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 Functions

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ABSTRACT

Dialyzed fetal bovine serum contains two distinct growth-controlling macromolecular fractions: one stimulates and the other inhibits proliferation of primary cultured differentiated fetal rat hepatocytes.

Both fractions are precipitated by ammonium sulfate (50% saturation, pH 7.4, 4°C). Serum fraction I (SFI, mol wt $\geq$120,000 daltons estimated by gel filtration with Bio-gel P200) appears to contain at least two factors which function, respectively, to initiate DNA synthesis (activity pH 4–10 stable) and to increase the rate at which initiated cells traverse the cell cycle (activity pH 4 and pH 10 labile). Intraperitoneal injections of SFI into adult rats have produced detectable stimulation of hepatic but not renal DNA synthesis. Serum fraction II (SFII, mol wt 40,000–80,000 daltons) suppresses in vitro incorporation of CH_3-[^{3}H]thymidine into DNA under conditions which diminish neither cell viability nor cell attachment.

Mixing experiments indicate that SFI and SFII mutually antagonize each other with respect to DNA synthesis and cell multiplication. Thus, both the relative and absolute serum levels of multiple factors control in vitro fetal hepatocyte proliferation.

INTRODUCTION

In vitro hepatocellular growth control studies have made use of either explant (1–5) or uncharacterized monolayer cultures (6, 7) but thus far, none of these systems has proved useful to assay for putative blood-borne hepatotrophic mitogens.

Problems encountered with liver and differentiated tissue culture systems (8, 9) have recently been obviated by using fetal rat hepatocytes plated according to enrichment culture techniques (10) which select against prolonged survival and multi-

plication of nonparenchymal cells (11). The resultant euploid (11) hepatocellular population in these long-term primary cultures retains prominent specialized[1] functions, such as the capacity to synthesize arginine and albumin (8), alpha$_1$-fetoprotein (14), and haptoglobin and hemopexin.[2] These cultured hepatocytes also respond to serum from partially hepatectomized rats by enhanced DNA and protein synthesis (10, 16) and display an obligatory requirement of conditioned medium for proliferation (8, 11, 17).

Previous findings (8, 10, 11, 16) that both heterologous and homologous sera stimulate in vitro fetal hepatocyte growth have indicated that growth-promoting activity is not entirely species-specific. For this reason and because of the availability of fetal bovine serum, it has been used for continued fractionation studies (11), the results of which suggest, as described here, that a multiplicity of serum factors control in vitro hepatocellular proliferation. In particular, it appears that serum factors required to initiate DNA synthesis are distinct from those which control continued traverse through the cell cycle. Moreover, inhibitors also are detectable in fractionated serum which antagonize one or more of the stimulatory factors. Thus relative as well as absolute levels of both stimulatory and inhibitory serum factors control proliferation of these cultured hepatocytes.

MATERIALS AND METHODS

Reagents

Collagenase (C0130), *dl*- or *l*-ornithine, Cohn Fraction II, and crystalline BSA[3] were purchased from Sigma Chemical Corp., St. Louis, Mo. Highly purified Fetuin preparations were obtained from Grand Island Biological Co., Grand Island, N. Y. Different batches of fetal bovine sera were supplied by Kam Laboratories, Inc.; Grandview, Mo., and Grand Island Biological Co. Crystalline ammonium sulfate was obtained from Mann Research Labs, Inc., New York. [^{3}H]dT (sp act 20 Ci/mmol) was purchased from New England Nuclear, Boston, Mass. Kodak AR10 stripping film was used for autoradiography (Eastman Kodak Co., Rochester, N. Y.).

Cell Cultures

The method of culturing fetal rat liver cells has been described in detail elsewhere (8, 11, 14). Freshly prepared cell pellets were washed twice with serum-free medium before final distribution into plating medium. Rapid cell plating into large numbers of dishes was accomplished by using presterilized flow syringes (Arthur H. Thomas Co., Philadelphia, Pa.). Liver cell culture medium was composed without arginine and supplemented with *dl*-ornithine (0.4 mM)[4] or *l*-ornithine (0.2 mM). Plastic 30-mm diameter tissue culture dishes (NUNC, Roskilde, Denmark) were used for all experiments. Initial seeding densities, serum concentrations (percent volume/volume), and culture medium volumes are described in the respective figure legends.

Mouse 3T3 and SV3T3 fibroblasts were obtained from Dr. M. Vogt (Salk Institute) and were used for control studies. These cells were passaged and grown as previously described (8).

Both liver cell and fibroblast cultures were checked and found to be free of pleuropneumonia-like organism contamination by labeling the cells with [^{3}H]dT and performing autoradiography as previously described (8).

Preparation of Serum and Serum Fractions

This report describes studies done only with dFBS, prepared as previously described (11). The concentration of serum protein ranged between 25 and 30 mg/ml as determined with the Folin reagent (19) using BSA as standard.

Serum was fractionated to 50% saturation with ammonium sulfate at 0–4°C with constant stirring. The precipitate was redissolved in 0.05 M sodium-potassium phosphate buffer, pH 7.4 ("standard buffer"). Both the precipitate- and supernate-derived fractions representing about 35% and 65% of the total protein, respectively, were dialyzed exhaustively against standard buffer. Recovery of A_{280} nm material was 90–95%. For some experiments (see Fig. 3 D), the precipitate-derived fraction (9 A_{280} units/ml) or pooled P200 column fractions

[1] Although many specialized functions differ between proliferating fetal and quiescent adult rat liver cells (12), there is reason to believe that humoral control(s) postulated to operate during adult liver regeneration also may affect proliferation of fetal liver cells late in gestation (13). Moreover, alpha-fetoprotein biosynthesis is at least one specialized rat liver function common to both the growing fetal and the regenerating adult liver (14, 15).

[2] H. Leffert, U. Mueller-Eberhard, and S. Sell. Manuscript in preparation.

[3] *Abbreviations used in this paper:* bovine serum albumin, BSA; CH_3-[^{3}H]thymidine, [^{3}H]dT; dialyzed fetal bovine serum, dFBS; serum fraction I (defined in Results) SFI; serum fraction II (defined in Results), SFII; trichloroacetic acid, TCA.

[4] Proliferative responses of cells cultured with *dl*-ornithine (0.4 mM) did not differ significantly from those of cells cultured with *l*-ornithine (0.2 mM). This would be expected from a previous report that amino acid *d*-enantiomers do not inhibit growth in the presence of adequate concentrations of the *l*-enantiomers (18).

nos. 10–12 (see Fig. 4 A) were dialyzed at 4°C for 24 h against either 0.2 M sodium acetate buffer, pH 4.0, or 0.2 M sodium bicarbonate buffer, pH 10. This was followed by exhaustive dialysis against standard buffer, pH 7.4. No detectable precipitate formed under these conditions.

Gel chromatography of ammonium sulfate fractions was performed by using a 2 × 120-cm siliconized glass column packed to a bed volume of 350 ml with Biogel P200, 100–200 mesh (Bio-Rad Laboratories, Richmond, Calif.), and then equilibrated and eluted at 4°C with standard buffer at a hydrostatic pressure of 15 cm water. Approximately 60 6.2-ml fractions were collected using a drop counter (model LKB, Bromma, Sweden) during a period of 168 h. Fractions to be rechromatographed were pooled, dialyzed exhaustively at 4°C against water, lyophilized, resuspended in standard buffer, and run on a 1.5 × 90-cm siliconized glass column packed to a bed volume of 150 ml, and then equilibrated and eluted as described above. Approximately 70 2-ml fractions were collected during a period of 96 h. Columns were calibrated with a mixture consisting of 1 mg/ml blue dextran, 10 mg/ml crystalline BSA, and 1 mg/ml phenol red. Recoveries of A_{280} nm material were 90–98%.

Serum or serum fractions used for various growth assays were filter sterilized (0.4 μm Swinnex, Millipore Corp., Bedford, Mass.) and stored at −20°C. Under these conditions, serum growth-promoting activity was stable for periods up to 6 mo.

Preparation of Conditioned Medium

Conditioned medium (CM) was prepared by plating 2 × 10^6 freshly isolated, washed (17) fetal hepatocytes together with 2 ml arginine-free, *dl*-ornithine-supplemented (0.4 mM) medium in 30-mm diameter tissue culture dishes. At the time of plating, the following additions were made: 0.2 ml isotonic NaCl; precipitate- or supernate-derived ammonium sulfate fraction equivalent to 10% vol/vol whole serum. CM was harvested at 44 h postplating, centrifuged, and sterilized as described elsewhere (14, 17).

Assays

Serum or serum fractions to be tested were added in different amounts to each 30-mm diameter culture dish in final amount ≤0.3 ml. Freshly prepared fetal hepatocytes were suspended in serum-free medium and plated at a concentration of 1 × 10^6 cells/ml, 2 ml per dish, together with the added fractions. DNA synthesis-initiating activities of conditioned media prepared with both ammonium sulfate fractions, as well as pH 4 and pH 10 treated serum fractions, were assayed using quiescent hepatocyte cultures prepared as described elsewhere (14, 17).

Measurements of cell multiplication and DNA synthesis (incorporation of [^{3}H]dT into DNA, autoradiography) were carried out as described in detail elsewhere (11, 14, 17). For all assays, duplicate or triplicate determinations were made. The measurement errors ranged between ±5 and 10%.

Serum-immunoreactive insulin was determined by radioimmunoassay with the Schwarz/Mann kit (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.).

RESULTS

Growth Cycle Studies with Cultured Fetal Rat Hepatocytes

It was previously reported that ornithine stimulated the proliferation of cultured, slowly growing fetal rat hepatocytes plated into arginine-free medium (11). Therefore, ornithine (0.4 mM *dl*-enantiomer, or 0.2 mM *l*-enantiomer) was routinely added to the plating medium to increase the sensitivity for detecting growth-promoting stimuli in various serum fractions.

It was found that under these conditions: (*a*) cells plated *together* with dFBS were capable of markedly enhanced proliferation; and (*b*) the recovery efficiency (8) 24 h postplating was inversely proportional to the percent volume/volume initial serum concentration. These results are shown in Figs. 1 A and 4 C, respectively. Consistent with previous findings (11), it was observed that fetal rat hepatocytes grew at rates and attained final cell densities in proportion to the initial amount of dFBS added to the medium (0–10% vol/vol). This proportionality also applied to the percentages of cells recruited to synthesize DNA before the onset of and *during* logarithmic growth (Fig. 1 B). At 10% vol/vol serum, autoradiography with extended pulse times of 12-h duration (less than 0.5 the population doubling time) indicated that greater than 80% of the cells were synthesizing DNA 65–77 h postplating (data not shown). Similar results were obtained with two different batches of dialyzed fetal bovine serum obtained from two different commercial sources.

The kinetics of cell attachment were studied in separate experiments and it was found that by 24 h postplating, although floating, nonviable cells and debris remained in the culture medium (11), further cell attachment was minimal. The attached cell population at this time was found to consist of at least 90% viable cells (trypan-blue exclusion [11]), independent of the initial serum concentration. Similar results were obtained 36 h postplating, however, these cells were more adherent to the

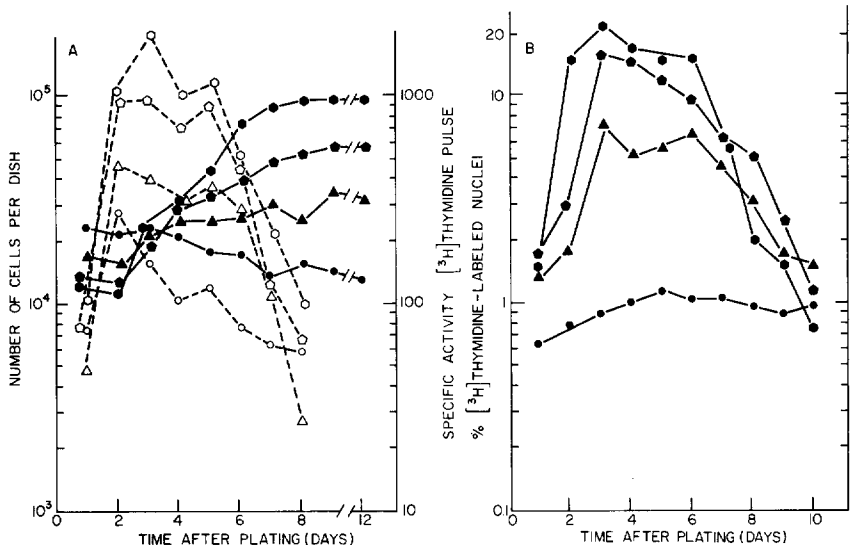

FIGURE 1 Growth cycle studies with fetal rat hepatocytes cultured in L-ornithine-supplemented (0.2 mM) medium. (A) Growth curves and DNA synthesis rates as a function of initial serum concentrations. Fetal rat hepatocytes were plated (2×10^5 cells/dish) with arginine-free, L-ornithine-supplemented medium (2 ml/dish), containing the following initial quantities (percent vol/vol) of dialyzed fetal bovine serum: 0.0 (circles), 0.5 (triangles), 2.0 (pentagons), and 10.0 (hexagons). At varying times after plating, cell multiplication (solid lines) was determined by counting the number of attached cells recovered by trypsinization. DNA synthesis rates (dashed lines) were determined by pulse labeling the cultures for 2 h with [^{3}H]dT (1.25 μCi/ml, 3×10^{-6} M dT) and measuring the quantity of radioactivity incorporated into TCA- (5% vol/vol) insoluble material. Abscissa: time after plating (days). Left ordinate: number of cells per dish. Right ordinate: TCA-insoluble cpm per 10^5 cells per 2-h pulse. (B) Proportion of DNA-synthesizing cells as a function of initial serum concentrations. Parallel cultures were established under conditions identical to those indicated above (Fig. 1 A). At varying times after plating, the proportion of DNA-synthesizing cells per culture was determined by pulse labeling the culture for 2 h with [^{3}H]dT (1.25 μCi/ml) after which autoradiography was performed using stripping film. 1,000 cells per culture were scored. Abscissa: time after plating (days). Ordinate: percent labeled nuclei per culture.

plastic substratum in comparison to 24-h cultures, as measured by the capacity of simple washing procedures to remove attached cells. Cells plated without added serum were observed (phase microscopy, $\times$ 100) to be more uniformly distributed over the substratum, although individual cells tended to remain refractile and rounded up. When serum or stimulatory serum fractions were present, the cells migrated on the plastic substratum and spread into monolayer aggregates (8) about 48 h postplating (phase time-lapse microcinematography, $\times$ 125).

Serum Fractionation Studies

When hepatocytes were plated into ornithine-supplemented media together with ammonium sulfate fractions equivalent to 10% vol/vol whole serum, the cells multiplied faster (threefold) and reached proportionately higher population densities in the fraction derived from precipitated serum (50% salt saturation) than in the supernatant fraction (material remaining soluble at 50% salt saturation). This is shown in Fig. 2 A.

It appears that of the two ammonium sulfate fractions, precipitate-derived material enhances growth because it contains greater quantities of one or more factors which stimulate the *rate* of DNA synthesis and/or continuation through the cell cycle (S $\rightarrow$ M traverse). Both fractions, however, contain material required to increase the proportions of DNA-synthesizing cells ($G_{0,1} \rightarrow$ S traverse). This implies that at least two factors control proliferation.

A "two-factor" hypothesis is supported by the observations that: (*a*) initial DNA synthesis rates (46–48 h postplating) of cells cultured with precipi-

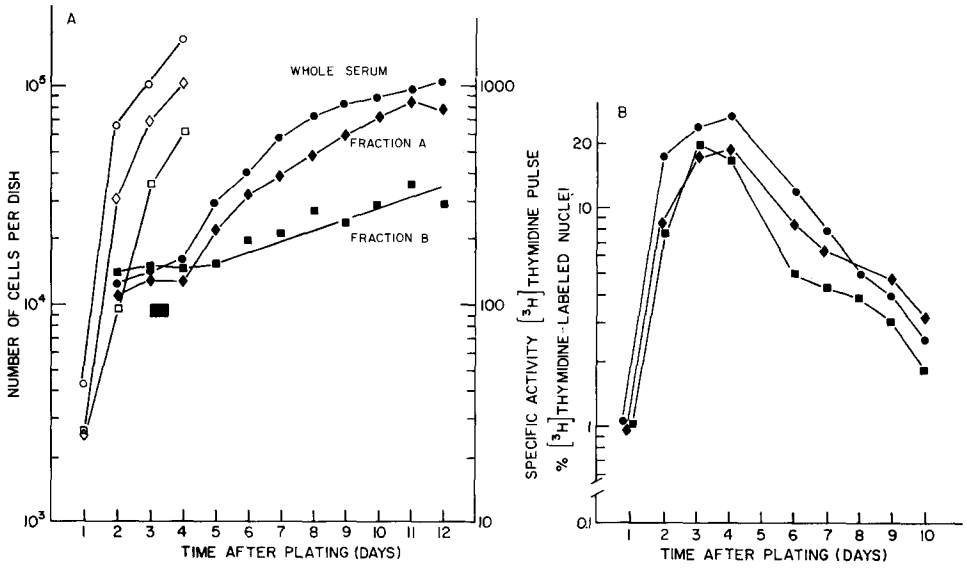

FIGURE 2 Growth cycle studies with fetal rat hepatocytes cultured in L-ornithine-supplemented (0.2 mM) medium together with ammonium sulfate fractions of dialyzed fetal bovine serum. (A) Growth curves and DNA synthesis rates. Fetal rat hepatocytes were plated (2×10^5 cells/dish) together with arginine-free, ornithine-supplemented medium (2 ml/dish) containing the following initial quantities of either dFBS (10% vol/vol [circles]) or material precipitated by ammonium sulfate at 50% saturation equivalent to 10% vol/vol unfractionated serum (diamonds); or material soluble in ammonium sulfate at 50% saturation equivalent to 10% vol/vol unfractionated serum (squares). At varying times thereafter, cell multiplication (solid points) was determined by counting the numbers of attached cells recovered by trypsinization. DNA synthesis rates (open points) were determined by pulse labeling the cultures for 2 h with [^{3}H]dT (1.25 μCi/ml, 3×10^{-6} M dT) and measuring the quantity of radioactivity incorporated into TCA- (5% vol/vol) insoluble material. Abscissa: time after plating (days). Left ordinate: number of cells per dish. Right ordinate: TCA-insoluble cpm per 10^5 cells per 2-h pulse. (B) Proportion of DNA synthesizing cells. Parallel cultures were established under conditions identical to those indicated above (Fig. 2 A). At varying times after plating, the proportion of DNA-synthesizing cells per culture was determined by pulse labeling the culture for 2 h with [^{3}H]dT (1.25 μCi/ml) after which autoradiography was performed using stripping film. 1,000 cells per culture were scored. Abscissa: time after plating (days). Ordinate: percent labeled nuclei per culture.

tate-derived material were usually two to threefold greater than rates observed with supernate-derived material (Fig. 2 A) although equal numbers of cells had been recruited during a 2-h [^{3}H]dT pulse to synthesize DNA in the presence of either fraction (Fig. 2 B); (*b*) fourfold increases of prevailing DNA synthesis rates resulted from the addition of increasing amounts of precipitate- but not supernate-derived material (Fig. 3 A); (*c*) cellular viability (determined by trypan-blue exclusion) and attachment were quantitatively similar in the presence of either fraction during the time interval 24–96 h postplating (Fig. 2 A) and no detectable preferential cellular detachment was observed thereafter in the presence of supernate-derived material as determined by pulse-chase DNA-labeling experiments (11) with [^{3}H]dT (Fig. 3 B); (*d*) supernate-derived material did not inhibit DNA synthesis since the addition of increasing amounts to cultures of cells already engaged in DNA synthesis produced small proportionate increases in DNA synthesis rates (Fig. 3 A); (*e*) culture media conditioned independently in the presence of either precipitate- or supernate-derived material were identical in their capacity to initiate (14, 17) DNA synthesis (Fig. 3 C); (*f*) dialysis of precipitate-derived material against pH 4 or pH 10 buffers, or direct acidification or alkalinization at 4°C for 24 h followed by neutralization, failed to diminish activity required to promote initiation of

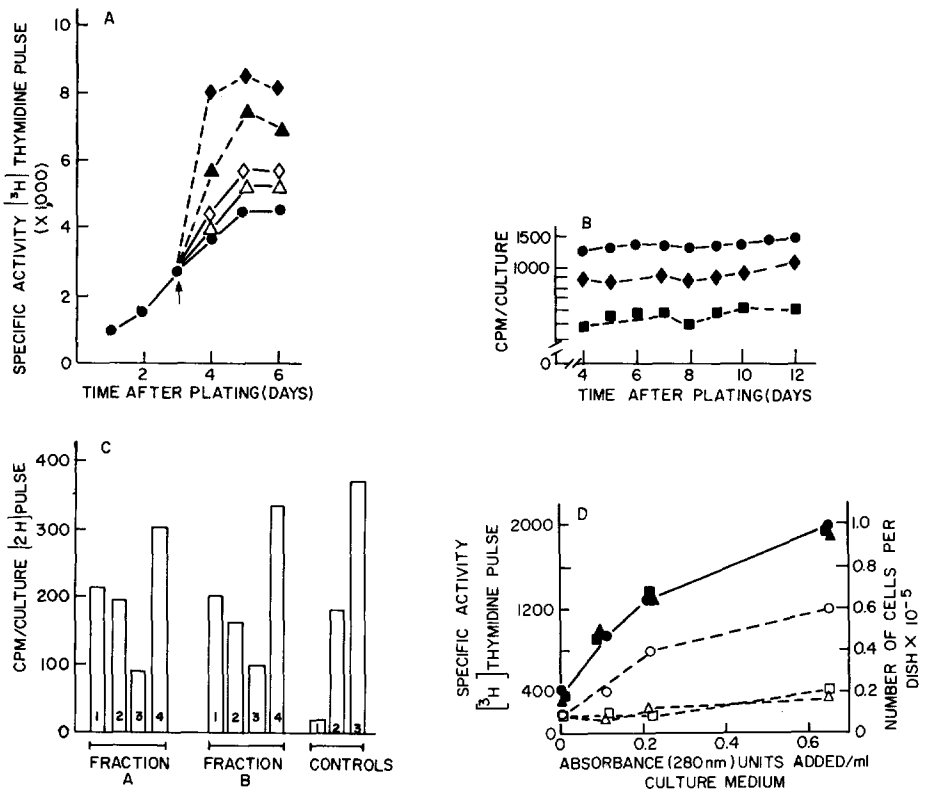

FIGURE 3 Comparisons between ammonium sulfate fractions obtained from dialyzed fetal bovine serum with respect to various cell culture parameters. (A) Growth inhibitory activity. Fetal rat hepatocytes were plated (2×10^5 cells/dish) together with 2 ml arginine-free, L-ornithine-supplemented (0.2 mM) medium containing the supernate-derived ammonium sulfate fraction (equivalent to 5%, vol/vol unfractioned serum [circles]). At 72 h postplating, the following additions were made (arrow): 0.2 ml standard buffer (circles); the precipitate-derived ammonium sulfate fraction (▲---▲, equivalent to 1.25%, vol/vol unfractionated serum; and ◆--- ◆, equivalent to 5%, vol/vol unfractionated serum); and the supernate-derived ammonium sulfate fraction (equivalencies of 1.25%, vol/vol, Δ—Δ; and 5%, vol/vol, ◇ —◇). At varying times thereafter, cultures were pulse labeled for 8 h with [^{3}H]dT (1.25 μCi/ml, 3×10^{-6} M dT), and TCA-insoluble radioactivity per culture was determined. Abscissa: time after plating (days). Ordinate: TCA-insoluble cpm per 10^5 cells per 8-h pulse. (B) Cellular detachment. Fetal rat hepatocytes were plated (2×10^5 cells/dish) under conditions identical to those indicated in Fig. 2 A. Identical parallel cultures were plated simultaneously from which conditioned media (CM) were collected (44 h postplating), worked up as described in Materials and Methods, and subsequently supplemented with dT (3×10^{-6} M). At 72 h postplating, all cultures were pulse labeled for 12 h (shaded rectangle, Fig. 2 A) with [^{3}H]dT (1.25 μCi/ml, 3×10^{-6} M dT) after which the radioactive media were aspirated. The cultures were washed twice with 2 ml fresh, serum-free, arginine-free, L-ornithine-supplemented (0.2 mM) medium and the appropriate CM were added to the corresponding dishes, dFBS, 10% vol/vol (circles); material precipitated by ammonium sulfate at 50% saturation equivalent to 10% vol/vol unfractionated serum (diamonds); and material soluble in ammonium sulfate at 50% saturation equivalent to 10% vol/vol unfractionated serum (squares). At varying times thereafter, TCA-insoluble radioactivity per culture was determined. Abscissa: time after plating (days). Ordinate: cpm per culture. (C) Conditioning factor production. Media conditioned by cultured fetal rat hepatocytes in the presence of ammonium sulfate fractions (precipitate-derived, fraction A; supernate-derived, fraction B) were prepared as described in Materials and Methods and 2 ml added under various conditions to 9-day old quiescent fetal hepatocyte cultures (0.29×10^5 cells/dish) prepared as described elsewhere (14, 17). The various CM additions were: undialyzed CM (*1*); 12 h dialysand of CM (17), (*2*); 12 h dialysate of CM, diluted 50% with fresh arginine-free, L-ornithine-supplemented (0.2 mM)

DNA synthesis but abolished almost 70% of the activity required to permit continued cell multiplication (Fig. 3 D); and (*g*) preparative gel chromatography (not shown) of both ammonium sulfate fractions under nearly identical conditions (ca. 20 ml each, $A_{280} \cong 14.5$ units/ml, 4 × 60-cm siliconized glass column, Bio-gel P200, 0.05 M phosphate buffer, pH 7.4) indicated that each fraction contained macromolecular material ≥120,000 daltons which stimulated, either alone, or if mixed, in an additive fashion, both cell multiplication and DNA synthesis. On the basis of protein content, however, precipitate-derived material yielded macromolecular fractions containing three to four times the amount of protein- and cell multiplication-stimulating activity as did parallel gel column fractions obtained from supernate-derived material.

Rechromatography of pooled lyophilized gel fractions of mol wt ≥120,000 daltons from precipitate-derived material showed that: (*a*) only one molecular weight class (≥120,000 daltons) of growth-promoting activity could be detected under these conditions, although this material was now about 30–40% less active; and (*b*) considerable cross contamination of both high and lower molecular weight materials (40,000–80,000 daltons) had occurred which masked the inhibitory activity present in the lower molecular weight fraction. Inhibitory activity was not detectable after similar rechromatography of pooled gel fractions of mol wt ≥120,000 daltons from supernate-derived material.

The resolution of both stimulatory and inhibitory activities is clearly shown in Fig. 4 A. Macromolecular serum fractions (designated SFI) exerted a positive stimulatory effect since increasing amounts produced stimulation of both DNA synthetic rates and cell multiplication (Figs. 4 B and 5 A, respectively). Separate experiments indicated that increasing amounts of SFI equivalent to 1–20% vol/vol dFBS also increased the proportions of DNA-synthesizing cells (29–65%) as measured by autoradiography 120–128 h postplating. These results suggested that SFI also contained at least two factors. Further evidence for this was obtained by exposing SFI to conditions of low (pH 4) and high (pH 10) pH and then performing experiments similar to those described and shown in Fig. 3 D, which gave similar results.

In contrast, the lower molecular weight serum fractions, designated SFII, exerted inhibitory effects, apparently not due to a deficiency of required factors since increasing amounts added to the culture medium produced more inhibition of DNA synthesis and, at higher levels, equivalent to 10% vol/vol dFBS, cell detachment and death (Figs. 4 C and 5 B).

Neither SFI nor SFII alters the pH of incubated culture medium (0–15% vol/vol). Their specific activities are, respectively, 12- and 15-fold greater than those of untreated dialyzed serum (Figs. 4 B and 4 C, respectively).

Mutually Antagonistic Actions of SFI and SFII

Mixtures of SFI (peak I, fraction no. 10, Fig. 4 A) and SFII (peak II, fraction no. 20, Fig. 4 A) were tested for their capacity to control DNA

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medium (*3*) and supplemented with 10% vol/vol dFBS (*4*). Controls received: 0.2 ml buffer (*1*); undiluted serum-free CM (17), (*2*) and supplemented with 10% vol/vol dFBS (*3*). DNA synthesis rates were determined by pulse labeling the cultures for 2 h (22–24 h postaddition) with [^{3}H]dT (1.25 μCi/ml, 3×10^{-6} M dT) and measuring the radioactivity incorporated into TCA-insoluble material. (D) Susceptibility of serum growth factors to differential pH treatment. Material precipitated by ammonium sulfate at 50% saturation (A_{280}, 9 units per ml) was dialyzed separately against buffer pH 4, pH 7.4, or pH 10, as described in Materials and Methods. Activity required to initiate DNA synthesis was assayed by using 9-day old quiescent cultures (0.5×10^5 cells/dish, basal cpm/dish, 80) prepared as described elsewhere (14, 17) and determining DNA synthesis rates as described in the legend to Fig. 3 C. Activity required to permit continued cell multiplication at detectable rates was assayed by adding similarly treated fractions to slowly growing cultures (plating density, 2×10^5 cells/dish; 2 ml arginine-free medium supplemented with 0.2 mM *l*-ornithine and 0.3% vol/vol dFBS) 2 days postplating (1×10^4 cells/dish) and counting the number of attached cells recovered by trypsinization 5 days later. Abscissa: absorbance (280 nm) units of fraction added per milliliter of basal culture medium. Left ordinate: specific activity of thymidine incorporation (cpm/10^5 cells/2-h pulse). Right ordinate: attached number of cells per dish, 7 days postplating.

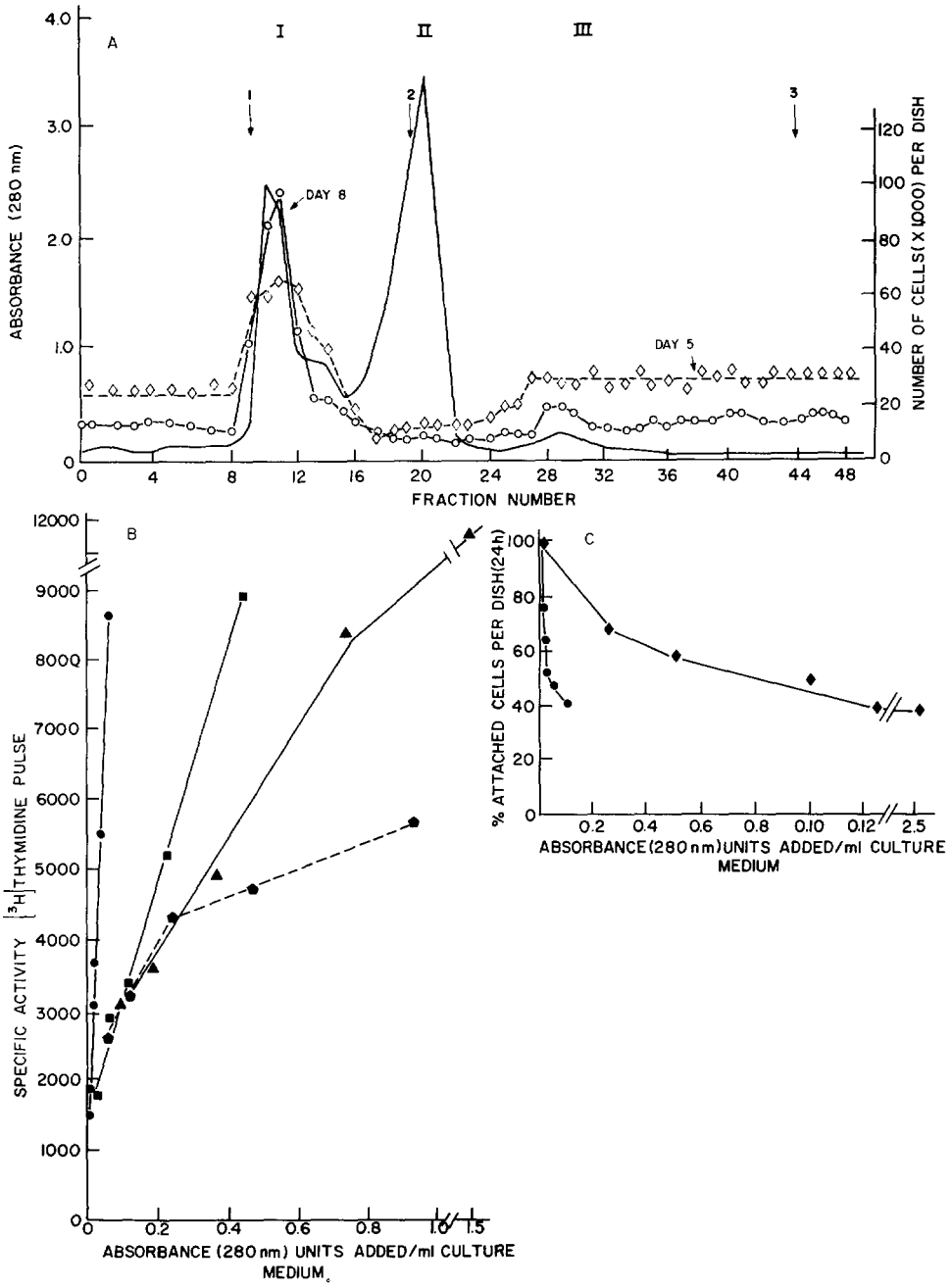

FIGURE 4 Simultaneous occurrence of growth-stimulatory and growth-inhibitory material in the precipitate-derived ammonium sulfate fraction of dialyzed fetal bovine serum. (A) Gel filtration and cell multiplication assays. A resolving column (2 × 120 cm) of Bio-gel P200, 100-200 mesh, was set up and run as described in Materials and Methods. Material precipitated by ammonium sulfate at 50% saturation (8 ml, 127 A_{280} units) was layered on to the column and eluted with 0.05 M phosphate buffer, pH 7.4, I, 0.05. Absorbance at 280 nm of each fraction (6.2 ml) (left ordinate, continuous solid line) was monitored with a Zeiss spectrophotometer; arabic numbers 1, 2, 3 represent, respectively, the following markers: blue

synthesis and cell multiplication. The results of these experiments, shown in Figs. 5 C and 5 D, indicated that each of the serum fractions contained one or more substances whose growth-controlling effects upon cultured fetal rat hepatocytes were mutually antagonistic.

For example, when SFI was present in the culture medium in excess (5% vol/vol, Fig. 5 C), increasing amounts of SFII inhibited DNA synthesis and cell multiplication but did not produce detectable cellular detachment. When SFII was present in the culture medium in excess (Fig. 5 D), increasing amounts of SFI failed to elevate DNA synthesis rates to those levels achieved by SFI alone (Fig. 5 A); however, the toxic effects of SFII were counteracted, as indicated by increased numbers of cells remaining attached to the dish (day no. 6 postplating). The capacity to counteract SFII toxicity appeared to be a specific property of material present in SFI because neither a commercial preparation of the gamma globulin fraction II of Cohn nor crystalline albumin, tested in concentrations ranging from 10–5000 μg/ml, was capable of replacing SFI.

Cell detachment alone would not be sufficient to explain the reduced DNA synthesis rates stimulated by SFII, since a 75% decrement of [^{3}H]dT incorporation could be observed under conditions where less than 15% of the attached cells had been lost from the dish (Fig. 5 C). Similarly, the DNA synthesis rate 4 days postplating in the presence of 5% vol/vol of both SFI and SFII was about 2.5-fold less than the observed rate for cultures exposed to 5% vol/vol SFI alone (Figs. 5 D and 5 A, respectively), although the numbers of attached cells at this time in the two different cultures were similar (1.5×10^5 cells/dish). In separate mixing experiments, when DNA synthesis was monitored by autoradiography, it was found that increasing amounts of SFII also reduced the proportions of DNA-synthesizing cells.

A series of control experiments was carried out in which SFII was incubated for 2 h at 37°C with 2.5 μCi of [^{3}H]dT, or added at a concentration of

dextran (mol wt $\geq 2 \times 10^6$ daltons), bovine albumin (mol wt 67,000 daltons), and phenol red (mol wt 354 daltons). 1 ml of each fraction is equivalent to 2.5 ml serum.[5] Abscissa: fraction number.

Fetal rat hepatocytes were plated (2×10^5 cells/dish) into 2 ml arginine-free, L-ornithine-supplemented (0.2 mM) medium together with the fractions (0.13 ml/dish equivalent to 15% vol/vol serum). At varying times thereafter, cell multiplication was determined by counting attached cells recovered by trypsinization. Right ordinate: number of cells ($\times$ 1,000) per dish. (B) DNA synthesis rates: dose response curves. Fetal rat hepatocytes were plated (2×10^5 cells/dish) into 2 ml arginine-free, L-ornithine-supplemented (0.2 mM) medium together with the fractions (0.1 ml/dish) to be tested (●—●, [peak I] pooled fractions 10–12, Fig. 4 A; ■—■, ammonium sulfate fraction derived from material precipitable at 50% salt saturation; ▲—▲, dFBS; shaded pentagons, material soluble in ammonium sulfate at 50% saturation). Beforehand, the fractions were adjusted by dilution with standard buffer so that their addition to each culture represented the following serum equivalents (percent, vol/vol): 0.32, 0.64, 1.25, 2.5, and 5.0. Control addition was standard buffer (0.1 ml/culture, "zero" absorbance). DNA synthesis rates were determined by pulse labeling the cultures for 8 h (64–72 h postplating) with [^{3}H]dT (1.25 μCi/ml, 3×10^{-6} M dT), and measuring the radioactivity incorporated into TCA-insoluble material. The number of attached cells was also determined at this time. Abscissa: absorbance (280 nm) units added per milliliter culture medium. Ordinate: cpm radioactivity incorporated per 10^5 cells per 8-h pulse. (C) Cytotoxicity of peak II: dose response. Fetal rat hepatocytes were plated into 2 ml arginine-free, L-ornithine-supplemented (0.2 mM) medium together with the fractions (0.1 ml–0.2 ml/dish) to be tested (●—●, fraction no. 21, Fig. 4 A; and ◆—◆, dialyzed fetal bovine serum). Beforehand, the fractions were adjusted by dilution with buffer so that their addition to each culture represented the following serum equivalencies (percent, vol/vol): 0.63, 1.25, 2.50, 5.0, and 10.0. Control addition was standard buffer (0.1 ml per culture). 24 h postplating, the number of attached cells per culture was determined by counting attached cells recovered by trypsinization (100% = control = 22,000 cells per culture). Abscissa: absorbance (280 nm) units added per milliliter culture medium. Ordinate: percent attached cells per dish, 24 h postplating.

[5] Since Fraction A (8 ml) is twice concentrated, i.e., was derived from 16 ml unfractionated serum, it follows that each gel filtration fraction (6.2 ml) represents approximately a quantity of material of a given molecular weight present in 16 ml initial serum.

10% vol/vol to serum-deficient conditioned medium (17) in order to determine whether inhibition of DNA synthesis had resulted from binding of either thymidine or conditioning factors to material present in SFII. In both cases, binding was unlikely since it was found that (*a*) greater than 99.9% of the radioactivity could be removed from SFII by dialysis against isotonic saline; and (*b*) 100% of the obligatory conditioning activity could be recovered by serial dialyses against equal volumes of fresh serum-free media which were tested by using DNA synthesis initiation assays with serum-supplemented quiescent cultures (14, 17). This latter observation also minimized the possibility that SFII had completely inactivated an obligatory nutritional component of the medium (20).

Because fetuin has been found to comprise as

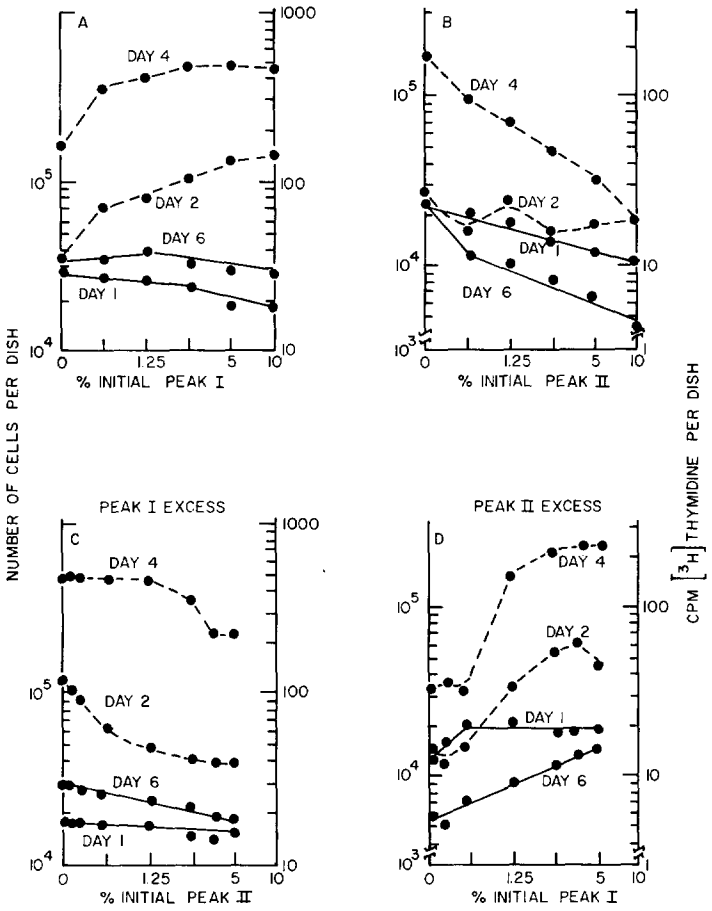

FIGURE 5 Mutual antagonism between SFI and SFII with respect to cell multiplication and DNA synthesis. Fetal rat hepatocytes were plated (2×10^5 cells/dish) into 2 ml arginine-free, L-ornithine-supplemented (0.2 mM) medium togenter with the fractions (0.1-0.3 ml per culture; SFI, fraction no. 10, Fig. 4 A; SFII, fraction no. 20, Fig. 4 A) to be tested. Beforehand, all fractions were adjusted (by dilution with buffer) so that their additions to the cultures represent vol/vol equivalencies to whole serum. Additions of "excess" fraction are equivalent to 5% vol/vol. At varying times after plating, either the number of cells/culture (solid lines), or the DNA synthesis rate (dashed lines, 2-h pulse) was determined as described above. The panels represent: (A) dose response to SFI (from peak I, Fig. 4A); (B) dose response to SFII (from peak II, Fig. 4A); (C) dose response to SFII in the presence of "excess" SFI; and (D) dose response to SFI in the presence of "excess" SFII. Absicssa: percent initial concentration (vol/vol) of either fraction no. 10 or no. 20. Left ordinate: number of cells/dish. Right ordinate: TCA-insoluble [^{3}H]dT incorporated/culture/2-h pulse.

much as 25–30% of the total protein of fetal bovine serum (21), to be precipitable by ammonium sulfate at 50% salt saturation (22), and to have a mol wt of 48,000 daltons (23), it was likely that the bulk of the protein present in SFII was fetuin. However, two different batches of highly purified (21) commercial fetuin preparations were found, when tested in concentrations ranging from 5–5,000 μg/ml, to be neither cytotoxic nor growth-inhibitory, nor growth-stimulatory to cultured fetal hepatocytes, but only after these preparations had been further purified by dialysis against EDTA (0.1 mM) or fractionated on carboxymethyl cellulose columns, pH 4.5, in order to remove bound Zn^{++}. These results suggest, then, that growth-inhibitory and/or toxic activity present in SFII was the effect of material other than fetuin.

Effects of SFI and SFII on Other Cultured Cell Types

The growth responses of two established cell lines, mouse 3T3 fibroblasts and their virally transformed derivative, SV3T3 cells, were studied by plating these cells with fractions (0.1 ml per dish) from the column shown in Fig. 4 A (the conditions for plating and for assaying were identical to those used for fetal rat hepatocytes except that the medium was supplemented with L-arginine [0.4 mM] to permit cell viability and multiplication). 3T3 cells were observed to synthesize DNA and to multiply slowly in proportion to the amount of protein present in all fractions from the column, that is, their multiplication-stimulating activity spread throughout the entire profile of column fractions. These results were similar to those previously reported (24) which showed that prior acidification of serum was necessary effectively to separate some of the 3T3 growth factors from the larger fraction of serum protein. There was no indication that 3T3 cells were either detached or inhibited from growing on the tissue culture dishes by material in SFII. In contrast, SV3T3 cells responded to SFI and SFII, as well as to mixtures of the two fractions, in a manner similar to that observed with fetal hepatocyte cultures.

Preliminary Animal Experiments

Preparations of SFI have been injected into intact adult Fisher/344 rats weighing 150–200 g. Animals received two intraperitoneal injections at 0900 h in divided doses of 35 mg protein each, 4 h apart. 22 h after the first injection, the animals were pulsed for 1 h with [^{3}H]dT (sp act 50 Ci per mmol, 250 μCi per kg body wt) administered intraperitoneally. After this, the animals were sacrificed by cervical dislocation, and their right lateral hepatic lobes and kidneys prepared for microscopic tissue sections and autoradiography by routine histological procedures and the stripping-film method (16), respectively. Animals which received the serum fractions showed, on the average, 8–10-fold as many labeled nuclei (40–50 per 3,000 scored) as did the controls (sham-hepatectomized or saline-injected animals, 5 per 3,000), although in comparison to partially hepatectomized animals (600 per 3,000) the response was considerably reduced. Kidney tissue sections were similarly scored and the results were similar to those of the controls. Other tissues reported to proliferate subsequent to partial hepatectomy, such as corneal epithelium (25) and testis (26), were not scored.

DISCUSSION

Growth control studies reported here demonstrate that the growth of primary cultured differentiated fetal rat hepatocytes is regulated by at least two distinct macromolecular serum fractions which have been partially purified from dialyzed fetal bovine serum and which separately exert dose-dependent stimulatory and inhibitory effects upon DNA synthesis and cell division. Combinations of these fractions are mutually antagonistic, which suggests that a ratio of substances contained within native serum controls the growth of these cultured cells.

The two serum fractions have been obtained after ammonium sulfate fractionation (0–50% saturation) and gel chromatography using Biogel P200, 0.05 M phosphate buffer, pH 7.4. They have been designated SFI and SFII and correspond to the first and second protein peaks, respectively, eluted from the P200 column shown in Fig. 4 A.

SFI consists of 12-fold purified material of $\geq$120,000 daltons mol wt. DNA synthesis rate and short-pulse (2 h) autoradiographic labeling studies, combined with pH lability experiments, tend to indicate that SFI is a mixture of at least two factors: one appears to promote $G_{0,1} \rightarrow S$ transi-

tion; the other, transition S → M, thereby diminishing transit time in the cell cycle. Whether or not these factors represent a single pleotypic material (27) or a dissociable complex susceptible to pH extremes cannot be determined until further purification studies are performed. The fact that acidification to pH 4 of stimulatory serum fractions fails to abolish DNA synthesis-initiating activity also suggests that acid-stable growth factors such as insulin (28) or somatomedin (29) may have similar activity. Some evidence for this is presented in an accompanying report (30) although neither peptide alone can account for the entire DNA synthesis-initiating activity of SFI. An additional observation (H. Leffert, unpublished observations) has been the finding of detectable immunoreactive insulin in peak fractions of SFI (75–100 microunits/mg protein peak fractions, Fig. 4 A) at about 10 times the amount detectable in peak fractions of SFII (Fig. 4 A). The possible significance of this is under current investigation, especially in light of the suggestive evidence that both insulin and somatomedin may be associated with macromolecules (29, 31, 32).

The findings reported by others (33, 34), using fibroblast systems, that 4–10 h of serum-exposure "commits" a fraction of the cells to one round of DNA synthesis and mitosis is not inconsistent with a "two-factor" hypothesis for cultured hepatocytes. Either both classes of stimulatory factors act during the prereplicative period, or "traverse" material is capable of affecting cultured hepatocytes subsequent to the initiation of DNA synthesis (S → M). A "commitment" phenomenon has also been demonstrated with the hepatocyte system (30) and may have as its in vivo correlate the known requirement for 10–12-h continuous blood mixing between a partially hepatectomized animal and its cross-circulated intact partner (35).

SFII consists of 15-fold purified material of 40,000–80,000 daltons mol wt. It is found in different batches of fetal bovine serum obtained from different commercial sources, which lessens the possibility of its being an artifact arising from problems of quality control (36). By itself, this fraction is cytotoxic to cultured fetal hepatocytes, whereas when it is mixed with SFI, the cells survive but are inhibited from multiplying and display both reduced DNA synthesis rates and percentages of DNA-synthesizing cells. Proteins which might be present as major or minor contaminants of this fraction, such as fetuin and albumin, respectively, are not cytotoxic, nor can they, like SFI, overcome SFII-mediated growth inhibition. However, SFII does not appear to be cell-specific because it also inhibits the growth of SV3T3 cells and kills them. These observations suggest that SFII contains specific material which preferentially antagonizes rapidly growing cells, presumably at some common metabolic pathway required for initiating DNA synthesis and cell cycle traverse. Alternatively, primary cultured diploid fetal hepatocytes may share certain growth characteristics with at least one virally transformed cell line.

Despite the apparent lack of cell specificity in vitro with SFII, there is reason to believe that under special conditions, in vivo hepatocellular DNA synthesis may be inhibited by glucagon (37). It is unlikely that the inhibitory effects of SFII observed in these in vitro studies are due to glucagon, owing to its low molecular weight (3000 daltons) (38), lack of evidence that it is carried, in its intact form, by serum proteins (39), and serum instability (40). However, physiological concentrations of glucagon (≥ 0.006 μg/ml) have been found to suppress insulin-stimulated DNA synthesis in this system using chemically defined medium and quiescent cultures (30). The possible significance of these findings is discussed in an accompanying report (30).

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