CULTIVATION OF MAMMALIAN CELLS IN DEFINED MEDIA WITH PROTEIN AND NONPROTEIN SUPPLEMENTS

GEORGE M. HEALY and RAYMOND C. PARKER

From the Connaught Medical Research Laboratories, University of Toronto, Toronto, Canada

ABSTRACT

An improved chemically defined basal medium (CMRL-1415) has been used to advantage in studying the effects on trypsinized, newly explanted mouse embryo cells of certain glycoproteins from plasma and serum, certain nonprotein macromolecules, and various combinations of these, in stationary cultures. When protein and nonprotein fractions were separated from fetal calf serum, the entire growth activity was found to be associated with the protein. When 100 mg % of dialyzed, freeze-dried, supernatant solution of Cohn's fraction V (method 6) from human plasma was used as a supplement for CMRL-1415, there was considerable improvement in the cultures; and seromucoid prepared from calf serum had a similar effect. Supernatant V was further fractionated by gel filtration to give a threefold concentration of growth activity in a single, highly purified α_1-acid glycoprotein (orosomucoid). Starch gel electrophoresis of horse serum that was used to supplement the basal medium revealed a decrease of both α_1-acid glycoprotein and α_2-macroglobulin during the cultivation of mouse embryo cells. When horse serum was fractionated on DEAE-cellulose columns, the only fraction that showed growth activity was a slow α_2-globulin. When the α_2-macroglobulin of Schultze was prepared from horse serum by salt precipitation, it was equally effective. When the α_2-macroglobulin from horse serum was tested (at 100 mg %) in combination with α_1-acid glycoprotein from Supernatant V, seromucoid from calf serum, or unfractionated Supernatant V, the growth response was greatly in excess of that produced by any of these supplements tested separately. The α_2-macroglobulin from horse serum could be replaced by certain nonprotein macromolecules (e.g., dextran or Ficoll). Thus, dextran (mol. wt. 100,000 to 200,000) had no visible effect on the cells when used alone at 0.1 or 1%. But when these levels of dextran were used in combination with low molecular weight glycoproteins (e.g., unfractionated Supernatant V at 100 mg %), the cultures remained active and healthy for unusually long periods.

Although one of our earlier media, CMRL-1066 (22), will support the continuous multiplication of certain established cell lines, it will not support the continuous multiplication of newly explanted tissue cells unless supplemented with protein, which is usually added as whole serum. Over the past several years, investigators in many laboratories have attributed growth-promoting or other desirable properties to several fractions of blood serum and to various nonprotein macromolecules. In the present report, evidence is presented to indicate that certain high and low molecular weight glycoproteins from blood serum, when added separately to chemically defined media, confer considerable benefit upon newly explanted mouse embryo cells in stationary cultures, as do also certain nonprotein macromolecules. More important, there is evidence that certain combina-

tions of these high and low molecular weight glycoproteins and combinations of certain of these and nonprotein macromolecules, notably dextran, have a marked effect in promoting the long-continued multiplication and attachment of newly explanted mouse embryo cells, with little or no cellular degeneration. An improved chemically defined basal medium, CMRL-1415, that has been especially designed for use with these and other supplements is described in the accompanying paper.

MATERIALS AND METHODS

The procedures followed in developing populations of newly explanted cells from trypsinized, whole mouse embryos, in preparing test cultures from trypsinized cell suspensions in plastic flasks fitted with silicone stoppers, and in preparing the cultures for cell counts or photography, are described in the accompanying paper.

The biochemical procedures used in preparing fractions of plasma and serum are described in subsequent sections of the report.

Except for a few selected experiments in which population-doubling time has been estimated, the results are presented photographically. The most convincing results were often obtained in experiments in which cells in the defined basal media serving as controls would begin to degenerate within a few days whereas replicate cultures containing useful supplements would remain healthy and active for several weeks. This situation almost always arose in experiments in which the cells did not seem as healthy as usual or when the inoculum size was smaller than optimal. Conversely, when the appearance of the cells and the inoculum size were optimal, cultures sometimes had to be carried for many weeks in order to detect appreciable differences between them. Often, under these conditions, the effects of useful modifications in the medium were revealed more clearly by improved cellular morphology and mode of growth than by appreciable differences in population density. In some instances, experiments had to be discarded after several weeks because cells in the control cultures in unsupplemented defined medium CMRL-1415 remained so healthy and vigorous that it was impossible to detect any real advantage from the supplements being tested.

EXPERIMENTS AND RESULTS

Effect of Protein and Nonprotein Fractions of Serum

1. SEPARATED BY SIMPLE DIALYSIS WITHOUT RETAINING THE DIALYSATE: In a typical experiment, 100 ml of pooled fetal calf serum (Hyland Laboratories, Los Angeles) were placed in a cellophane dialysis sac (1⅛-in. diam.; pore size, 48-A units) that was immersed in running tap water for 48 hr and then dialyzed a further 24 hr at 4°C against 10 liters of 0.12 M NaCl, with continuous stirring to dissolve the precipitated globulins. The original volume increased to 128 ml and was concentrated to 100 ml by pervaporation. When the resulting dialyzed serum was added to CMRL-1066 at a level of 15% by volume and tested on newly explanted mouse cells, it showed growth activity equivalent to that obtained with the same level of undialyzed serum.

2. SEPARATED BY CONTINUOUS ASEPTIC DIALYSIS WITH CONSERVATION OF THE DIALYSATE: In another type of experiment, a continuous closed dialysis system was assembled from standard-taper Pyrex glassware. The system consisted of a 1-liter boiling flask (with a bottom outlet) fitted with Claisen connecting tubes and a Liebig condenser. The condenser was connected to a Soxhlet extractor provided with a return tube to the bottom of the boiling flask. Four-hundred ml of ion-exchange water were introduced into the boiling flask and heat was applied without vacuum and without cooling the condenser jacket. After the system had been thoroughly steamed out, heat was reduced, a sterile dialysis sac containing 75 ml of fetal calf serum was introduced into the extractor, vacuum was applied, and cold water was passed through the condenser jacket. The distilled water was cycled continuously through the extractor and over the serum sac for 48 hr during which time the internal temperature did not exceed 56°C. After 48 hr, the dialysate was concentrated by vacuum distillation to 75 ml. The dialyzed serum residue was dialyzed a further 24 hr against 5 liters of 0.12 M NaCl, stirred continuously to dissolve precipitated globulins, and concentrated to 75 ml by pervaporation. The dialyzed serum residue and the dialysate were added to separate samples of CMRL-1066 at levels of 15% by volume and tested on newly explanted mouse cells. The cell growth obtained with the dialysate was comparable to that obtained with CMRL-1066 alone, whereas the dialyzed serum residue showed considerable growth stimulation.

3. SEPARATED BY SALT PRECIPITATION: Total serum protein and protein-free extracts from the same sample of serum were prepared by the method of Deutsch et al. (8). One-hundred ml of

fetal calf serum were mixed with 40 g of anhydrous Na_2SO_4 at 56°C, which precipitated all the protein. The mixture was filtered by suction through a warm Buchner funnel into a filtration flask cooled in cracked ice. The filtrate, which contained all the low molecular weight materials present in the original serum, was freed of Na_2SO_4 by the addition of a calculated amount of $CaCl_2$. The precipitated $CaSO_4$ was filtered off and the filtrate was made isotonic by dilution with water. When this material was added to CMRL-1066 at a level of 15% by volume, it showed no growth-promoting activity on newly explanted mouse cells. But when the protein residue was dissolved in water and was dialyzed free of Na_2SO_4 by running tap water, made 0.14 M with respect to NaCl, and reprecipitated according to the method of Michl (38) with $(NH_4)_2SO_4$, it yielded an active α-globulin fraction that stimulated the growth of newly explanted mouse cells from the same suspension.

4. SEPARATED BY GEL FILTRATION WITH SEPHADEX G-25: A Sephadex column (45 × 2.5 cm; Pharmacia, Uppsala, Sweden) was filled with a slurry of Sephadex G-25 gel that had been allowed to swell for a few hours in ion-exchange water. A 50-ml sample of fetal calf serum was applied to the column and desalted according to the procedure of Flodin (17). The serum proteins were recovered in the first portion of the effluent and all the low molecular weight constituents were recovered from 400 ml of the remaining effluent. Both fractions were concentrated by pervaporation to 50 ml, adjusted to 0.14 M NaCl, added to separate samples of CMRL-1066 at levels of 15% by volume, and tested on newly explanted mouse cells. The growth activity was found to reside in the protein fraction, not in the low molecular weight material.

Effect of Low Molecular Weight Glycoproteins from Pooled Human Plasma (Supernatant Fraction V)

In 1954, we tested Cohn (7) fractions I to V, inclusive, that had been prepared from human serum (method 6) by Dr. A. F. Charles of these Laboratories. When these fractions were tested singly or in combination, as supplements for defined medium 858 (21), they had little or no growth-promoting activity for L-strain cells from mouse; in fact, fractions II and III (which included the gamma globulins) were somewhat toxic. Eagle (10) reported similar findings with the same fractions prepared at the Harvard Medical School. Because these observations led us to question the wisdom of attempting any further purification of Cohn's clinically useful fractions, we turned our attention to the seromucoids (51). These low molecular weight glycoproteins, that are not coagulable by heat and are not precipitated by sulfosalicylic acid, have sedimentation constants of approximately 3S and are known to be present in CMRL's supernatant solution of Cohn's fraction V. These proteins represent about 1 to 2% of the total serum protein and have the following electrophoretic composition (46): 50% albumin, 35% α_1-, 11% α_2-, 4% β_1- and β_2-globulins.

Supernatant V, which is the solution remaining after the removal of fraction V (albumin) by Cohn's method 6 (7) for the fractionation of human plasma, was concentrated about 15-fold in vacuo, dialyzed 24 hr against running tap water, and the dialyzed Supernatant V concentrate was dried in vacuo from the frozen state. The product, a light brown fluffy powder, was the starting material for the separation of glycoproteins and had the following proximate analysis (by weight): albumin 40% and glycoproteins 60% (including hexoses 15.5%, hexosamines 14.5%, sialic acid 9%, and fucose 1%, as prosthetic groups). Twenty ml of a 4% solution of dried Supernatant V in 0.005 M sodium phosphate buffer (pH 7.0) were applied to a chromatographic column (45 × 2.5 cm) filled to a height of 40 cm with a suspension of Sephadex G-50 (medium) or G-75 (fine) in the same buffer. After the sample had entered the gel, a constant head of 1 m of 0.005 M phosphate buffer (saturated with respect to chlorobutanol) was applied to the column from a Mariotte bottle. The albumin appeared in the first portion of the effluent, as revealed by testing successive portions of it with 20% trichloroacetic acid. After passage of the albumin, which was discarded, the effluent was collected on an automatic fraction collector, the optical density of the fluid in each tube was determined in a Hilger spectrophotometer (Hilger & Watts, Ltd., London, England) at a wave length of 280 mμ, and the contents of adjacent tubes showing UV absorption were combined and lyophilized. In a typical experiment with Sephadex G-50, a sharply resolved peak (*a*) and a partially-resolved peak (*b*) were obtained. In this experiment, the following recoveries were obtained from the original 800 mg

of protein in the solution applied to the column: albumin, 320 mg; peak *a*, 350 mg; and, peak *b*, 130 mg, of freeze-dried materials. When these fractions and the original Supernatant V were subjected to starch gel electrophoresis (49), peak *a* was found to contain α_1-glycoprotein and peak *b* a mixture of α_2-glycoproteins and β-globulin. With Sephadex G-75, three separate peaks of the same proteins were obtained in the same elution order. The material from the first peak contained the principal glycoprotein of Supernatant V (orosomucoid or α_1-acid glycoprotein) and a small amount of an α_2-glycoprotein.

When 100 mg% of dialyzed, freeze-dried Supernatant V was used as a supplement for CMRL-1415 (to give CMRL-1415-SCV[1]), there was considerable improvement in the appearance of newly explanted mouse embryo cells (Figs. 1 and 2). When the Sephadex subfractions of Supernatant V had been prepared and tested (Fig. 3), it was found that all the growth-promoting activity of Supernatant V was contained in the material eluted first from G-50 and G-75 columns (i.e., in G-50-*a* and G-75-*a*). When either of these two subfractions of Supernatant V was tested at 35 mg% in combination with the α_2-macroglobulin of Schultze et al. (48) at 100 mg% (Figs. 5 and 6), they showed a far greater growth response than when tested alone. When unfractionated Supernatant V was tested in combination with the α_2-macroglobulin (Fig. 7), the growth response was as striking as that obtained by combining the Sephadex subfractions with the α_2-macroglobulin. By treatment with neuraminidase from *Vibrio cholerae*, bound sialic acid was removed from Supernatant V without loss of growth activity. Further hydrolysis with mild mineral acid or trypsin resulted in progressive loss of growth activity.

[1] Registered trademark.

Effect of α_2-Macroglobulin from Horse Serum

The importance of the α_2-macroglobulin (48, 47) portion of normal serum supplements was indicated by two types of experiments: (*A*) During column chromatographic studies of normal horse serum, a series of fractions were separated on a diethylamino ethylcellulose (DEAE) column by the method of Robinson et al. (44). These authors obtained 6 major peaks (I to VI) from human serum, but with horse serum the first peak was resolved into two (at 280 mμ), the first at about 500 ml of effluent followed by an inflection point and a second higher maximum at about 750 ml. Because of this difference, the major peak I of Robinson et al. was treated by us as two independent peaks and were designated peaks Ia and Ib. Adjacent tubes of effluent corresponding to each peak were combined, dialyzed, and freeze-dried to give 7 fractions. When these fractions were tested as supplements for CMRL-1415 in the proportions in which they occur in whole horse serum, only fraction Ib showed activity in promoting the growth of newly explanted mouse embryo cells. When this fraction was subjected to starch gel electrophoresis, it proved to be mainly a slow α_2-globulin together with a trace of γ-globulin, which was eluted in fraction Ia.

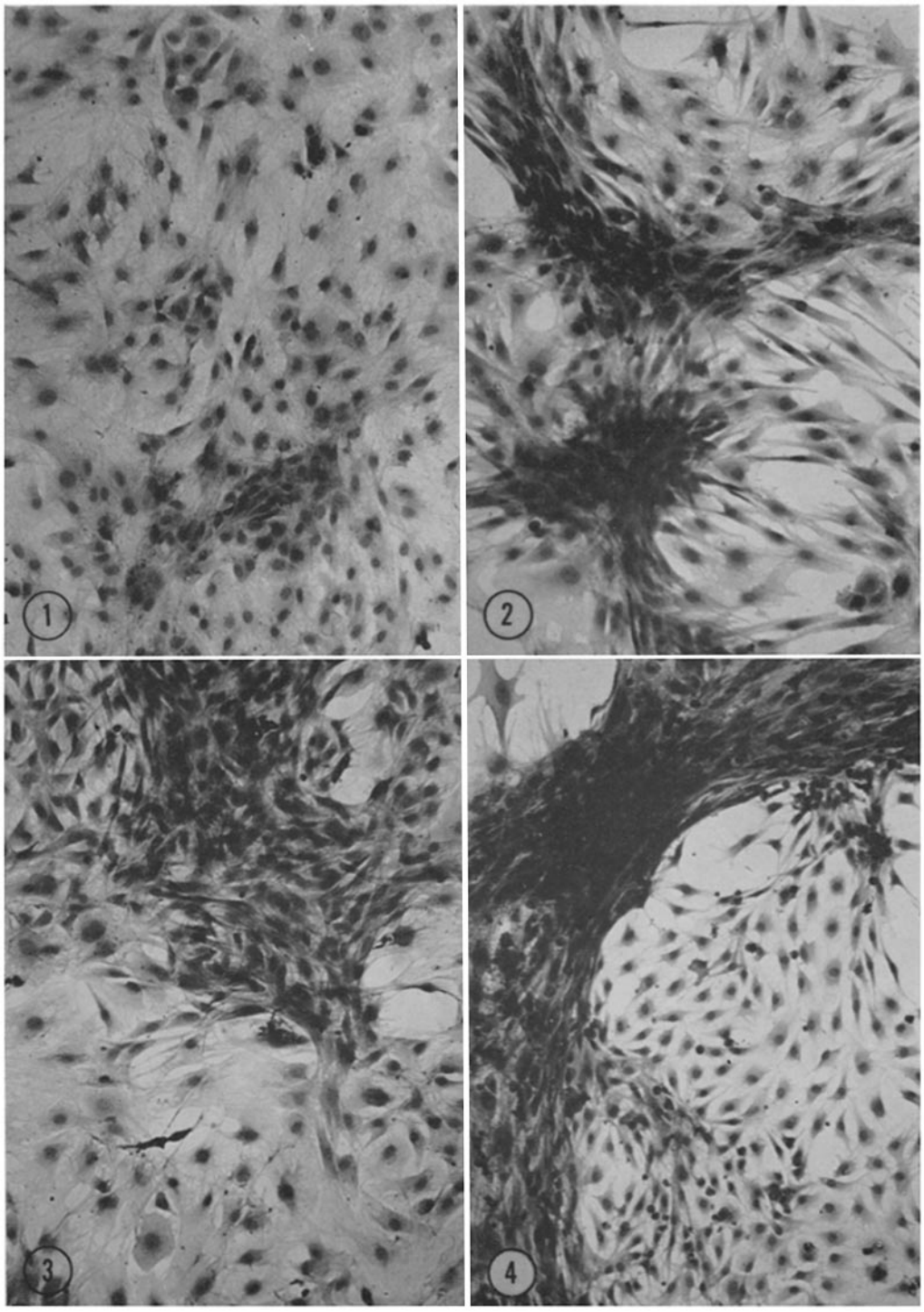
(*B*) A comparison of the protein patterns obtained by starch gel electrophoresis of normal horse serum and of the same serum that had been used

FIGURE 1 Ten-day-old culture (34226-1) of mouse embryo cells in medium CMRL-1415; MsE 194:T3(6). (Embryos from mouse No. 194; culture is one of a series prepared after 3rd trypsinization of cells, 6 days after tissues were explanted from embryos). × 120.

FIGURE 2 Ten-day-old culture (34226-17) in medium 1326-5 (CMRL-1415 supplemented with 100 mg % Cohn's dialyzed, freeze-dried Supernatant V from human plasma); MsE 194:T3(6). × 120.

FIGURE 3 Thirty-five-day-old culture (34172-7) in medium 1328-6 (CMRL-1415 supplemented with 35 mg % Sephadex G-50-*a* fraction of Cohn's Supernatant V from human plasma); MsE 190:T4(9). × 120.

FIGURE 4 Twenty-eight-day-old culture (33873-4) in medium 1309-3 (CMRL-1415 supplemented with 100 mg % α_2-macroglobulin from horse serum and 35 mg % seromucoid from calf serum); MsE 171:T5(29). × 120.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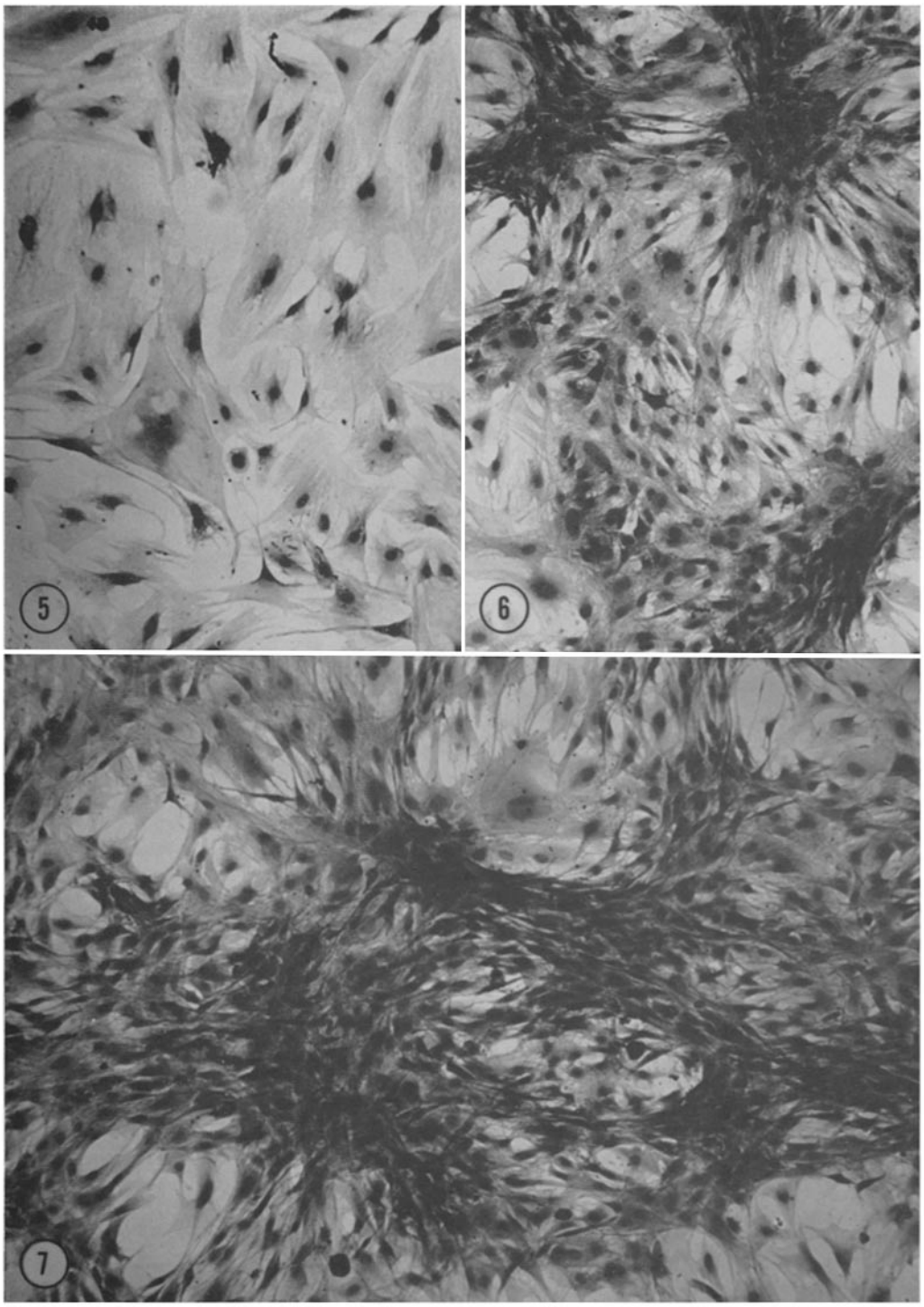


FIGURE 5 Eighteen-day-old culture (34107-1) in medium CMRL-1415; MsE 187:T4(9). × 120.

FIGURE 6 Eighteen-day-old culture (34107-3) in medium 1312-6 (CMRL-1415 supplemented with 100 mg % α_2-macroglobulin from horse serum and 35 mg % Sephadex G-50-*a* fraction of Cohn's Supernatant V from human plasma); MsE 187:T4(9). × 120.

FIGURE 7 Eighteen-day-old culture (34107-7) in medium 1335-1 (CMRL-1415 supplemented with 100 mg % α_2-macroglobulin from horse serum and 100 mg % Cohn's dialyzed, freeze-dried Supernatant V from human plasma); MsE 187:T4(9). × 120.

as a supplement for the cultivation of newly explanted mouse cells revealed a decreased intensity of the electrophoretic bands corresponding to the prealbumin (orosomucoid or α_1-acid glycoprotein) and the α_2-macroglobulin of Schultze et al. (48). Consequently, α_2-macroglobulin (mol. wt. about 850,000) was isolated from horse serum on a preparative scale by salt precipitation according to the method of Schönenberger et al. (47) for human serum. The product was electrophoretically homogeneous (starch gel) and had growth-promoting activity similar to fraction Ib of the DEAE column material. But this activity depended upon its integrity, which was destroyed by mild acid hydrolysis or gentle tryptic digestion.

The response of newly explanted mouse embryo cells to the α_2-macroglobulin of Schultze in combination with various low molecular weight glycoproteins is illustrated in Figs. 4 to 7.

Effect of Seromucoid from Calf Serum

The seromucoids (51) include several water soluble, low molecular weight glycoproteins that are not coagulable by heat, are not precipitated by sulfosalicylic acid, and migrate electrophoretically with the α_1- and α_2-globulin fractions; and a few of them have been isolated in pure form, notably orosomucoid or α_1-acidic seromucoid (53), 3.5S α_1-glycoprotein, and haptoglobin. Because there is considerable evidence supporting the identical nature of orosomucoid and Schmid's α_1-acid glycoprotein (45) from Cohn's Supernatant V,[2] we prepared this seromucoid from calf serum by salt precipitation (55). When this material was added to the basal medium (CMRL-1415) at 35 mg%, its effect on the growth and survival of newly explanted mouse embryo cells was comparable to that of Supernatant V. When it was tested in combination with α_2-macroglobulin (Fig. 4), it has as pronounced an effect as did Supernatant V with this same macromolecular supplement (Figs. 5 to 7).

Orosomucoid was then separated from the seromucoid complex of Supernatant V by chromatography on DEAE cellulose according to the procedure of Kalous and Poncavá (26). Later, a purer preparation (electrophoretically) of orosomucoid was obtained by applying Supernatant V to a DEAE cellulose column and then by applying the eluate to a carboxymethyl cellulose column (2). Attempts are now being made to evaluate the growth-promoting activity of these fractions.

[2] Pentex, Inc. (Kankakee, Illinois), distributes seromucoid fractions that are almost identical with the dialyzed, freeze-dried supernatant solution of Cohn's fraction V, are prepared by salt precipitation (55) from the serum of various species, and are designated glycoprotein (Cohn Fraction VI).

Supplementation of Basal Media with Nonprotein Macromolecules in Combination with Low Molecular Weight Glycoproteins

When it was found that the α_2-macroglobulin from horse serum in combination with certain low molecular weight glycoproteins (Figs. 6 and 7) had a much greater effect on newly explanted mouse cells than any of these fractions tested separately, we explored the possibility of substituting certain high molecular weight polymers for the macroglobulin. Although certain polymers (e.g., dextran, polyvinylpyrrolidone, and methyl cellulose) showed little or no effect when tested by us 10 yr ago in stationary cultures of established cell lines in medium CMRL-1066, dextran and Ficoll (Pharmacia, Uppsala, Sweden) now showed very dramatic effects on newly explanted mouse cells when tested in combination with the glycoproteins prepared in the present study. When Ficoll (mol. wt. approx. 400,000) was added to basal medium CMRL-1066 at levels of 5 to 10% (w/v), it produced many large and densely granular round cells with abnormally small nuclei; and many of the cells contained one or more cytoplasmic vacuoles. But when it was tested at these same levels in combination with seromucoid from calf serum, or the Sephadex G-50(a) fraction of Supernatant V, cell growth was greatly stimulated and more nearly normal in appearance than with Ficoll alone, although there were still many abnormally large cells with small nuclei. When Ficoll was reduced to 2 to 5 per cent and tested in combination with fractionated and unfractionated Supernatant V, growth was more normal in appearance (Figs. 8 and 9).

When dextran with a molecular weight of 100,000 to 200,000 (Nutritional Biochemicals Corp., Cleveland) was tested in basal medium CMRL-1415 at levels of 0.1 to 2.0%, the cells were usually indistinguishable from those in basal medium alone (Figs. 12 and 13). At 5% there was little or no growth stimulation, but there were were many large vacuolated cells (Fig. 10), as with Ficoll. At 7% the cell population was more dense and more normal in appearance, and the majority

of the cells multiplied in linear progression to form whorls that often surrounded heavier centers of increased cellular proliferation (Fig. 11). A few large cells, densely granular cells, and cells with cytoplasmic vacuoles were also seen.

When 0.1 or 1% dextran in CMRL-1415 (to give CMRL-1415-D[1]) was tested on mouse embryo cells in combination with the low molecular weight seromucoid from calf serum, with dialyzed, freeze-dried preparations of the supernatant solution of Cohn's fraction V of human plasma (to give CMRL-1415-DSCV[1]), or with Sephadex subfractions of Supernatant V, a pronounced stimulation of growth occurred that was greatly in excess of that obtained with the glycoproteins alone, and there was little evidence of the cellular abnormalities seen with Ficoll. Dextran with a molecular weight of 100,000 to 200,000 in combination with the low molecular weight glycoproteins just mentioned (Figs. 14 to 16) yielded healthy, active cultures that remained completely free of visible degeneration for long periods. In a typical experiment in which mouse embryo cells were cultivated in CMRL-1415-DSCV containing 1% dextran (the recommended level) and 100 mg% Supernatant V, the approximate population-doubling time was 2½ days, whereas in replicate cultures prepared with unsupplemented CMRL-1415 it was 4⅛ days.

The usual serum supplement for defined media is 5 to 20% by volume, which increases substantially the total solids, specific gravity, and viscosity, and contributes a colloid osmotic pressure (COP) that is usually absent in defined media. The addition of 1% dextran (mol. wt. 100,000 to 200,000) to CMRL-1415 results in expected low increments in total solids and specific gravity, whereas the COP and relative viscosity approach the accepted values for human plasma (as an 8% protein solution) as follows: COP, 19.3 mm Hg (plasma = 20 to 28 mm Hg); viscosity, 1.6 (plasma = 1.7 to 2.0).

The normal appearance of mouse embryo cells in CMRL-1415 supplemented with 0.1 to 2% (w/v) dextran (Fig. 13), the abnormally large cells in 5% dextran (Fig. 10), and their unexpected return to near normal appearance in 7% dextran (Fig. 11), is related to the peculiar osmotic properties of these solutions. We have measured the colligative properties of 1 to 10% (w/v) aqueous dextran solutions and have found that the COP increases exponentially as a function of concentration with a sharp discontinuity at a critical concentration of 5%. At this concentration, the COP is 50% less than the COP of 1% dextran. This unusual behavior results in the passage of water in the direction opposite to that anticipated from ordinary osmotic considerations so that cells in CMRL-1415 supplemented with 5% dextran become greatly enlarged.

DISCUSSION

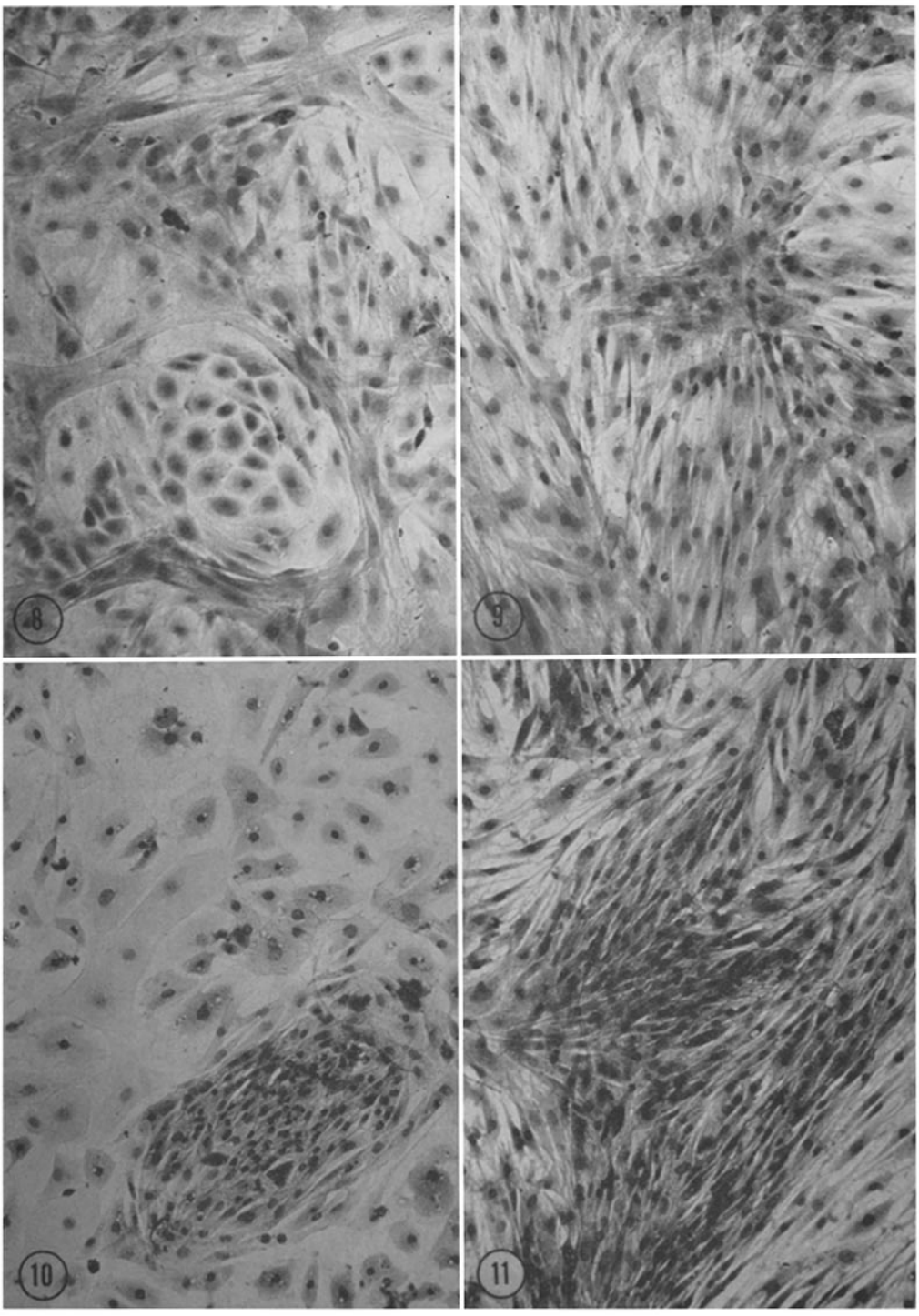
Part of the early difficulty experienced by ourselves and others in attempting to assess the nutritive value of serum fractions was due almost certainly to the use of established cell lines in the test cultures. Although our first defined media (40) were developed for freshly explanted chick embryo cells, we eventually turned to Earle's L strain cells from mouse as an easy means of making replicate cultures and assessing the results quantitatively. Eventually, however, it became evident that this and other established cell lines become adapted, very easily, to the more elaborate defined media and lack the sensitivity that is so necessary in testing minor changes made in the medium as well as materials added as supplements. For the work reported here we chose newly explanted

FIGURE 8 Twenty-four-day-old culture (34211-1) in medium CMRL-1415; MsE 193:T3(6). × 120.

FIGURE 9 Twenty-four-day-old culture (34211-5) in medium 1363-1 (CMRL-1415 supplemented with 2% Ficoll and 100 mg% Cohn's dialyzed, freeze-dried Supernatant V from human plasma); MsE 193:T3(6). × 120.

FIGURE 10 Twenty-one-day-old culture (34217-3) in medium 1375-1 (CMRL-1415 supplemented with 5% dextran, mol. wt. 100,000 to 200,000); MsE 193:T4(9). × 120.

FIGURE 11 Twenty-one-day-old culture (34217-5) in medium 1368-1 (CMRL-1415 supplemented with 7% dextran, mol. wt. 100,000 to 200,000); MsE 193:T4(9). × 120.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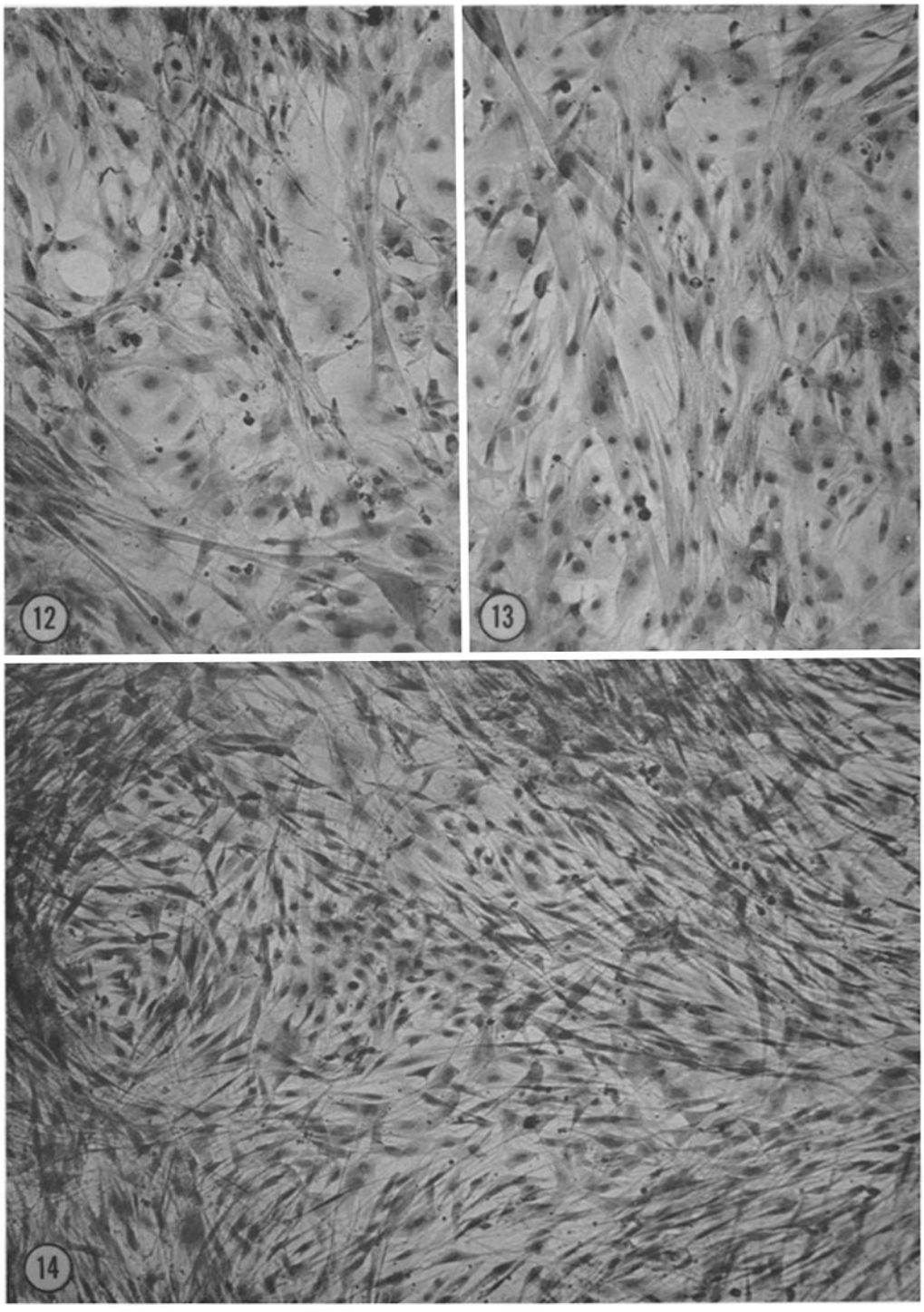

FIGURE 13 Forty-two-day-old culture (34338-3) in medium 1386-4 (CMRL-1415 supplemented with 1% dextran, mol. wt. 100,000 to 200,000); MsE 202:T3(7). × 120.

FIGURE 14 Forty-two-day-old culture (34338-5) in medium 1393-6 (CMRL-1415 supplemented with 1% dextran, mol. wt. 100,000 to 200,000, and 100 mg % Cohn's dialyzed, freeze-dried Supernatant V from human plasma); MsE 202:T3(7). × 120.

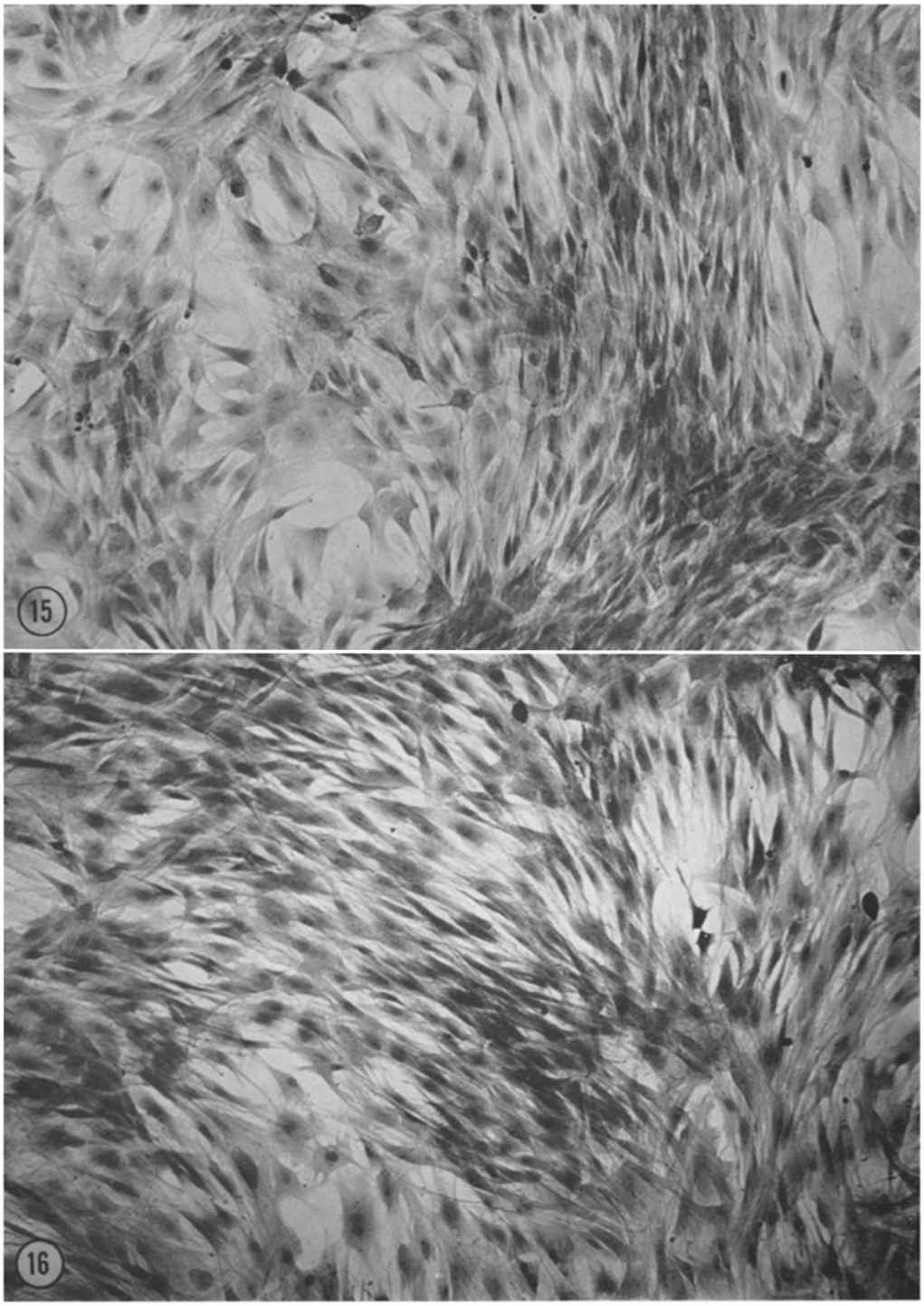

FIGURE 15 Fifteen-day-old culture (34267-6) in medium 1394-1 (CMRL-1415 supplemented with 0.1% dextran, mol. wt. 100,000 to 200,000, and 100 mg % Cohn's dialyzed, freeze-dried Supernatant V from human plasma); MsE 197:T3(6). × 120.

FIGURE 16 Fifteen-day-old culture (34267-10) in medium 1393-2 (CMRL-1415 supplemented with 1.0% dextran, mol. wt. 100,000 to 200,000, and 100 mg % Cohn's dialyzed, freeze-dried Supernatant V from human plasma); MsE 197:T3(6). × 120.

mouse embryo cells that had been subcultured a sufficient number of times (2 to 3 times in 6 to 9 days) to render them a bit more homogeneous than freshly explanted cells, but not often enough for them to lose their initial sensitivity to life outside the organism.

Fischer and his associates (13) worked extensively with dialyzed serum and stressed the importance of low molecular weight growth factors. Later, Harris (20) concluded that the defined media then existing were all deficient in one or more factors that occur in serum dialysate and are essential for growth; and, Eagle (11) found that serum does, in fact, contain one or more low molecular weight growth factors, either bound to serum protein or formed from it on proteolysis. In 1951, Jacquez and Barry (25), working with rat cells in washed plasma clots, found that the entire growth-promoting activity of exhaustively dialyzed human placental cord serum was associated with the non-dialyzable fraction. More recently, Lockart and Eagle (35) found dialyzed serum as effective as whole serum for heavily seeded cultures but not for cloning experiments with the same cell strains. Metzgar and Moskowitz (37) cultivated various cell lines in protein-free dialysates of horse serum; and Gwatkin (19) reported that CMRL-1066 supplemented with a dialysate of horse serum increased the plating efficiency of L cells to a degree comparable with that obtained when the supplement consisted of whole serum. It is likely that these dialysates contained substantial amounts of protein that remained undetected by the sulfosalicylic acid and biuret protein tests that were used. In any event, it has been our experience that simple dialysis uncomplicated by the addition of proteolytic enzymes does not reduce the growth-promoting activity of dialyzed serum when used as a supplement for CMRL-1066 or CMRL-1415, under the condition of the present experiments. It should be remembered that the degree of complexity of a basal medium to which test supplements are added determines the response of the cells to these supplements, i.e., a relatively simple basal medium such as 199 or Eagle's minimal essential medium makes greater demands on a protein supplement than a more completely adequate medium such as CMRL-1066 or CMRL-1415.

In 1923, Carrel and Ebeling (5) obtained a growth-promoting factor for chicken fibroblasts by supplementing Tyrode's solution with globulins that had been precipitated from adult fowl serum with CO_2. But despite renewed interest in the last decade, knowledge of the growth-promoting properties of serum proteins is still meager. Quite recently, the importance of low molecular weight globulins was demonstrated by reports from other laboratories of a serum factor that was said to promote flattening and adherence of certain strain cells to glass. Fetuin (42, 9) is an α_1-glycoprotein (mol. wt. 48,400) that accounts for about 45% of the proteins of fetal calf serum. Lieberman and Ove (33, 34) obtained a glycoprotein from bovine serum that was said to promote the attachment of human appendix cells to glass. Fisher et al. (15) concluded that fetuin had this same effect on HeLa cells. Later, the same authors (16) reported the quantitative growth of single HeLa cells on glass in a defined medium supplemented with serum albumin and fetuin, but Lieberman et al. (32) made further purification studies and decided that purified fetuin lacks biological activity. A year later, Spiro (50) also reported a highly purified fetuin from fetal calf serum that was inactive, although it is possible that in both instances the activity may have been destroyed by the purification procedures (14). More recently, Marr et al. (36) used the preparative ultracentrifuge to separate fractions from fetal calf serum and found growth activity in fetuin and another glycoprotein, α-macroglobulin. They attributed the growth activity to common chemical groupings found in both glycoproteins, to a common trace protein, or to a low molecular weight material bound by both proteins. Earlier, L. Weiss (54) studied the adhesion of human cells to glass and found that cells that had lost their adhesive properties after trypsin treatment could regain them if whole serum or serum fraction G2 of Kekwick and Mackay (29) was added to the medium. Fraction G2, which contains macroglobulin and other α-globulins (4, 43), was said to be interchangeable with whole serum for adhesion of cells to glass but not for nutrition. In 1951, Jacquez and Barry (25) prepared albumin and globulin fractions from human placental cord serum by ammonium sulfate precipitation and found all the growth activity of the original serum was present in the globulin fraction. When subfractions were prepared from the globulin fraction, some activity was found in all of them. Later, Katsuta et al. (27) found that growth activity for Yoshida ascites tumor cells of the rat was asso-

ciated with the α-globulin and albumin fractions as obtained by zone electrophoresis, cold ethanol, or ammonium sulfate precipitation of *dialyzed* horse serum.

A growth-active globulin has also been isolated by Michl (38, 39) by ammonium sulfate precipitation. The addition of this globulin to a defined medium containing serum albumin or methyl cellulose supported maximal growth of freshly explanted rabbit heart and kidney cells and various established cell lines. Tozer and Pirt (52) studied the macromolecular growth-promoting fractions of dialyzed calf serum separated by electrophoresis on a cellulose powder column, by continuous-flow paper curtain electrophoresis, and by gel filtration on Sephadex G-200 columns. Because these investigators felt the "attachment factor" may be separate from that for growth, they tested their fraction on suspension-type cultures of L- and ERK-strain cells in a defined medium containing bovine serum albumin, insulin, thyroxine, and catalase, and concluded that the activity of dialyzed serum was associated with albumin and with an α-macroglobulin. Holmes and Wolfe (23, 24) separated two components (1 and 2) from dialyzed bovine serum by curtain electrophoresis. Both components stimulated the growth of certain human cell lines but only component 1 promoted cellular attachment and spreading. The growth-stimulating material was associated with the α-globulins during electrophoresis.

In our experience, attachment and spreading of cells has been possible in all protein-free media reported from this laboratory since 1950, often without prolonged growth and multiplication. It doesn't seem realistic at present, therefore, to attribute these various qualities to separate and distinct factors (or serum fractions). In any event, it has been known in tissue culture laboratories for many years that attachment and spreading of cells can be blocked indefinitely, even in the presence of fetal calf serum, by placing them in culture vessels with unsuitable surfaces.

There is general agreement on the decrease of α_2-globulins in serum supplements used in the cultivation of cells and tissues, as revealed by electrophoresis (30, 31, 18). In this laboratory, we have confirmed the disappearance of α_2-globulins from the medium and have established that it is the α_2-macroglobulin that decreases in horse serum used to supplement CMRL-1066 during the cultivation of newly explanted mouse embryo cells. Because α_2-macroglobulin has the same mobility as other α_2-globulins during paper and moving boundary electrophoresis, the specific disappearance of α_2-macroglobulin was not apparent until it was revealed by starch gel electrophoresis. Similarly, α_1-acid glycoprotein (orosomucoid), which has a distinct and separate mobility from other α_1-globulins during starch gel electrophoresis, was also taken up by the cells, but to a lesser extent.

Over the years, the most adequate media for animal cells in vitro have consisted of plasma or serum and tissue extracts, in suitable diluents. By analogy, the ideal chemically defined medium should contain the essential nutrients and possess the physicochemical properties of a transudate of blood plasma such as lymph or interstitial fluid. Some investigators (1, 52) have supplemented defined media with well characterized proteins such as insulin and catalase, but the amounts used were quantitatively insignificant. Others (12, 6, 28, 3, 41) have tested methyl cellulose, dextran, polyvinylpyrrolidone (PVP), Ficoll, serum albumin, ovalbumin, acacia, and alginic acid, as supplements for various types of media, some of them defined. Thus, Katsuta et al. (28) used alginic acid, dextran, and PVP in 7-day stationary cultures of rat ascitic tumor cells and found alginic acid and dextran (mol. wt. 50,900) to be greatly inferior to 0.1% PVP (mol. wt. 70,000), which they reported could replace 99.5% of the dialyzed serum protein present in a control medium that included 20% dialyzed bovine serum and 0.4% lactalbumin hydrolysate in saline. More recently, Moskowitz and Schenck (41) reported that the addition of human serum albumin to CMRL-1066 protected cells of various established lines from growth-inhibiting factors that were separated by dialysis, along with growth-promoting factors, from an extract of porcine pancreas; and Brinster (3) studied the ability of certain nonprotein polymers to replace the physical properties of a bovine serum albumin supplement (0.1%) during the development of mouse ova in a defined medium. He found that various methyl cellulose preparations were ineffective substitutes for albumin whereas PVP (mol. wt. 150,000), acacia, dextran, and Ficoll responded favorably.

In general, recent work in this and other laboratories indicates that two separate α-globulins in serum are at least partly responsible for its growth-promoting effects. They are both glyco-

proteins and are further characterized by their molecular weights. The low molecular weight α-globulin (mol. wt. 40,000 to 50,000) occurs in seromucoid and in Cohn's supernatant solution V (method 6). The high molecular weight α-globulin (mol. wt. about 850,000) is the α_2-macroglobulin that was first isolated from human serum by Schultze et al. (48). We have found that these α-globulins are synergistic and can substitute for whole serum, at least for a time, if they are used as supplements for suitably designed chemically defined media. And while the low molecular weight α-globulin seems to be essential, the α_2-macroglobulin required as a supplement may be replaced by certain nonprotein polymers of moderately high molecular weight.

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