), insulin (Δ — Δ , 1.0 $\mu\text{g/ml}$), or SFI (□—□, equivalent to 5% vol/vol whole dFBS) and then added to quiescent cultures (0.41×10^5 cells/dish). At varying times (abscissae), cumulative TCA-soluble (ordinate in A) and cold TCA-precipitable (ordinate in B) radioactivity per culture were determined as described in Materials and Methods.

the molar concentration of exogenously added ornithine (Fig. 4 D).

DISCUSSION

CM harvested from primary monolayer cultures of differentiated fetal rat hepatocytes (1, 4) has been analyzed to determine the manner by which it regulates growth of these cells. These studies were facilitated by preparing CM without exogenously added serum ($\leq 0.00005\%$ vol/vol) and by using quiescent cultures which do not condition the medium to assay for growth-stimulatory material.

CM contains material obligatory for growth in addition to that which is functionally equivalent to one or more serum factors (4, 8). The former material has been identified as arginine, at least 80% of which is synthesized from ornithine and released by the cells into the culture medium. This aspect of the conditioning effect is, therefore, similar to that described for a variety of undifferentiated, cloned mammalian cell lines capable of constitutively synthesizing amino acids required for growth (13–15). The possibility has not been excluded that ornithine is also a precursor to additional growth-stimulatory material distinct from arginine. Compounds which might otherwise spare the ornithine requirement (16), such as putrescine and uridine, are unlikely, however, because neither obviated the growth requirement for CM, or was capable of producing active CM.

Conditioning material distinct from arginine, which promotes the initiation of DNA synthesis is extracted by ethyl ether, stable to acid (pH 2) and alkali (pH 10) treatment, and chromatographs on thin-layer silica gel plates in hexane-ether (4:1) with a peak of activity corresponding to a $R_f \sim 0.5$. This material, therefore, is unlikely to be identical to putrescine (17), or to the somatomedin-like peptides released by a liver-derived cell line (18) which lacks arginine-biosynthetic capacity (1), or to the tripeptide (gly-lys-his) isolated from human serum which promotes survival, but not DNA synthesis, of uncharacterized adult liver cell suspensions (19). Rather, a nonpolar lipid or lipid-containing material is implicated. The nature of the nondialyzable material appearing in CM (Table II) as well as its possible identity with ether-extractable material and other previously reported macromolecular conditioning (20, 21) and serum (4) factors remains to be determined.

The probability that cultured fetal rat hepatocytes enter or leave G_0 appears to be controlled by the exogenous levels of serum factors, at least three amino acids, arginine, ornithine, and cysteine, and probably additional, as yet to be determined, nutritional components of the medium. Limitation of either the serum factor or the obligatory nutritional component limits the initiation of DNA synthesis. How interaction between these factors regulates postmitotic and early prereplicative events in cultured hepatocytes is poorly understood, although the present evidence suggests (Fig. 5, Table II) that SFI (4), or insulin, or possibly lipid-like conditioning material promotes cellular utilization and/or synthesis (3) of small molecules,

such as arginine, which limit growth (8, 22-26). The results further implicate, elevated protein synthesis rates as at least one of the early responses to "shift-up" conditions (27, 28). The kinetics of insulin- or SFI-stimulated arginine utilization (Fig. 5) could be taken to mean that exogenous [^3H]-L-arginine entered protein faster than the free intracellular pool, a result consistent with the interpretation that exogenous amino acids are preferentially selected for protein synthesis at a membrane site (29), perhaps related to findings in other cell systems of possible serum-factor regulation of amino-acyl tRNA synthetase-membrane complex formation (30, 31).

Amino acids have been reported to regulate protein synthesis in the perfused liver (32) and, under special conditions, to exert insulin-like effects (32) and to promote the initiation of *in vivo* hepatocellular DNA synthesis (34), although this latter phenomenon also may be related to indirect effects (8). It remains to be determined whether or not observations made with this *in vitro* hepatocyte system, that DNA synthesis may be stimulated by fresh arginine-supplemented, serum-free medium (5), bear any relation to these other findings.

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