Growth-Active Globulins from Calf Serum Tested on Cultures of Newly Isolated Mouse Embryo Cells (34665)

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In earlier papers (1, 2) we reported chemically defined medium CMRL-1415[1] and described the preparation and use of certain macromolecular supplements, namely, α_1-acid glycoprotein (orosomucoid) from the supernatant solution of Cohn's fraction V (method 6), sometimes referred to as fraction VI, of human plasma together with α_2-macroglobulin from horse serum or with commercial dextran (mol wt 100,000–200,000). The latter combination (CMRL-1415-DSCV[1]) yielded a population doubling time of 2.5 days for newly-isolated mouse embryo cells, whereas in replicate cultures prepared with unsupplemented CMRL-1415 it was 4.33 days.

Although many established cell lines have been adapted to serum-free, chemically defined media, newly-isolated mammalian cells in continuous culture require certain proteins that are usually supplied in the form of a native or dialyzed serum supplement in a suitable defined medium. The nature of these proteins and their nutritional role have not been fully elucidated. It is generally agreed that normal sera contain several proteins that greatly influence cell growth; and these proteins include both activators (3, 4, 6–10) and inhibitors (3–6) of growth.

In view of the multiplicity of these factors in serum, no single technique such as column chromatography or gel filtration will resolve them without certain preliminary separations. It is the purpose of this report to present a method for the separation of growth-active globulins from fetal calf serum. All operations were performed at room temperature unless otherwise specified. A few crystals of chlorobutanol were added as a preservative to all solutions during fractionation (except during the Rivanol[1] treatment). The method of separation is outlined in Table I.

Bentonite adsorption removes certain lipoproteins and clotting components (11, 12). The potassium phosphate precipitates the globulins, leaving most of the albumin in solution (13). After saturation with glutam-

TABLE I. Isolation of Growth-Active Globulins.

Fetal calf serum	Undiluted, pH 7.5–8.0; adsorbed with 2% bentonite for 30 min
Supernatant	Diluted 1:1 with water; added 2 vol 3.5 *M* potassium phosphate, pH 6.8
Precipitate	Dissolved in 0.14 *M* NaCl, dialyzed 48 hr in tap water, concentrated to approx original vol by pervaporation. Redissolved the euglobulins by making 0.14 *M* with NaCl; added L-glutamic acid to pH 4.1, stirred at 4° for 1 hr
Supernatant	Dialyzed against tap water for 12 hr, redissolved euglobulins in 0.15 *M* sodium lactate. Adjusted protein to approx 1% and pH 8.0 with $NaHCO_3$. Made solution 0.003 *M* with respect to Rivanol
Supernatant	Added 5% solid NaCl to precipitate excess Rivanol
Supernatant	Adsorbed last traces of Rivanol on polyurethane sponge. Eluted proteins from sponge and freeze-dried the protein solution
Redissolved proteins	Applied to hydroxylapatite column; eluted stepwise with sodium phosphate buffers at pH 6.8. Growth-active fraction eluted in 0.1 *M* buffer

[1] Registered trademark.

ic acid, more residual albumin, α_1-antitrypsin, α_2-globulins, transferrin, and hemopexin are precipitated (14). Rivanol (6, 9-diamino-2-ethoxyacridine) 0.003 *M*, at pH 8.0, flocculates all but a trace of albumin, the prealbumin, α_1-easily-precipitable glycoprotein, tryptophan-poor α_1-glycoprotein, Gc-globulins, inter-α-trypsin-inhibitor, haptoglobin, α_2-macroglobulin and β_1A-globulin (11, 12, 15). As revealed by agarose gel immunoelectrophoresis, the freeze-dried product at this stage consisted of α- and β-globulins with only a trace of albumin and no immunoglobulins. Calcium phosphate (hydroxylapatite) chromatography (16, 17) of a solution of this material in sodium phosphate buffer, at pH 6.8, further resolved the proteins into three fractions. The third fraction, eluted with 0.1 *M* phosphate, contained the growth activity. Agarose gel immunoelectrophoresis of freeze-dried fraction 3 from the hydroxylapatite column revealed only an α-globulin and a trace of albumin.

Two growth-active globulins (2) isolated earlier in this laboratory are eliminated by these procedures. The relationship of the α-globulin referred to above to fetuin is not clear. Electrophoresis of fraction 3 from the hydroxylapatite column, at pH 4.2, in acetate buffer, according to Oshiro and Eylar (18), resulted in only three bands. Unmodified fetuin would give rise to eight bands under these conditions.

In a typical experiment, the population doubling time in replicate cultures of newly isolated mouse embryo cells in basal medium CMRL-1415 was 4.75 days, whereas the doubling time of cells in sister cultures containing 25 mg% of dialyzed, freeze-dried fraction 3 from the hydroxylapatite column was 1.33 days. When fraction 3 from the hydroxylapatite column was combined with 35 mg% α_1-acid glycoprotein (2), the cells remained more healthy in appearance for longer periods. For many purposes, it would probably not be necessary to make the final separation of the α- and β-globulins on hydroxylapatite.

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