

Growth Control in Cultured 3T3 Fibroblasts

II. Molecular Properties of a Fraction Enriched in Growth Inhibitory Activity

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ABSTRACT Treatment of sparse, proliferating cultures of 3T3 cells with medium conditioned by exposure to density-inhibited 3T3 cultures resulted in an inhibition of growth and division in the target cells when compared to similar treatment with unconditioned medium. This growth inhibitory activity was fractionated by ammonium sulfate precipitation and gel filtration, yielding one fraction that was 35-fold enriched in specific activity. Analysis of the chemical and biological properties of this highly active fraction indicated that: (a) it is an endogenous cell product, synthesized by the 3T3 cells and shed into the medium; (b) it is a protein and its activity is sensitive to treatment with pronase; (c) the constituent polypeptide chains have molecular weights of 10,000 and 13,000; and (d) it is not cytotoxic and its effect on target cells are reversible. These results suggest that we have partially purified from conditioned medium an endogenous growth regulatory factor that may play a role in density-dependent inhibition of growth in cultured fibroblasts. We propose the term Fibroblast Growth Regulator to describe this class of molecules.

The growth rate of normal cells in culture decreases as the number of cells approaches a critical cell density to form a monolayer. This phenomenon, which has been termed density-dependent inhibition of growth (1), was first described and most extensively studied in the 3T3 cell line (2, 3). From analysis performed in a number of laboratories, three major hypotheses have been proposed to account for density-dependent inhibition of growth. The first hypothesis suggests that cell-to-cell contact is a necessary requirement for growth inhibition (3, 4). A second hypothesis suggests that depletion of growth factors, in the bulk medium (5) or in a micro diffusion barrier surrounding the cells (6), causes the cessation of growth. The third hypothesis postulates that soluble growth inhibitors are released into the bulk medium and that these inhibitory factors accumulate to a sufficiently high concentration to act on the target cells.

Although these three theories are not mutually exclusive, several recent studies have provided evidence implicating the role of soluble inhibitory factors (7-10). For example, it has been demonstrated that growth and phosphate metabolism in sparse cultures of 3T3 cells were inhibited when the cells were placed in a petri dish containing dense cultures of the same cells (10). The ability of dense cultures of 3T3 cells to inhibit the proliferation of sparse cultures, separated from each other

but sharing a common growth medium, suggests the depletion of medium or the presence of a soluble inhibitor as a possible mechanism for growth control in fibroblasts.

In a previous communication, we demonstrated that medium conditioned by exposure to cultures of density-inhibited 3T3 cells contained a growth inhibitory activity that acted on sparse, proliferating cultures of the same cell line (11). In this paper, we describe the isolation and characterization of a growth inhibitory fraction that yields two major protein components on polyacrylamide gel electrophoresis analysis. In addition, we demonstrate that the inhibitory activity is not cytotoxic and that its effects on cell growth are reversible. The results suggest that we have established a system for studying the mechanism of density-dependent growth control at the level of interactions of an endogenous growth inhibitor with its target cells.

MATERIALS AND METHODS

Cell Culture and Preparation of Conditioned Medium

Swiss 3T3 cells (American Type Culture Collection, Rockville, Md. CCL 92) were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) (K.C. Biological, Lenexa, Kans.) containing 10% calf serum (Microbiological Associates, Walkersville, Md.). The detailed protocol for the preparation of serum-free Condition Medium [CM(SF)] has been previously described (11). The following

modification of the previous protocol were made to minimize the concentration of serum proteins in our CM(SF). Growth medium from confluent 3T3 source cells was removed and replaced with fresh medium. After 24 h, the growth medium was removed, the cells were washed twice with phosphate-buffered saline (PBS), and then DMEM (10 ml/150 cm² of growth area) was added to the cultures. CM(SF) was collected from the cultures 24 h later and processed as previously described (11).

For experiments that required the source cells to be incubated with radioactive compounds, the labeled precursor was added to the medium during the production of CM(SF). The ¹⁴C-labeled precursors were added to a final concentration of 3.3 μCi/ml for [2-¹⁴C]thymidine (51.4 mCi/mmol, New England Nuclear, Boston, Mass.), [2-¹⁴C]uridine (58 mCi/mmol, Schwarz-Mann, Orangeburg, N. Y.), and to 5 μCi/ml for L-[U-¹⁴C]amino acid mixture (1.87 mCi/mg, ICN Pharmaceuticals, Cleveland, Ohio). D-[2-³H]mannose (20–30 mCi/mmol, New England Nuclear) was used at a final concentration of 1 mCi/ml. To prepare CM(SF) labeled with [³⁵S]methionine, source cells were labeled with DMEM containing 3 μg/ml unlabeled methionine (one-tenth of the concentration normally found in DMEM) and 10 μCi/ml of [³⁵S]methionine (1014 Ci/mmol, New England Nuclear).

Assays of Growth Inhibitory Activity

Target cells were seeded at a density of 5×10^3 cells/cm² in a 24-well culture dish (2 cm²/well, Costar, Cambridge, Mass.) in DMEM containing 3 μg/ml unlabeled methionine and 10% calf serum. The cells were then incubated in [³⁵S]methionine (1 μCi/well) for 24 h. The radioactive medium was then removed and the fraction (0.45 ml) to be assayed for its growth inhibitory activity was placed on the well along with 50 μl of calf serum. After 24 h, the cells were pulsed with 1 μCi/well of [³H]thymidine (1.9 Ci/mmol, Schwarz-Mann) for 3 h at 37°C. The cells were then rinsed twice with cold PBS, once with 10% trichloroacetic acid (TCA), and then 0.55 ml of 1% sodium dodecyl sulfate (SDS) in 0.05 M NaOH was added. After incubation at 37°C for 10 min, an aliquot (0.5 ml) was removed and added to 5 ml of scintillation cocktail (21 g of 2,5-diphenyloxazole; 1 liter of Triton X-100; and 2 liters of toluene) containing 20 μl of 50% TCA. Autoradiographic analysis of cells labeled with [³H]thymidine was carried out as previously described (11).

The growth inhibitory activity of a CM-derived fraction was determined by comparing the amount of [³H]thymidine incorporation on a per cell basis in cultures treated with the fraction to the amount in UCM-treated cultures (percent inhibition, 100% – (CM/UCM) × 100). The number of cells in the target cultures treated with CM-derived fractions or with UCM was determined by the amount of [³⁵S]methionine incorporated into the target cells before the treatments with the fractions to be tested for activity. It should be noted that because the CM(SF) was collected from ³⁵S-labeled cells, the CM-derived fractions that are added to the target cultures for tests of activity also contained radioactivity due to ³⁵S whereas the UCM control cultures did not. We have found, however, that the additional ³⁵S carried by the CM-derived fractions did not affect our determination of the number of target cells. This is mostly because the ³⁵S label carried by the CM-derived fractions was <5% of the level of ³⁵S radioactivity in the target cells and because much of this label could be removed by the PBS washing steps immediately after the [³H]thymidine incorporation.

Assays of Cell Viability

The viability of cells treated with UCM and CM was determined while the cells remained attached to the plastic growth surface. After removal of growth medium, the cells were incubated with trypan blue (0.08% in PBS) for 10 min at room temperature. The staining solution was then removed and the viable cells were counted using an inverted microscope.

Target cells were also labeled with ¹⁴C-amino acids (2 μCi/culture, 58 Ci/matoms of ¹⁴C, Amersham Corp., Arlington Heights, Ill.) for 24 h. After washing, these cells were treated with UCM(SF), CM(SF), or fractions derived from CM(SF) that contained growth inhibitory activity. At various times thereafter, the cells were washed with PBS and were dissolved in 100 μl of 1% SDS. The amount of labeled target cells remaining after the treatment with growth inhibitors was determined by counting the ¹⁴C radioactivity in each culture (11).

Colony Formation Assay

3T3 cells were seeded in a very sparse configuration (150 cells on 10 cm² of growth surface) in 35-mm dishes (Falcon Labware, Oxnard, Calif.). The cultures were incubated overnight to allow for cell attachment. The medium was then removed and parallel cultures were treated with 0.81 ml of UCM(SF), CM(SF), or FGR-s fraction plus 0.09 ml of calf serum. After 48 h, the media were again removed and the cultures were refed with a fresh batch of corresponding media and incubated for another 48 h. The cultures were then washed and stained with 1% crystal violet (Sigma Chemical Co., St. Louis, Mo.) in 50% aqueous ethanol

for 1 h. The cultures were washed three times with PBS. The number of colonies and the number of cells per colony were then counted under an inverted microscope.

Fractionation and Gel Electrophoretic Analysis

CM(SF) (60 ml) was precipitated by the addition of ammonium sulfate to a final concentration of 80% saturation. The precipitate, obtained after centrifugation at 12,400 g for 15 min, was redissolved in 1.5 ml of DMEM. Gel filtrations of the precipitated material were carried out at 4°C on a Sephadex G-50 column (90 x 1 cm) and a Sephadex G-15 column (27 x 1 cm) equilibrated with DMEM. Several different assays were carried out on the individual fractions from the columns. First, the inhibitory activity of each fraction was assayed on growing 3T3 target cells by measurements of [³H]thymidine incorporation. Second, 50-μl aliquots of each fraction were taken for the determination of total radioactivity. Third, the TCA-precipitable radioactivity, representing labeled macromolecular components, was determined by the addition of 100 μl of each fraction onto GF/A (Whatman) filters, washing twice with 10% TCA and once with absolute methanol. Finally, two different methods were used to determine the protein content of the fractions. In most experiments, the method described by Lowry et al. (12) was used, with bovine serum albumin as a calibration standard. In some experiments, the much more sensitive method of Schaffner and Weissman (13) was used, with ribonuclease A as a standard.

Protein fractions were labeled with ¹²⁵I using the method of Greenwood et al. (14). After the labeling reaction, noncovalently bound ¹²⁵I was removed by gel filtration on a column (38 x 2 cm) of Bio-Gel P-2 (Bio-Rad Laboratories, Richmond, Calif.) equilibrated with PBS.

Polyacrylamide gel electrophoresis in SDS was performed according to the procedure of Laemmli (15), using 10% or 15% acrylamide running gel and 5% acrylamide stacking gel. β-Mercaptoethanol (4%) was included in the sample buffer. The gels were fixed and then stained with Coomassie Brilliant Blue. After destaining, the gel was subjected to fluorographic treatment as described by Bonner and Laskey (16), using Kodak X-Omat R (XR-5) film. Alternatively, the gels were sliced immediately after electrophoresis, solubilized by digestion with 30% hydrogen peroxide (60° for 5 h), and then subjected to scintillation counting. In certain gels containing ¹²⁵I-labeled samples, direct autoradiography was performed instead of fluorography; Kodak X-Omat RP (XRP-5) film and intensification screens were used in these autoradiographic procedures.

Sensitivity of Growth Inhibitory Activity to Hydrolytic Enzymes

The sensitivity of the growth inhibitory activity to various enzymes was tested using proteases and nucleases coupled to solid supports. Pronase coupled to carboxymethyl-cellulose beads and ribonuclease A bound to polyacrylamide beads were purchased from Sigma Chemical Co. Bovine deoxyribonuclease I (Worthington Biochemical Corp., Freehold, N. J.) coupled to Sepharose beads was prepared according to the method of Lazarides and Lindberg (17).

All enzyme digestion experiments were carried out in pyrex, silicized test tubes precoated with BSA to minimize nonspecific adsorption of the proteins to the glass surface. The tubes were incubated with BSA (2 mg/ml) for 1 h at 37°C. They were then washed two times with PBS and three times with DMEM. For the pronase-bead experiments, 0.40 ml of UCM(SF), CM(SF), or FGR-s was added to tubes containing 0.06 U of enzyme, and the mixtures were incubated at 37°C for up to 18 h. The highest concentrations tested for ribonuclease-beads and deoxyribonuclease-beads were 0.01 U/ml and 0.014 U/ml, respectively.

After the incubation, the reaction mixtures were centrifuged at 1470 g for 5 min and the supernatant was removed from the pelleted beads for tests of growth inhibitory activity and for gel electrophoretic analysis. These procedures, allowing us to effectively remove the hydrolytic enzymes after the reaction period, have obviated the many problems associated with the addition of proteases and/or protease inhibitors to the target cells, which in turn often changed the proliferative properties of the cells.

RESULTS

Fractionation of the Growth Inhibitory Activity in Conditioned Medium

We have reported previously the optimized parameters for the collection of CM(SF) from source cells and for the assay of the growth inhibitory activity on target cells (11). We have now carried out fractionation of the growth inhibitory activity using ammonium sulfate precipitation and gel filtration on a column of Sephadex G-50 (Fig. 1). In these experiments, CM(SF) was

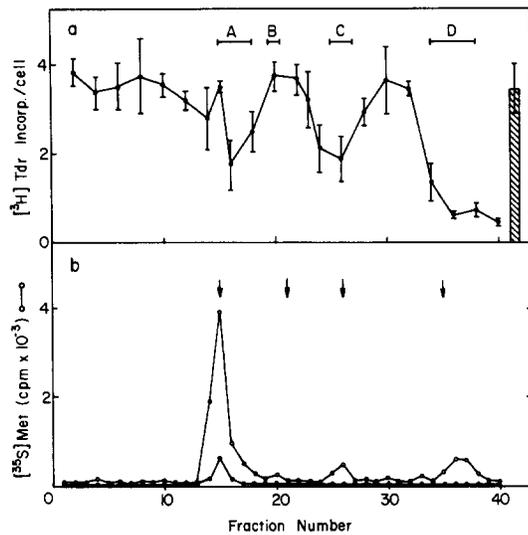


FIGURE 1 Chromatography of the growth inhibitory activity derived from the ammonium sulfate precipitate of CM(SF) on a column (90 × 1 cm) of Sephadex G-50 equilibrated with DME. The CM(SF) was collected from six flasks (150 cm² growth surface) of source cells that had been cultured in the presence of [³⁵S]methionine for 24 h. 1.6-ml fractions were collected. (a) Profile of the growth inhibitory activity assayed by the inhibition of [³H]thymidine ([³H]Tdr) incorporation in target cells. The data on the ordinate axis are expressed as [³H]Tdr incorporation per cell in the target cultures. The horizontal bars containing the letters A, B, C, and D denote fractions which were pooled for analysis by PAGE. The fractions in pool D lysed the target cells, resulting in no incorporation of [³H]-Tdr. The data represent the averages of triplicate determinations ± the standard error of the mean (SEM). The vertical hatched bar at the right represents the average level of [³H]Tdr incorporation (± SEM) in target cells treated with fractions from the column before the void volume (fractions 2-14). This level of incorporation corresponded to that obtained in target cells treated with UCM(SF). (b) Profile of the protein content assayed by the Lowry method (●) or assayed by counting the TCA-precipitable radioactivity due to [³⁵S]methionine ([³⁵S]Met; ○). The vertical arrows indicate the positions of elution of molecular weight markers: bovine serum albumin (68,000), myoglobin (17,000), ribonuclease T (11,000), and bacitracin (1,400).

collected from source cells (six flasks, 10 ml/150 cm² growth area) that had been cultured in the presence of [³⁵S]methionine. Thus the protein components that were secreted or shed from the source cells and accumulated in the CM(SF) were labeled with radioactivity. After each step of the fractionation protocol, aliquots of material were taken for the following assays: (a) determination of the growth inhibitory activity (column B, Table I); (b) determination of the protein content by quantitating the total TCA-precipitable radioactivity due to ³⁵S (column D, Table I); and (c) gel electrophoretic analysis in the presence of SDS. These data were used in the calculations of total activity, specific activity, and percent recovery of the fractionated samples (columns C, E, and F, Table I).

On the basis of these assays, we found that the largest increase in specific activity (~35-fold) was obtained by fractionating the ammonium sulfate precipitate on a column of Sephadex G-50 (Fig. 1). The inhibitory activity was partitioned into two major components (components A and C, Fig. 1a), component A (Fig. 1a), which was eluted at the void volume of the column, contained the major portion of the protein of the sample. This conclusion was obtained both by determining the protein content using the Lowry method and by quantitat-

ing the amount of TCA-precipitable radioactivity due to [³⁵S]methionine (Fig. 1b).

In contrast, a second component of growth inhibitory activity (component C, Fig. 1a) was associated with a minute amount of protein material. When TCA-precipitable ³⁵S radioactivity was assayed, a small but reproducible peak was observed in the fractions containing the growth inhibitory activity (Fig. 1b). However, we could not detect any protein material in these fractions using the Lowry assay. Therefore, this component C (Fig. 1a) represents a fraction enriched in terms of specific activity (Table I). Moreover, these data also suggest that the inhibitory activity associated with Component C was a product synthesized by the 3T3 source cells and not some serum-derived protein. The position of elution of component C on Sephadex G-50 suggested that the material contained polypeptide chains with mol wt of ~12,000.

We have also assayed for the growth inhibitory activity at the level of individual cells by autoradiography after incorporation of [³H]thymidine. The percent inhibition in this assay was determined by comparing the fraction of labeled nuclei in CM-treated cultures with the corresponding value in UCM-treated cultures. In UCM(SF)-treated cultures, ~50% of the nuclei were labeled, consistent with the value calculated on the basis of the cell cycle time (22 h [see reference 18]), length of S-phase (8 h), and the length of the [³H]thymidine pulse period

TABLE I
Activity, Specific Activity, and Recovery of Fibroblast Growth Regulator at Various Stages of the Fractionation Protocol

Purification step	(A)	(B)	(C)	(D)	(E)	(F)
		Inhi- bi- tion*	Total activ- ity*	Total pro- tein‡	cific activ- ity	Re- cov- ery
		%		cpm × 10 ⁻⁵	× 10 ⁴	%
CM(SF)§		53.3	3198	301	1.1	—
(NH ₄) ₂ SO ₄ precipitate		20.0	192	15.9	1.2	6.0
Sephadex G-50 component A (Fig. 1)	33.1	238	9.6	2.5	7.4	
Sephadex G-50 component C (Fig. 1)	37.2	357	0.94	38.0	11.1	

* The growth inhibitory activity of CM-derived fractions is expressed as percent inhibition of DNA synthesis due to 1 ml of the fractionated material. Percent inhibition is defined as the amount of [³H]thymidine incorporation on a per cell basis in cultures treated with CM-derived fractions as compared to UCM-treated cultures. The number of cells in the target cultures treated with CM-derived fractions and with UCM is determined by the amount of [³⁵S]methionine incorporated into the target cells before the treatments with the fractions to be tested for activity. Total activity is calculated by multiplying the percent inhibition of the fraction by the total volume of the fraction.

‡ The protein content of a fraction is quantitated in terms of the total trichloroacetic acid-precipitable radioactivity due to ³⁵S in the CM-derived fractions. In these experiments, CM was collected from source cells that had been cultured in the presence of [³⁵S]methionine.

§ When unfractionated, ³⁵S-labeled CM(SF) was assayed for inhibitory activity, the amount of ³⁵S radioactivity due to CM(SF) was too high to allow an accurate determination of the number of target cells prelabeled with [³⁵S]methionine. In this case, the inhibitory activity was determined simply by comparing the levels of [³H]thymidine incorporation in CM(SF)-treated and UCM(SF)-treated target cells, without normalization to the total number of target cells. Target cultures prelabeled with [³⁵S]methionine and then treated with CM(SF) and UCM(SF) for 24 h retained the same amount of ³⁵S-label (see Fig. 9).

|| The growth inhibitory activity of the ammonium sulfate precipitate fraction could not be measured directly because the high salt concentration lysed the target cells (see reference 11 for details). Therefore, this fraction was first desalted on a Sephadex G-15 column before the activity was determined.

(3 h). In contrast, the fractions of labeled nuclei in cultures treated with CM-derived fractions were significantly lower. The percent inhibition observed for CM(SF) and for components A and C (Fig. 1) were 33%, 19%, and 26%, respectively. Therefore, the inhibition of [³H]thymidine incorporation into target cells treated with CM-derived fractions reflects a true reduction of the percent of cells undergoing DNA synthesis rather than alterations of the transport or pool sizes of the label.

Gel Electrophoretic Analysis and Identification of the Polypeptides Associated with the Growth Inhibitory Activity

The materials containing growth inhibitory activity at various stages of fractionation were subjected to polyacrylamide gel electrophoresis in SDS. After electrophoresis, the gel was stained with Coomassie Blue; in addition, the gel was subjected to fluorography to reveal ³⁵S-labeled protein components. The gel revealed a large number of proteins, as detected by Coomassie Blue staining and by fluorography, for CM(SF), ammonium sulfate precipitate and component A (Fig. 1a) from the Sephadex G-50 column (lanes 1–3, Fig. 2a and b). In contrast, component C (Fig. 1a) yielded no Coomassie Blue-positive material (lane 5, Fig. 2a) and only two major bands on the fluorogram (lane 5, Fig. 2b). Identical results were obtained in the presence and absence of β-mercaptoethanol during the electrophoresis. The mol wt estimated for the two bands (lane 5, Fig. 2b) in component C (Fig. 1a) were 10,000 and 13,000. These results suggest that, under both gel filtration and SDS gel electrophoresis conditions, the two polypeptides migrate without any evidence of covalent linkage to each other.

The experiment described above used CM(SF) material derived from six flasks of 3T3 source cells. When the experiment was scaled up 10-fold, the pooled material corresponding to component C (Fig. 1) yielded two Coomassie Blue-stained bands on polyacrylamide gel electrophoresis (Fig. 3). The electrophoretic mobilities of these polypeptides were identical to those of the labeled bands (M_r : 10,000 and M_r : 13,000).

Densitometric analysis of the fluorogram of component C (lane 5, Fig. 2b) indicated that the two bands accounted for 98% of the radioactive material in the gel. In addition, a parallel experiment was carried out in which the gel sample was not fixed and stained but was sliced into fractions immediately after electrophoresis. These fractions were then solubilized and subjected to scintillation counting (Fig. 4). More than 97% of the radioactivity was recovered in the gel slices corresponding to the two bands observed in the fluorogram of component C (lane 5, Fig. 2b). This result argues against the possibility that low mol wt components, representing major contaminants, could have diffused from the gel during the fixation and staining procedures and, therefore, would have escaped detection in the fluorogram.

Finally, the material in component C was labeled with ¹²⁵I and subjected to gel electrophoretic analysis. It was found that 98% of the radioactivity was associated with two bands (Fig. 5), corresponding to those observed in the gels of the ³⁵S-labeled sample (lane 5, Fig. 2b). This result suggests that there was no other protein species in component C that was previously undetectable by Coomassie Blue staining or [³⁵S]methionine labeling. All of these results, coupled with the data shown in Table I, strongly suggest that component C represents a highly purified preparation enriched in terms of specific

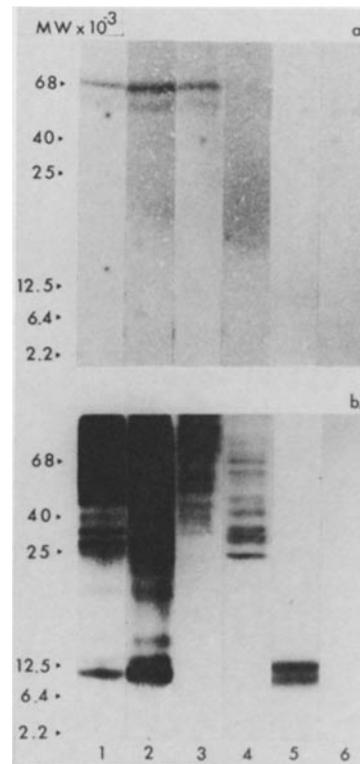


FIGURE 2 SDS PAGE of CM(SF) and various components derived from CM(SF) after fractionation. The acrylamide concentration of the running gel was 15%. The CM(SF) was collected from source cells that had been cultured in the presence of [³⁵S]Met for 24 h. (a) Photographs of Coomassie Blue-stained gels. (b) Fluorographs of ³⁵S-labeled components in the same gels. In both a and b, the panels are: (1) CM(SF), (2) precipitate fraction of CM(SF) after ammonium sulfate fractionation, and (3) component A, (4) component B, (5) component C, and (6) component D, respectively, of Fig. 1. In panels 4–6, the maximal amount of ³⁵S radioactivity available (~6,000 cpm/gel) was used. In panels 1–3, ~70,000 cpm/gel of ³⁵S radioactivity was used; this amount was chosen such that any minor components, particularly those in the molecular weight range of 10,000–15,000, could be revealed. The arrowheads at the left indicate the positions of migration of molecular weight markers: bovine serum albumin (68,000), aldolase (40,000), chymotrypsinogen A (25,000), cytochrome c (12,500), bovine pancreas trypsin inhibitor (6,400), and insulin A chain (2,200).

activity. At present, we do not have any information on whether the two bands in component C (Fig. 1a) constitute a single activity for growth inhibition or whether they are in any way related. Therefore, it is possible that only one of the polypeptides is responsible for the bioactivity. With this qualification in mind, we shall hereafter refer to this material (component C, Fig. 1a) as Fibroblast Growth Regulator—soluble form (FGR-s).

Chemical Characterization of FGR-s

Although we have identified the polypeptide chains associated with FRG-s, the question of whether other chemical components may be associated with the inhibitory activity remained to be addressed. To determine the composition of the purified inhibitory fraction, parallel cultures of source cells were grown in the presence of ¹⁴C-labeled amino acids, thymidine, uridine, and D-[2-³H]mannose. When CM(SF) was prepared from source cells pre-labeled with ¹⁴C-amino acids

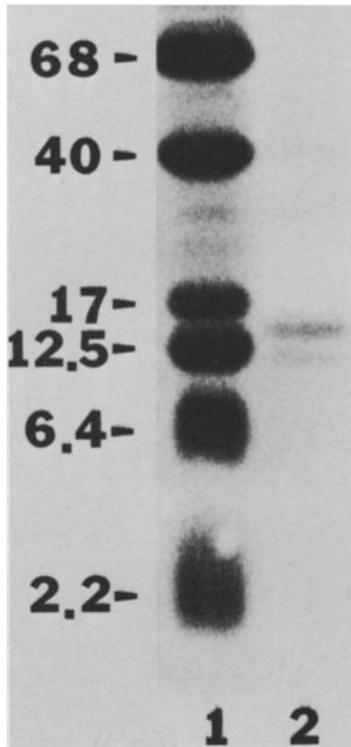


FIGURE 3 SDS PAGE of component C (Fig. 1) derived from a large scale preparation of CM(SF) (60 flasks, 10 ml each). The acrylamide concentration of the running gel was 15%. Both panels 1 and 2 are photographs of Coomassie Blue-stained gels. (1) Molecular weight markers: bovine serum albumin (68,000), aldolase (40,000), myoglobin (17,000), cytochrome c (12,500), bovine pancreas trypsin inhibitor (6,400), and insulin A chain (2,200). (2) Pooled material corresponding to component C (Fig. 1).

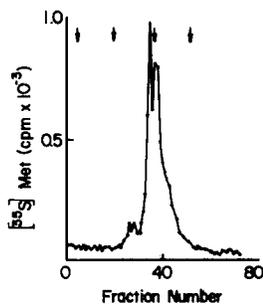


FIGURE 4 SDS PAGE of ^{35}S -labeled component C (Fig. 1). The acrylamide concentration of the running gel was 15%. The ^{35}S radioactivity profile was obtained by slicing the gel immediately after electrophoresis and then subjecting the solubilized individual slices to scintillation counting. The arrows indicate the positions of migration of molecular weight markers: bovine serum albumin (68,000), chymotrypsinogen A (25,000), cytochrome c (12,500), and bovine pancreas trypsin inhibitor (6,400).

and then fractionated, we found ^{14}C radioactivity in the fractions corresponding to components A and C (Fig. 1a) in the elution profile of the Sephadex G-50 column (Fig. 6a). These results corroborate the data obtained using the [^{35}S]methionine label.

In contrast to these findings, collection of CM(SF) from source cells prelabeled with [^{14}C]thymidine or [^{14}C]uridine did not yield any radioactive components corresponding to the FGR-s fractions (Fig. 6c and d). These results suggest that

there were no DNA or RNA molecules associated with the inhibitory activity. This conclusion was further corroborated by experiments in which the sensitivity of the growth inhibitory activity to various enzymes was tested.

To carry out these experiments, pronase was first immobilized on beads. The enzyme-bead complexes were then incubated with the fractions to be tested for enzyme sensitivity. After the incubation, the reaction mixtures were centrifuged and the supernatant was removed from the pelleted beads for tests of growth inhibitory activity on target cells and for gel electrophoresis. Incubation of FGR-s fraction with pronase-beads followed by gel electrophoretic analysis showed a decrease in the intensity of the radioactive bands (M_r : 10,000 and M_r : 13,000). This was paralleled by loss of growth inhibitory activity. After 18 h of incubation, no activity was detectable in our assay system (Fig. 7). In contrast, the activity was not affected by treatment with deoxyribonuclease-beads and ribonuclease-beads. Thus, the results of these enzyme sensitivity experiments provide data confirmatory to those obtained in experiments using radioactively-labeled precursors.

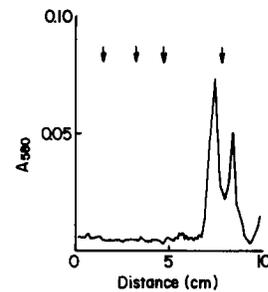


FIGURE 5 SDS PAGE of ^{125}I -labeled component C (Fig. 1). The acrylamide concentration of the running gel was 10%. The ^{125}I radioactivity profile was obtained as a densitometric tracing of the autoradiogram at 580 nm. The arrows indicate the positions of migration of molecular weight markers: bovine serum albumin (68,000), aldolase (40,000), concanavalin A (26,000), and ribonuclease A (13,000).

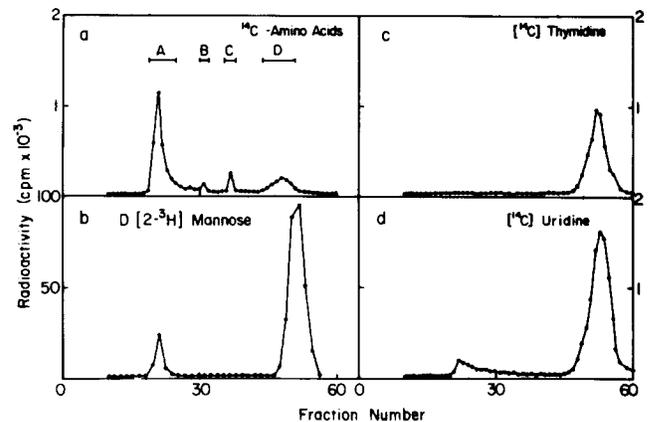


FIGURE 6 Chromatography of the growth inhibitory activity derived from several different preparations of CM(SF) on columns (60 \times 1 cm) of Sephadex G-50 equilibrated with DME. The CM(SF)s were collected from source cells that had been cultured for 24 h in the presence of: (a) ^{14}C -amino acids (5 $\mu\text{Ci/ml}$), (b) [$2\text{-}^3\text{H}$]mannose (1 mCi/ml), (c) [^{14}C]Tdr (3.3 $\mu\text{Ci/ml}$), and (d) [^{14}C]uridine (3.3 $\mu\text{Ci/ml}$). 1-ml fractions were collected and 100- μl aliquots of each fraction were assayed for radioactivity. The horizontal bars containing the letters A, B, C, and D denote the fractions which correspond to those pooled in Fig. 1.

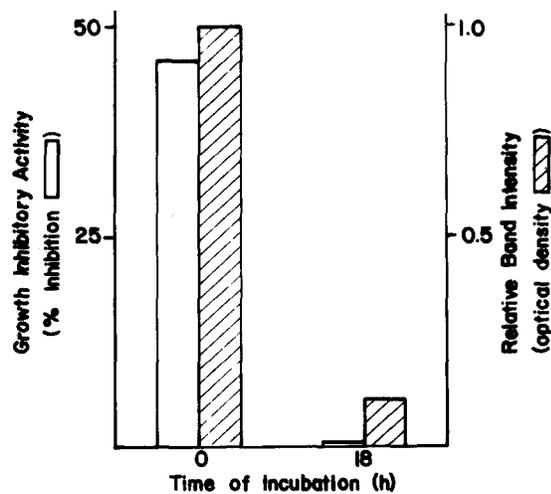


FIGURE 7 The effect of pronase treatment on the growth inhibitory activity of FGR-s (open bars) and on the total intensity of the FGR-s bands ($M_r = 10,000$ and $M_r = 13,000$) on polyacrylamide gels (hatched bars). ^{35}S -labeled FGR-s (0.45 ml) was treated with pronase coupled to carboxymethylcellulose beads (0.06 U). 1 U of enzyme activity hydrolyzes 1 μmol of *N*-benzoyl-L-arginine ethyl ester per minute at pH 7 and 30°C. Samples were tested for growth inhibitory activity and were subjected to PAGE and fluorography as described in Materials and Methods. The intensities of the protein bands ($M_r = 10,000$ and $M_r = 13,000$) were obtained by densitometric tracing of the fluoro-gram at 580 nm.

When CM(SF) was collected from source cells cultured for 24 h in the presence of D-[2- ^3H]mannose, fractionation on the Sephadex G-50 column yielded radioactivity only in fractions corresponding to component A (Fig. 6b). No radioactivity was detected in fractions corresponding to component C, even when the incubation medium contained as much [2- ^3H]mannose as 1 mCi/ml. We have not tested as yet other precursors for labeling glycoproteins such as L-[^{14}C]fucose or D-[^{14}C]glucosamine.

The Effects of FGR-s on Target Cells: Dose-response, Viability and Reversibility

The inhibitory effect of FGR-s on [^3H]thymidine incorporation in target cells was dependent on the concentration of ligand added (Fig. 8). Above 100 ng/ml, there was a monotonic increase in inhibitory activity with increasing concentration of FGR-s added to the cultures. The concentration of inhibitor required for 50% inhibition was ~ 400 ng/ml. At the highest concentration tested (720 ng/ml), the percent inhibition achieved was $\sim 60\%$. We have recently demonstrated the binding of [^{35}S]methionine-labeled FGR-s to target 3T3 cells; the growth inhibitory effect of increasing concentrations of FGR-s paralleled the increase in the amount of binding observed.¹

Three series of experiments were performed to ascertain that the inhibition of [^3H]thymidine incorporation in target cells by FGR-s was due to a true suppression of cell growth rather than to any cytotoxic effects of the inhibitory fractions. First, the viabilities of the cells, assayed by the trypan blue exclusion tests, were identical for target cultures treated with FGR-s and with UCM(SF) for up to 48 h. Because nonviable cells could have been lost from the dishes, we pre-labeled the target cells by culturing them in the presence of ^{14}C -amino acids. After

¹ Voss, P. G., P. A. Steck, J. C. Calamia, and J. L. Wang. Growth control in cultured 3T3 fibroblasts. III. Binding interactions of a growth inhibitory activity with target cells. *Exp. Cell Res.* In press.

treatment with FGR-s for 24 and 48 h, the levels of ^{14}C label retained were found to be similar to those observed for control cultures (Fig. 9). The same results were also obtained when target cells were treated with CM(SF) and UCM(SF).

In another series of experiments, 3T3 cells were seeded onto culture dishes in a very sparse configuration such that individual cells would give rise to colonies. After overnight incubation, the medium was removed and parallel cultures were treated with UCM(SF), CM(SF), and FGR-s for 96 h. When the number of cells in each colony and the number of colonies were counted, the control culture (UCM(SF)) showed a population distribution ranging from 10 cells per colony to 150 cells per colony (Fig. 10a). The average numbers of cells per colony was 72. In contrast, when the sparsely distributed cells were treated with CM(SF) or isolated FGR-s fraction, the number of colonies with fewer than 50 cells per colony increased significantly and the distributions were centered around mean values of 44 and 48, respectively (Fig. 10b and c).

Student's *t* tests were used to compare the means of the distributions shown in the histograms of Fig. 10. The mean values for the distributions of UCM(SF) and CM(SF) were significantly different ($t = 6.17$, $df = 227$, $P < 0.0001$). Similarly, the mean values for UCM(SF) and FGR-s were significantly different ($t = 5.02$, $df = 246$, $P < 0.0001$). In contrast, the mean values for CM(SF) and FGR-s showed significant

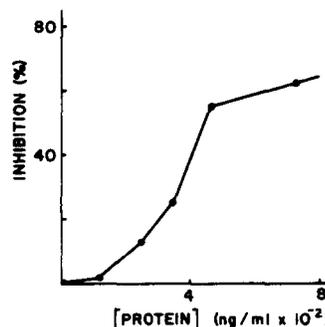


FIGURE 8 Dose-response curve of the growth inhibitory activity of FGR-s on 3T3 target cells (5×10^3 cells/cm²). The protein concentration of FGR-s was determined by the method of Schaffner and Weissman (13). The target cells were treated with FGR-s for 20 h and were then assayed for [^3H]Tdr incorporation as described in Materials and Methods. The data represent the averages of duplicate determinations.

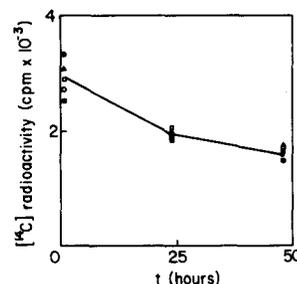


FIGURE 9 The effect of various CM(SF)-derived components on the viability of cells in target cultures as assayed by the retention of ^{14}C -amino acids previously incorporated into cellular proteins. Target cells were cultured in ^{14}C -amino acids (2 μCi /culture, 24 h), washed, and then treated with CM(SF) (○), UCM(SF) (●), FGR-s (△), and fractions 10 (□) and 16 (■) from the Sephadex G-50 column shown in Fig. 1. At the times indicated, the amount of labeled target cells was determined by counting the ^{14}C radioactivity in each culture. The data represent the averages of duplicate determinations.

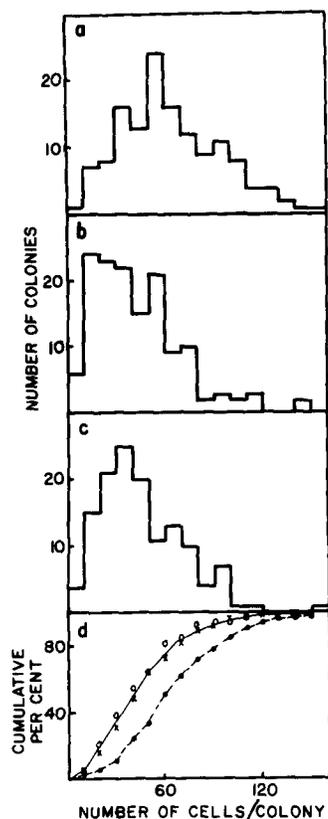


FIGURE 10 Cell count distributions for colonies of 3T3 cultures treated with (a) UCM(SF), (b) CM(SF), and (c) FGR-s. The following numerical data were obtained: (a) UCM(SF), 137 total colonies counted, average number of cells per colony, 72; (b) CM(SF), 138 total colonies counted, average number of cell per colony, 44; and (c) FGR-s, 133 total colonies counted, average number of cells per colony, 48. In *d*, the data of panels a-c are plotted in the form of a cumulative distribution curve of colony size. (●) UCM(SF) cultures, (○) CM(SF) cultures, (×) FGR-s cultures.

similarity ($t = 1.29$, $df = 268$, $P > 0.2$). A similar conclusion can be derived from a plot of the cumulative distribution of colony size (Fig. 10*d*). For example, 70% of the colonies in cultures treated with CM(SF) or FGR-s had 50 cells or less whereas the corresponding value for UCM(SF)-treated cultures was 30%.

Most importantly, we have found that there was no difference in the total number of colonies in dishes treated with UCM(SF), CM(SF), or FGR-s. All of these results suggest that CM(SF) and FGR-s inhibited the growth of cells, thereby decreasing the size of the colonies without affecting the overall number of the originally attached cells. Moreover, the data provide a confirmation of the growth inhibitory activity of FGR-s using an assay that is independent of [³H]thymidine incorporation.

To demonstrate that the inhibitory effect of FGR-s on DNA synthesis was reversible, parallel target cultures were treated with FGR-s for 24 h. The medium was then removed and replaced with an equal volume of fresh growth medium. At various times thereafter, DNA synthesis was assayed by the incorporation of [³H]thymidine. The data showed that the inhibition was reversible within 20 h after removal of FGR-s (Table II). Taken together with the demonstration that the viability of target cells was not affected by FGR-s, these results suggest that the inhibitory activity of FGR-s cannot be ascribed to cytotoxicity.

TABLE II
Reversibility of the Effects of CM(SF) and FGR-s

Conditions	24 h*	12 h after	24 h after
		reversal‡	reversal‡
		% of Control	
CM(SF)	56	105	125
FRG-s	54	130	127

* Target cells (5×10^3 cells/cm²) were treated for 24 h with CM-derived and UCM fractions. DNA synthesis was then measured by the incorporation of [³H]thymidine. The results are expressed as percent of control ([³H]thymidine incorporated in cultures treated with CM-derived fractions divided by [³H]thymidine incorporation in UCM cultures).

‡ After the cultures were treated for 24 h with CM-derived or UCM fractions, the medium was removed and replaced by an equal volume of fresh growth medium. DNA synthesis in these cultures was measured 12 h and 24 h later.

DISCUSSION

The results obtained in the present study indicate that we have partially purified, from the 3T3 fibroblast system, a growth inhibitory preparation enriched in specific activity. This preparation has the following key properties: (a) it is an endogenous cell product, synthesized by the 3T3 cells and shed into the medium; (b) it is a protein and its activity is sensitive to treatment with pronase; (c) the constituent polypeptide chains have mol wt of 10,000 and 13,000; and (d) it is not cytotoxic to the target cell and its effects on cell growth are reversible. We have also obtained evidence that at least one of these two polypeptides (M_r : 10,000) is stably bound by the 3T3 target cells.¹ Although it is still possible that the growth inhibitory activity is due to molecules present in minute amounts well below our detection levels, the best candidates for this activity appear, at the present, to be these two protein components. We propose to designate this factor FGR-s, which stands for Fibroblast Growth Regulator that is secreted or shed into the medium in a soluble form.

Growth inhibitory factors have also been studied in a number of other cultured cells that exhibit density-dependent inhibition of growth. Lipkin and Knecht have reported that a glycoprotein of mol wt 160,000 can be isolated from culture medium of contact-inhibited hamster melanocytes and that this factor can reversibly inhibit growth in vitro of a broad spectrum of malignant and normal cell types of ectodermal, mesodermal and endodermal origins (19-21). Moreover, an electrophoretically identical protein has been found in culture media of contact-inhibited fibroblasts and epidermal cells.

Holley and co-workers have reported (22) that the growth of the monkey epithelial cell line, BSC-1, is limited at high cell densities partly by the action of three inhibitors. Two of these inhibitors have been isolated from the culture medium of BSC-1 cells (23). At ng/ml concentrations, they reversibly arrest the growth of the same cells in the G₁ phase of the cell cycle. On the basis of the chromatographic behavior on Bio-Gel P-60 columns, the molecular weight of one of these inhibitors was estimated to be ~15,000.

Our most highly purified preparation of FGR-s yielded two major protein components on polyacrylamide gel electrophoresis in SDS (M_r : 10,000 and M_r : 13,000, panel 5, Fig. 2*b*). The growth inhibitory activity of this material may be due to (a) one of the polypeptides acting alone; (b) both of the polypeptides acting independently; or (c) a complex of the two polypeptides associated together, either one of which is not sufficient for activity. The resolution of these possibilities must await the successful separation of these two components under non-denaturing conditions so that the activity of each polypeptide and the reconstituted system can be compared.

Moreover, we have yet to determine the relationship of the two polypeptides to each other. For example, one (M_r : 10,000) may be a fragment derived from the other (M_r : 13,000). In this connection, it is also possible that one or both of the polypeptides in component C (Fig. 1) represent proteolytic products of larger precursors observed in component A (Fig. 1). This hypothesis is supported by preliminary experiments which indicate that prolonged incubation of CM(SF) at 37°C followed by chromatography on Sephadex G-50 generated more material eluting in the molecular weight range of component C (Fig. 1).

The possibility of a higher molecular weight precursor form of the FGR-s polypeptides may be particularly important in considering the biological significance of the isolated inhibitor fraction. It should be emphasized that the highest level of inhibition obtained with the FGR-s fraction is ~60%. Therefore, the proliferation of the target cells is not completely suppressed by the presence of the inhibitor. Similarly, the results of the colony formation assays indicate that, despite significant reductions in the size of the colonies, the presence of the FGR-s fraction still allowed some cell growth and division to occur. Finally, we have estimated that over 15 h, a time-period required for accumulation of appreciable activity in CM (11), each source cell yielded 1 pg of the FGR-s fraction and that, under such production rates, at least 30–40 source cells were required to inhibit one target cell. These estimates are obviously incomplete inasmuch as we have yet to take into account other variables such as: (a) the density-dependence of target cells in response to the inhibitory activity; (b) the degradation rate of the isolated activity; and (c) the presence of inhibitory activity in peak A (Fig. 1) of the preparation. All these considerations call for caution in interpreting the absolute significance of the isolated FGR-s as a growth regulatory factor and suggest that perhaps higher molecular weight precursors or membrane-bound forms of the molecule may yield higher levels of growth inhibition.

These considerations also emphasize the limitations of the inhibitor concept as a complete explanation for density-dependent inhibition of growth in cultured cells. As we had discussed previously (11), many laboratories have contributed evidence in support of three major hypotheses accounting for the phenomenon of density-dependent inhibition of growth: (a) cell-to-cell contact effects; (b) depletion of growth stimulatory factors; and (c) release of soluble inhibitors. These three mechanisms are not mutually exclusive and may in fact be synergistic with each other. Thus, the isolated FGR-s fraction of our present study may act in direct antagonism with growth stimulatory factors or in concert with cell contact effects.

In this connection, Whittenberger and Glaser have shown that the growth of 3T3 cells can be reversibly inhibited by a surface membrane fraction from the same cells (24) and that the inhibitory components can be solubilized by the nonionic detergent octylglucoside (25). Natraj and Datta have also shown that an inhibitor of DNA synthesis can be extracted from 3T3 cells by treatment with 0.2 M urea in PBS (26, 27). It was suggested that these surface membrane molecules which inhibit cell proliferation may be the same molecules that are responsible for contact-dependent growth regulation.

In both of these studies on the membrane-bound growth regulatory factor(s), no purified preparation of the inhibitory molecule has, to date, been reported. It would be of obvious interest to establish the relationship between the FGR-s from

CM and the similar activity observed in the membrane fractions. It is possible, for example, that the same molecule can exert its effects either anchored on the cell surface or released into the medium. The isolation of FGR-s and the characterization of its polypeptide chains represent the first step in the parallel lines of experiments on membrane-bound and soluble forms of growth regulatory factors that mediate density-dependent inhibition of growth.

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REFERENCES

1. Stoker, M. G. P., and H. Rubin. 1967. Density-dependent inhibition of cell growth in culture. *Nature (Lond.)*, 215:171–172.
2. Todaro, G. J., and H. Green. 1963. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J. Cell Biol.* 17:299–313.
3. Todaro, G. J., H. Green, and B. D. Goldberg. 1964. Transformation of properties of an established cell line by SV 40 and polyoma virus. *Proc. Natl. Acad. Sci. U. S. A.* 51:66–73.
4. Todaro, G. J., G. K. Lazar, and H. Green. 1965. The initiation of cell division in a contact-inhibited mammalian cell line. *J. Cell. Comp. Physiol.* 66:325–334.
5. Holley, R. W., and J. A. Kiernan. 1968. "Contact inhibition" of cell division of 3T3 cells. *Proc. Natl. Acad. Sci. U. S. A.* 60:300–304.
6. Stoker, M. G. P. 1973. Role of diffusion boundary layer in contact inhibition of growth. *Nature (Lond.)*, 246:200–203.
7. Rheinwald, J. G., and H. Green. 1975. Formation of a keratinizing epithelium in culture by a cloned cell line derived from a teratoma. *Cell*, 6:317–330.
8. Canagaratna, M. C. P., and P. A. Riley. 1975. The pattern of density dependent growth inhibition in murine fibroblasts. *J. Cell. Physiol.* 85:271–282.
9. Harel, L., and M. Jullien. 1976. Evaluation of proximity inhibition of DNA synthesis in 3T3 cells. *J. Cell. Physiol.* 88:253–254.
10. Harel, L., M. Jullien, and M. DeMonti. 1978. Diffusible factor(s) controlling density inhibition of 3T3 cell growth: a new approach. *J. Cell. Physiol.* 96:327–332.
11. Steck, P. A., P. G. Voss, and J. L. Wang. 1979. Growth control in cultured 3T3 fibroblasts. Assays of cell proliferation and demonstration of a growth inhibitory activity. *J. Cell. Biol.* 83:562–575.
12. 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.
13. Schaffner, W., and C. Weissman. 1973. A rapid, sensitive, and specific method for the determination of protein in dilute solution. *Anal. Biochem.* 56:502–514.
14. Hunter, W. M., and E. C. Greenwood. 1962. Preparation of ¹²⁵I-iodine-labeled growth hormone of high specific activity. *Nature (Lond.)*, 194:495–496.
15. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*, 227:680–685.
16. Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46:83–88.
17. Lazarides, E., and U. Lindberg. 1974. Actin is the naturally occurring inhibitor of deoxyribonuclease. I. *Proc. Natl. Acad. Sci. U. S. A.* 71:4742–4746.
18. Paul, D., K. D. Brown, H. T. Rupriak, and H. J. Ristow. 1978. Cell cycle regulation by growth factors and nutrients in normal and transformed cells. *In Vitro (Rockville)*, 14:76–84.
19. Lipkin, G., and M. E. Knecht. 1974. A diffusible factor restoring contact inhibition of growth to malignant melanocytes. *Proc. Natl. Acad. Sci. U. S. A.* 71:849–853.
20. Knecht, M. E., and G. Lipkin. 1977. Biochemical studies of a protein which restores contact inhibition of growth to malignant melanocytes. *Exp. Cell Res.* 108:15–22.
21. Lipkin, G., M. E. Knecht, and M. Rosenberg. 1978. A potent inhibitor of normal and transformed cell growth derived from contact-inhibited cells. *Cancer Res.* 38:635–643.
22. Holley, R. W., R. Armour, and J. H. Baldwin. 1978. Density-dependent regulation of growth of BSC-1 cells in cell culture: growth inhibitors formed by the cells. *Proc. Natl. Acad. Sci. U. S. A.* 75:1864–1866.
23. Holley, R. W., P. Bohlen, R. Fava, J. H. Baldwin, G. Kleeman, and R. Armour. 1980. Purification of kidney epithelial cell growth inhibitors. *Proc. Natl. Acad. Sci. U. S. A.* 77: 5989–5992.
24. Whittenberger, B., and L. Glaser. 1977. Inhibition of DNA synthesis in cultures of 3T3 cells by isolated surface membranes. *Proc. Natl. Acad. Sci. U. S. A.* 74:2251–2255.
25. Whittenberger, B., D. Raben, M. A. Lieberman, and L. Glaser. 1978. Inhibition of growth of 3T3 cells by extract of surface membranes. *Proc. Natl. Acad. Sci. U. S. A.* 75:5457–5461.
26. Natraj, C. V., and P. Datta. 1978. Control of DNA synthesis in growing Balb/c 3T3 mouse cells by a fibroblast growth regulatory factor. *Proc. Natl. Acad. Sci. U. S. A.* 75:6115–6119.
27. Datta, P., and C. V. Natraj. 1980. Fibroblast growth regulatory factor inhibits DNA synthesis in Balb/c 3T3 cells by arresting in G₁. *Exp. Cell Res.* 125:431–439.