PREPARATION FROM HUMAN SERUM OF AN ALPHA-ONE PROTEIN WHICH INDUCES THE IMMEDIATE GROWTH OF UNADAPTED CELLS IN VITRO

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ABSTRACT

An alpha-one protein is separated from human serum on a microbead column. This nondialyzable protein induces the immediate growth of unadapted cells placed in chemically defined Medium A2 + APG. HeLa, conjunctiva and human heart cells, which stop growing if the protein is removed, continue to grow only if the protein is returned or the cells are permitted to adapt to the defined medium. A 90–120 day period is required for adaptation. The spreading and growth of fully adapted cells is also stimulated by the addition of the protein. As little as 0.4 μg per ml of medium is effective. The protein analyzed by paper, starch, and discontinuous acrylamide gel electrophoresis appears to be a single component. The protein is periodate-Schiff positive and readily binds small molecules which are removed, without loss of biological activity, by precipitating the protein in 50% saturated ammonium sulfate. The protein is adsorbed on the microbead column as a complex with the beta lipoprotein fraction of human serum; it cannot be separated from bovine or equine sera by this method; and it is not identical with fetuin. Its biological response is not duplicated by insulin, carbamyl phosphate, putrescine, or linoleic acid.

INTRODUCTION

An alpha-one type protein isolated from human serum has been shown to be equally effective in stimulating both the growth and spreading of cultured human cells. This protein is nondialyzable. The biological activity of the alpha-one protein was tested on monolayer cultures using three different human cell lines, HeLa (Gey), conjunctiva (Chang), and human heart (Girardi). Two findings with respect to its biological activity were noteworthy. First, the alpha-one protein could replace serum in those stock cultures which were maintained on the defined base medium plus serum. Second, those strains which had been adapted to the chemically defined base medium without serum and which have been demonstrated to grow indefinitely in this defined medium were stimulated to enhanced growth and spreading when treated with preparations of the alpha-one protein.

Electrophoretic patterns have shown that the material is not fetuin obtained from fetal calf serum by Fisher, Puck, and Sato (7), or the light and heavy sedimenting fractions described by Marr, Owen, and Wilson (20). Though biologically much more active than either of Michl's calf serum preparations (21, 22) by a factor of 250 and 80, respectively, it is not known whether this increase in activity is due to greater purity of the protein or the use of a more effective, defined base medium (14). In biological activity this

protein more nearly corresponds to the active material described by Lieberman and Ove (18) who used a base medium of greater complexity than that used by Michl; their report lacks the necessary data for comparison of electrophoretic properties. Since the human-derived alpha-one protein is intimately associated with the lipoproteins in the initial stages of purification, as will be described later, it was considered useful to show by electrophoretic and biological means that the protein differed from any lipoproteins, of a 1.20 density or less, prepared by ultracentrifugation. Finally, a biological comparison has been made which showed that its effect on the growth and spreading of human, cultured cells could not be duplicated by those small molecular weight substances (23) which have been suggested as replacements for fetuin.

MATERIALS AND METHODS

Preparation of Serum

Bovine and equine sera were obtained from Grand Island Biological Co., Grand Island, N. Y. Fresh human serum was prepared from whole blood which was allowed to clot in sterile, silicone-coated tubes. After standing overnight at 4°C the clear supernatant serum was withdrawn by pipette.

Human serum was also prepared from outdated, nonhemolyzed, ACD-preserved blood. The plasma was decanted from the red cells and then centrifuged for 15 min at 2,000 rpm to remove particulate matter. 1 volume of the plasma was then dialyzed overnight against 10 volumes of 0.6% sodium chloride. Clot formation in the dialyzed plasma was induced by adding calcium chloride to a concentration of 0.1%. The resulting serum, hereafter called processed serum, was squeezed from the clot through a cheese cloth bag. At this point, if necessary, the processed serum could be frozen and stored at −70°C until needed.

Preparation of Biologically Active Alpha-One Protein

The ground-glass column procedure of Carlson (2) was modified in the following respects. Soda-lime glass microbeads, Class IV, (Microbeads Div., Cataphote Corp., Jackson, Miss.) were substituted for crushed glass, sodium was substituted for potassium in the first two eluting electrolyte solutions, and the sequence of developing solutions was changed from that recommended by Carlson (2) to that described below.

5 kg of beads was boiled in aqua regia, rinsed with distilled water, and then washed with 1 liter of 0.6 M sodium bicarbonate solution adjusted to pH 8.0 with sodium hydroxide. A 5.8 × 40 cm column was filled to a depth of 24 cm with the washed beads and further conditioned with 200 ml more of the same bicarbonate solution just before adding the serum.

Either fresh or processed serum was adjusted to pH 8.0 with 0.1 N sodium hydroxide, and 20 ml, to which was added 1 mg of phenol red, was placed on the column. The elution sequence was as follows: (*a*) 200 ml of 0.6 M sodium bicarbonate solution, pH 8.0; (*b*) 100 ml of a mixture of 0.6 M sodium bicarbonate and 0.2 M sodium carbonate, at pH 9.5; (*c*) 100 ml of water; (*d*) 100 ml of a mixture of 0.6 M potassium bicarbonate and 0.2 M potassium carbonate with a permissible range of pH 9.6–9.8; (*e*) 100 ml of water; and (*f*) 200 ml of 0.2 M potassium carbonate. The column effluent was collected in 20 ml fractions. The column was operated at both room temperature and 35°C, at a flow rate not exceeding 125 ml per hr.

The presence of protein in each of the fractions was determined either by spotting the fraction on paper and staining the dried spots with 0.5% solution of bromophenol blue in ethanol saturated with $HgCl_2$ (15) or by the method of Daughaday, Lowry, Rosebrough, and Fields (4). Lipids were determined by the method of Sperry and Brand (29), using 10 ml of sample concentrated to 1 ml. Carbohydrates were determined by the method of Svennerholm (31). Fractions 23–27 contained the active substance (Fig. 1). These fractions were combined and dialyzed against water. The dialyzed solution of active material was adjusted to pH 8.0 with 0.01 N potassium hydroxide and sterilized by cold filtration through Morton glass filters (Corning Glass Works, Corning, N. Y.). (The filters, prior to use, were cleaned in hot concentrated sulfuric acid plus 5% (v/v) nitric acid, then washed by aspiration with distilled water, 0.1% sodium bicarbonate solution, and distilled water). A portion of the solution was dried to constant weight at 105°C for the determination of the solids content.

The above solution which was biologically as effective, volume for volume, as the original serum was further purified. For this purpose the dialyzed solution was adjusted to pH 7.2 in a carbon dioxide atmosphere, using phenol red as an indicator, and frozen. Immediately upon thawing, the cloudy solution was centrifuged and the biologically active precipitate dissolved in a small volume of water adjusted to pH 10 with dilute potassium hydroxide. The dissolved precipitate was centrifuged; the supernatant was dialyzed against water and concentrated by slow evaporization at 4°C. Fractions eluted at pH 8.9 and 35°C contained sufficient lipid material that the active protein removed by 50% saturated ammonium floated, like a lipoprotein, to the top of the

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centrifuge tube. In the development of these procedures, the retention of maximum biological activity guided each detail and each step.

Preparation of Lipoproteins

Fresh or processed serum was used to prepare the lipoproteins according to Lindegren, Nichols, and Freeman (19). The different fractions were recentrifuged to ensure that residual nonlipoprotein proteins were removed. This was accomplished by layering 3 ml portions of each fraction under 9.5 ml of KBr solution, 1.21 density, and recentrifuging the layered tubes for 22 hr at 38,000 rpm. The final fractions were dialyzed to remove salts and adjusted either to pH 7.2 for determination of biological effects, or to pH 8.0 for chromatography.

slide is shown in Fig. 2. The large area of the slide was covered with 18 ml of 7.5% acrylamide gel monomer mix, (see Table I) and the glass cover immediately installed, avoiding air bubbles. The box containing the slide was flushed with nitrogen. After 20 min the divider was removed and the remaining small area of the slide covered with 4 ml of 5% acrylamide gel monomer mix (Table I). Three sample slot molds were installed (Fig. 1), leaving a clearance of 1 mm between the bottom of each slot and the gel-glass interface. The mold for the slots was located 0.5 cm from the edge of the glass covering the 7.5% running gel. The box containing the slide was again flushed with nitrogen, and polymerization of both gels was allowed to progress to completion overnight.

The following morning the sample slot mold was

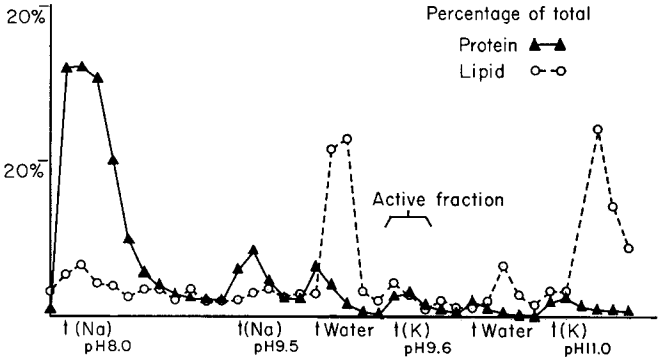

FIGURE 1 Separation of serum on glass microbeads at 25°C, showing the percentage of total protein and lipid material found in each fraction. All fractions were tested for biological activity (see text). Activity was located in fractions 23–27 inclusive.

Electrophoretic Analysis and Separations

(*a*) Starch gel electrophoresis and staining of the patterns with amido black 10B dye was carried out according to Smithies (28).

(*b*) Discontinuous thin layer acrylamide gel electrophoresis, an original method capable of separating human sera into 28 different protein components, based on the works of Raymond (27), Davis (5) and Ornstein (24), was carried out as follows A 3¼ × 4 inches glass slide was ground on one side with 600 gr carborundum powder (The Carborundum Company, Niagara Falls, N. Y.). The ground slide was cleaned in a 70°C bath ot sulfuric acid containing 5% nitric acid, rinsed, and dried. The slide was ringed (1 cm wide) with a thin layer ot 5% acrylamide gel (see Table I) which was allowed to dry for 6 hr. The ring acted at a later stage to cement the edges of the matrix gel to the glass.

A plastic divider was placed 2 cm from the end of the slide and two thin strips of plastic, ⅛ inch thick, were laid along each side to act as spacers for a glass cover. Both ends of the slide were rimmed with Vaseline and the slide placed in a level box which could be filled with nitrogen. The arrangement of the

removed and the slide was ready for use. A ¼ dilution of whole serum was used for serum patterns. Albumin crystalline human albumin (Pentex Inc., Kankakee, Ill.), and fetuins, according to Fisher, Puck, and Sato (7) (Colorado Serum Co., Denver, Colo.) as well as according to Deutsch (6) and Spiro (30) (Grand Island Biologicals), were prepared in 1% solutions. After filling each sample slot with protein solution, K150 dye mixture (Kensington Scientific Supply Co., Berkeley, Calif.) was spotted on top of the gel between each sample slot. Electrical contact to the plate was established using double layers of 3MM Whatman filter paper (H. Reeve Angel & Co., Clifton, N. J.). Both electrode vessels were filled with 0.1675 M boric acid adjusted to pH 8.6 with sodium hydroxide. The slide was developed (2½ to 3 hr) using 100 v DC until the blue marker in the K150 dye mixture had reached the far edge of the glass cover.

The glass cover was carefully removed from the top of the gel and the slide stained for 30 min in amido black 10B dye solution (28). The slide was washed clear of excess dye with several changes of 20% ethanol solution containing 2% of acetic acid. A few

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TABLE I

Composition of Acrylamide Gel Solutions

Aronsson's Buffer (1): 2-hydroxymethyl 2-amino 1-3 propanediol, 10.8 g; disodium ethylenediaminetetraäcetic, 1.3; Boric acid, 0.8; Water to 1 liter, pH 8.9

	5% acrylamide gel for ringing plate	7.5% acrylamide gel running gel	5% acrylamide gel stacking sampling
	ml	*ml*	*ml*
Solution* 1	2.5	7.5	2.5
Solution 2	2.5	5.0	—
Solution 3	—	—	2.5
Water	8.0	13.5	8.0
Deaerate before adding deaerated Soln. 4			
Solution 4	2.5	5.0	2.5

* Solution 1 15 g acrylamide plus 0.4 g bisacrylamide in 50 ml water.

Solution 2 1.0 ml tetramethyl ethylene diamine in 100 ml Aronsson's buffer.

Solution 3 0.5 ml tetramethyl ethylene diamine plus 8.4 ml Aronsson's buffer adjusted to pH 6.0 with acetic acid, final volume 50 ml.

Solution 4 0.4 ammonium persulfate in 50 ml of water.

drops of glycerin were added to the final wash solution, after which the slide was air dried.

(*c*) Paper electrophoresis has been described (15).

(*d*) Two nonionic surfactants, Igepal CO 730, and alkyl phenoxy polyoxethylene ethanol (General Aniline and Film Corporation, Dyestuff & Chemical Division, New York) and Renex 648, a polyethylene alkyl aryl ether (Atlas Chemical Industries Inc., Wilmington, Del.), which have been shown to prevent the trailing of lipoproteins (17), were used in conjunction with certain of the paper and starch gel electrophoretic experiments. Concentration of the surfactant added to the borate buffer was varied between 0.1 and 1.0%.

(*e*) Electrophoretic patterns were stained for periodate-Schiff-reactive material using the method of Green et al. (10).

Medium A2 + APG for Cell Cultures

Chemically defined Medium A2 (salts, carbohydrates, amino acids, cofactors, and vitamins) has been described in detail (14). In the present study, 1 mg of acetylcholine chloride, 10 mg of sodium pyruvate, and 10 mg of D-galactosamine hydrochloride were added to each 100 ml of Medium A2. This was designated Medium A2 + APG.

This medium may be prepared ahead of time if frozen and stored at −70°C, otherwise it must be used immediately or be oxidized rapidly. A satisfactory state of reduction exists when 10 ml of medium placed in an evacuated Thunberg tube (Corning Glass Works, Corning, N. Y.) reduce 0.5 ml of a 0.01% methylene blue solution at 37°C in less than 45 min.

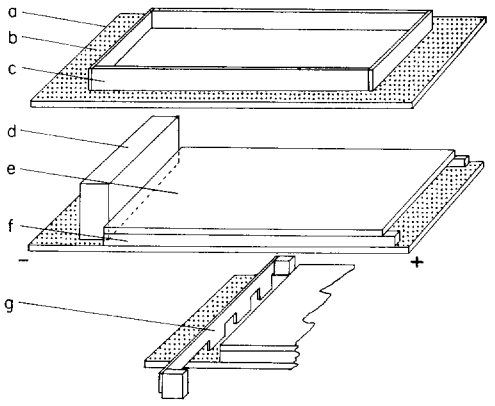

FIGURE 2 Design of discontinuous acrylamide gel plate: (*a*) ring, 1 cm wide, of 5% acrylamide gel; (*b*) ground glass projection slide 3¼ × 4 inches; (*c*) plastic form, removable; (*d*) plastic divider; (*e*) glass cover plate, covering 7.5% running gel; (*f*) plastic spacers ⅛ inch thick; (*g*) mold for sample slots set in 5% spacer gel.

Maintenance of Stock Cultures

Unadapted stocks of HeLa, conjunctiva, and human heart cells were carried as monolayer cultures in either glass or polystyrene flasks, with Medium A2 + APG supplemented with 10% dialyzed serum. The serum supplement consisted of human serum, from which the lipoproteins had been removed by ultracentrifugation at 1.20 density (3, 16) followed by dialysis against saline.

From the stock cultures, three strains were adapted to grow continuously as monolayer cultures in Medium A2 + APG without any serum supplement. The HeLa cell which was the first strain to be adapted has been grown for 6 yr (14). Conjunctiva and human heart strains were adapted later in the same manner as the HeLa strain, and have been under culture for 2 yr. Growth of cultures was measured by determining the change in total cell protein, by using the method of Oyama and Eagle (25).

EXPERIMENTAL

Separation and Properties of Alpha-One Protein

The method for separating biologically active alpha-one protein on glass microbeads is described under Materials and Methods. The distribution of

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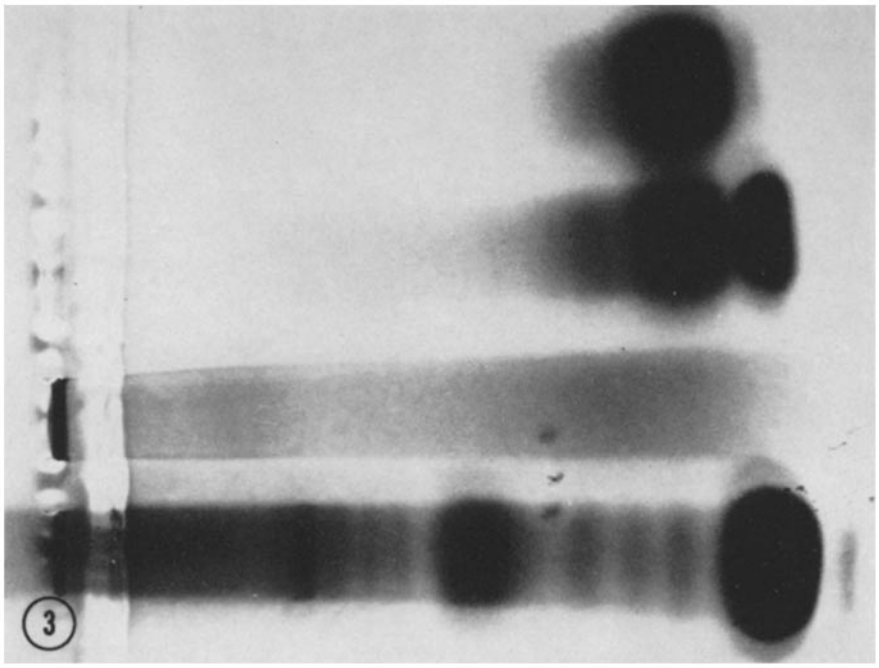

FIGURE 3 A comparative discontinuous 5% acrylamide gel electrophoretic pattern showing the active alpha-one protein, third from the top. The 5% gel has a sufficiently large pore size to permit running of the alpha-protein, but the pores are too large to give detailed separations of Spiro's and Deutsch's type fetuins, first and second patterns from the top. Nor is the gel adequate for separation of human serum, bottom pattern.

total protein and lipid material between the various fractions is shown in Fig. 1. The distribution of carbohydrate material, since it appeared to be identical with that of the protein, is not shown in Fig. 1. In Fig. 1 many of the protein peaks slightly precede the lipid peaks suggesting that some of the serum proteins, probably the lipoproteins, were adsorbed on the microbeads with lipid material interposed between the surface of the beads and the protein.

Fig. 3 shows a 5% acrylamide gel electrophoretic pattern in which the active material, prepared by freezing (see Materials and Methods), is compared with that of pooled human serum and two inactive fetuin preparations. The active material which appears as a broad band in the alpha-one position also exhibited considerable trailing. Patterns obtained on both starch gel (28) and paper were very similar. With 5% acrylamide the density of the gel is not sufficient to bring about the degree of separation than can be obtained by using a 7.5% acrylamide gel. A 7.5% gel pattern is shown in Fig. 4. In this pattern the bulk of the active fraction remains at the point at which the protein enters the 7.5% gel. The remainder of the pattern is free of any of the proteins seen in the serum patterns directly below, and free from any of the proteins in the fetuin patterns seen directly above. Since the active material does not readily enter the 7.5% gel and enters the 5% gel with considerable trailing, it would appear to be a relatively large molecule, possibly micellar in behavior.

Extraction of fractions 23–27 with either acetone or diethyl ether prior to electrophoresis did not reduce trailing. Addition of nonionic surfactants (see Materials and Methods) to either the sample or the developing electrolyte did not reduce trailing. It was of interest to note that none of these procedures impaired biological activity of the protein. Cholic acid did reduce the trailing and, as previously reported in abstract form (17), the electrophoretic mobility, on paper, of the main alpha-one band was increased so that it appeared

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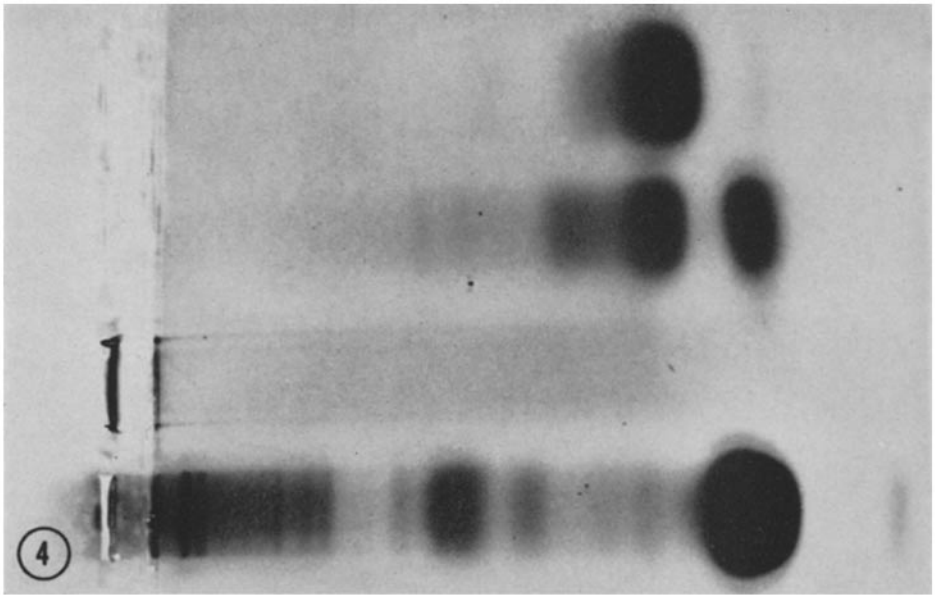

FIGURE 4 A comparative discontinuous 7.5% acrylamide gel electrophoretic pattern showing the active alpha-one protein, third from the top. The bulk of the alpha protein under these conditions remains at the 5% stacking gel–7.5% running gel interface. Traces of protein which run into the 7.5% gel have a reduced lipid content. Spiro's and Deutsch's fetuins are represented by the first and second patterns from the top. Serum is represented by the bottom sample.

in the position usually occupied by prealbumin. On eluting the alpha-one protein plus the small amount of cholic acid associated with it, and electrophorizing on paper, in a cholic acid–free system, trailing was again observed. Throughout this procedure, biological activity was also unimpaired.

As a result of further attempts to improve the electrolytic appearance of the alpha-one protein, new properties of the material were discovered. When the material isolated from the column fractions 23–27 was rechromatographed on the glass bead column, contrary to its initial behavior it no longer exhibited retardation but was found at the solvent front. This suggested the possibility that the retardation observed on the first passage was due to association with some other component of serum. Accordingly, a preparative run on the glass beadcolumn was stopped after development with the pH 9.5 sodium bicarbonate–carbonate solution and, instead of the usual water wash, the column was stripped with 0.2 M K_2CO_3 solution which removed all remaining protein and lipid material. The material in the K_2CO_3 effluent was precipitated from solution by saturation with CO_2, and the precipitate was collected by centrifugation. The supernatant was discarded and the solid pellet which dissolved in K_2CO_3 solution was dialyzed against water. Electrophoretic patterns on a portion of the dialyzed material on 5% acrylamide gel were stained for protein with bromophenol blue reagent and with Sudan black B. The major protein band appeared in the position of the alpha-one protein, and Sudan black B staining material was found in the position of beta lipoprotein. The remainder of the dialyzed material, containing both alpha-one and beta lipoprotein, was then subjected to the standard preparative procedure on the glass bead column. As is in the initial run, active alpha-one protein, free of beta lipoprotein, was found in fractions 23–27. From these experiments it was concluded that retardation of the alpha-one protein on the glass bead column was due to association with beta lipoprotein and that attachment to the glass bead surface took place in the presence of the beta lipoprotein.

The lipoproteins which were prepared from human serum by density gradient centrifugation were themselves biologically inactive and did not

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produce an active fraction on passage through the glass bead column.

Upon subjecting the alpha-one protein to paper chromatography, using a mixture of 45 vol butanol, 10 vol acetic acid and 45 vol water, several ninhydrin reactive spots were observed. On precipitating the protein with 50% saturated ammonium sulfate, these ninhydrin-reactive substances were removed without loss of biological activity.

Comparison of Fetuin with Alpha-One Protein

Fetuin from embryonic sera, prepared by the method of Fisher, Puck, and Sato, has been reported to exhibit biological properties resembling those of the alpha-one protein. Therefore fetuin and alpha-one protein were compared.

Starch and acrylamide gel electrophoretic patterns of fetuin (Colorado Serum Co., Denver, Colorado) were made. The discontinuous acrylamide gel patterns shown in Fig. 5 are to be compared with those of the alpha-one protein shown in Figs. 3 and 4. Compared to the alpha-one protein pattern with its single broad band (Fig. 3), the fetuin patterns shown in Fig. 5 had at least four major bands and a number of minor bands, the latter varying between batches. Trailing, characteristic of the human alpha-one protein, appeared to be absent in the fetuin patterns. The fetuin pattern shown in Fig. 5 exhibited characteristics identical with those described by Marr, Owen, and Wilson (20). Both the active human and fetuin fractions were stained with the periodate-Schiff reagents.

Some growth substances, besides being prepared from human sera, have also been prepared from bovine and equine sera (18, 14). Attempts, by means of a microbead column, were not successful in separating from either of these sera an alpha-one type protein, containing properties similar to those of the human alpha-one protein. In both cases biological activity was located in the fraction removed by sodium bicarbonate at pH 8.0. This fraction included the bulk of the serum proteins. However, although no active material was present in the potassium bicarbonate pH 9.6–9.8 fraction, one explanation of this result could be that no beta lipoprotein, which is essential for alpha-one protein adsorption, eluted from the column after the pH 9.6–9.8 fraction was removed.

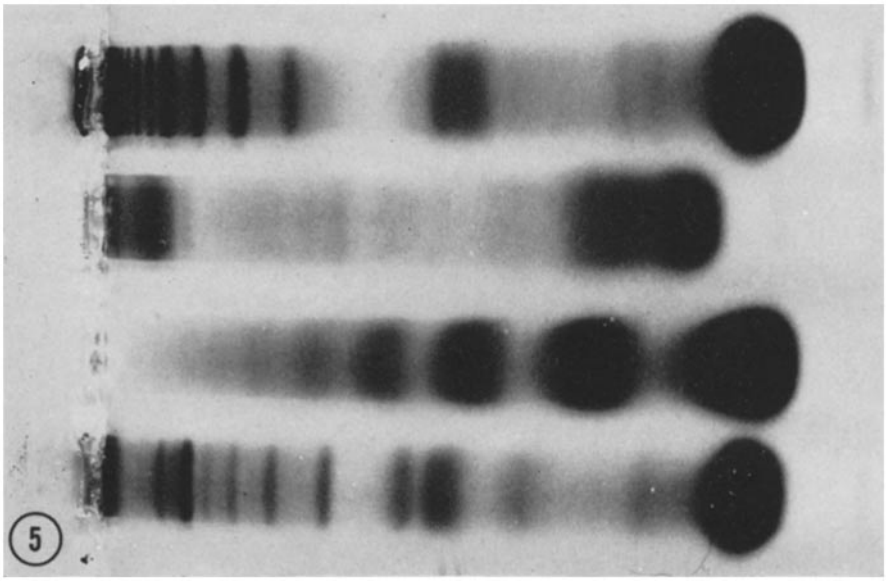

FIGURE 5 A comparative detailed discontinuous 7.5% acrylamide gel pattern of Fisher et al. (6) fetuin, second from the top. Note that this pattern shows at least four major components as well as a number of minor components. The top and bottom patterns illustrate the type of separation achieved using two different specimens of human serum. The third pattern from the top is that of crystalline bovine albumin obtained from Pentex; this material is homogeneous by immunoelectrophoresis (13).

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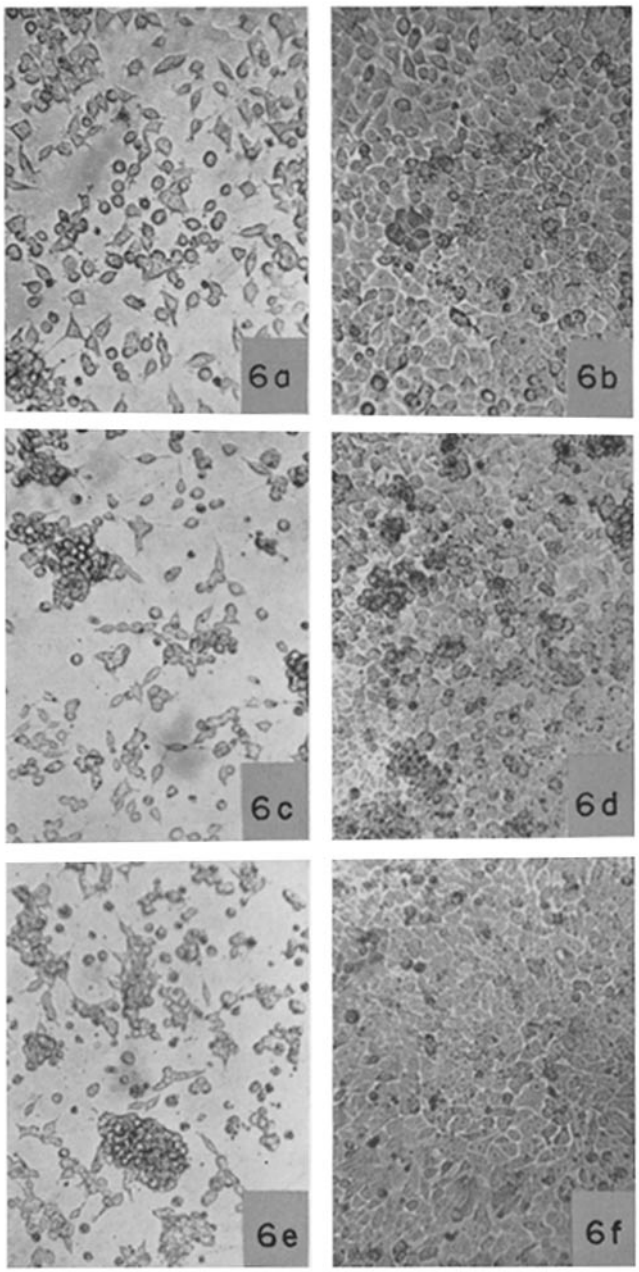

FIGURE 6 Effect of 2 μg/ml of purified alpha protein on replicate cultures of cells adapted to grow in chemically defined medium. *a*, *c* and *e* represent 24-hr-old cultures of HeLa, conjunctiva and human heart cells, respectively. *b*, *d* and *f* represent replicate 24-hr-old cultures of the same cells with alpha protein added to the medium.

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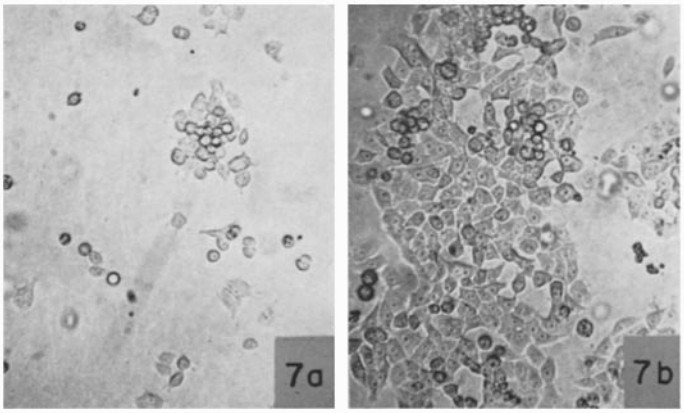

FIGURE 7 Effect of alpha-one protein on replicate cultures of unadapted human heart cells placed in chemically defined medium: *a*, 24-hr-old culture, no alpha-one protein added; *b*, 24-hr-old culture, 8 μg/ml of alpha-one protein added to the medium.

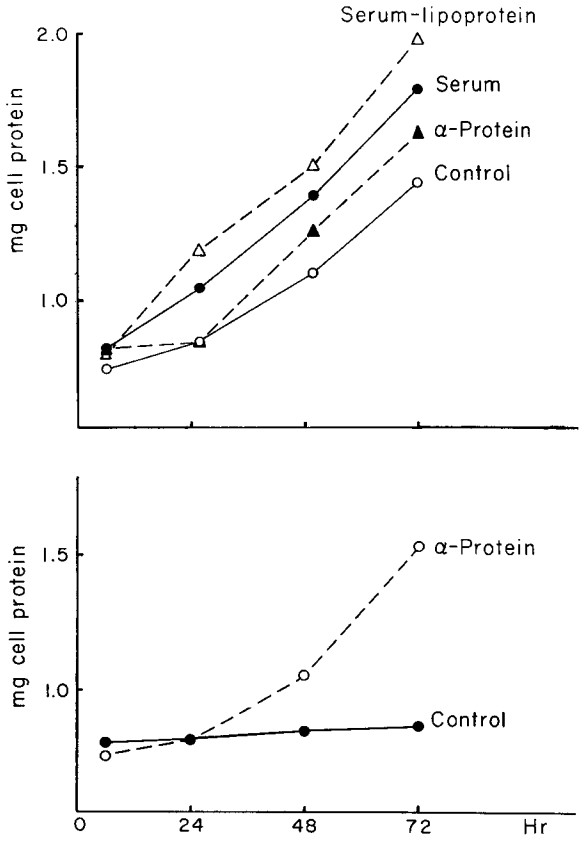

FIGURE 8 Growth of adapted and unadapted conjunctiva cells. Triplicate experiments were conducted with replicate cultures. Growth is reported as the average increase in total cell protein. The top set of four curves represents the growth of adapted cells in: medium containing 20% processed serum, lipoproteins removed; medium containing 20% processed serum lipoproteins, not removed; medium containing 8 μg/ml of alpha protein; and medium chemically defined with nothing added. The bottom set of two curves represents growth of unadapted cells in medium containing 8 μg of alpha protein and in medium chemically defined with nothing added.

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The Response of Tissue Cells to the Alpha-One Protein

The response to the alpha-one protein of strains of HeLa (Gey), conjunctiva (Chang), and human heart (Girardi) cells, which had been previously adapted to and were growing in defined medium, is shown in Fig. 6. The control cultures without added alpha-one protein are shown in Fig. 6 *a*, *c*, and *e*; HeLa cells, conjunctiva and human heart, respectively. Cultures of these cells supplemented with 2 μg of alpha-one protein per ml of medium are shown, respectively, in Fig. 6 *b*, *d* and *f*. The spreading and growth of the cell due to the alpha-one protein are apparent. Further tests showed that, in the case of the conjunctiva cells, as little as 0.4 μg of alpha-one protein per ml of medium initiated a like response. The other two cell lines required slightly more, between 0.8 and 1.0 μg of alpha-one protein.

The effect of alpha-one protein on unadapted cells, when first transferred to the medium, was also determined. With 8 μg per ml of medium, growth commenced within 24 hr after the cells were transferred from the stock medium containing serum to the defined medium containing no serum. Fig. 7 *a* shows a culture of unadapted human heart cells 24 hr after removal of the serum. Fig. 7 *b* shows a culture to which alpha-one protein was added in place of serum. The survival and growth of cells in the medium supplemented with alpha-one protein were high compared to those of the control. At the end of 10 consecutive subcultures, alpha-one protein was removed from the medium. Growth promptly stopped and was not resumed unless the alpha-one protein was returned to the medium. If the alpha-one protein was not returned, the cells continued through the customary 90–120 day period of adaptation to the defined medium.

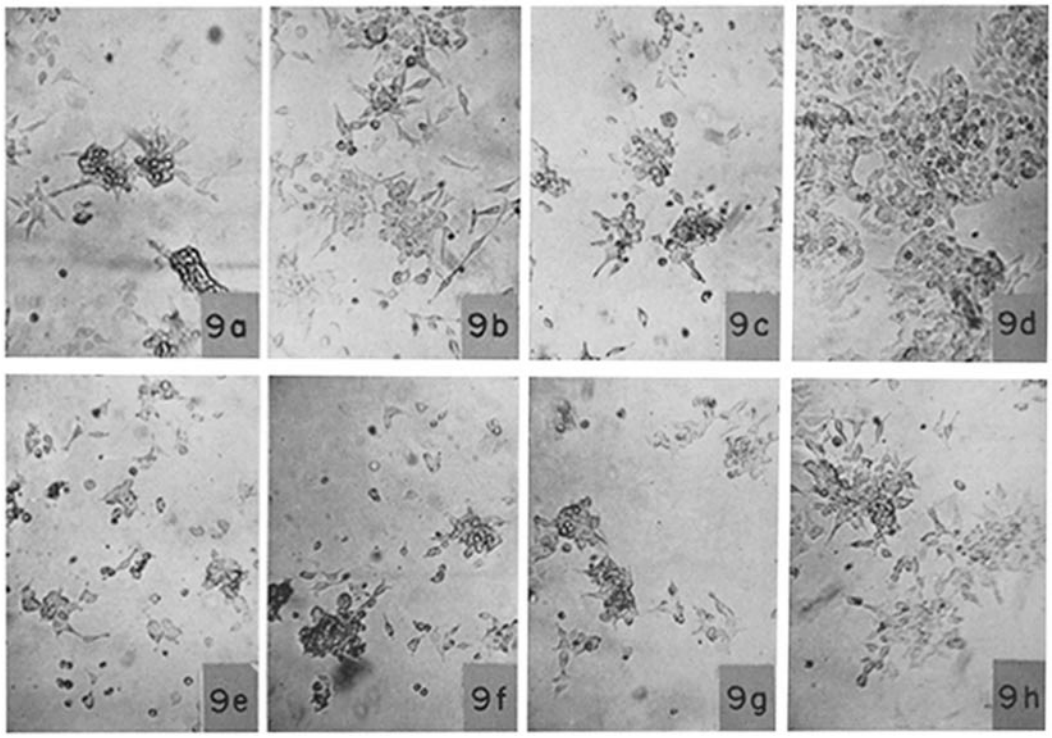

FIGURE 9 Replicate monolayer cultures of unadapted conjunctiva cells. Cultures incubated for 24 hr: *a*, Chemically defined Medium A2 + APG; *b*, 0.01 mg insulin per ml; *c*, 3×10^{-10} M linoleic acid per ml; *d*, 2 μg alpha-one protein per ml; *e*, 1×10^{-9} M putrescine per ml; *f*, 1×10^{-9} M plus 3×10^{-10} M linoleic acid per ml; *g*, 100 μg carbamyl phosphate per ml; *h*, 100 μg carbamyl phosphate plus 0.01 mg insulin per ml.

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Fig. 8 shows the actual growth rates of conjunctiva cells under the above conditions. The top set of curves were obtained with fully "adapted" cells. The highest rate was obtained in medium supplemented with 20% processed human serum (see Materials and Methods) from which the lipoproteins had been removed by ultracentrifugation. Progressively decreasing rates were obtained with processed human serum which still contained the lipoproteins, the alpha-protein supplemented medium, and the chemically defined medium without any supplement.

The bottom set of growth curves, shown in Fig. 8, were obtained with "unadapted" cells. Whereas these cells grew very slowly in the defined medium, the growth rate of a replicate set was stimulated by the alpha protein.

A number of substances of known composition which had been reported stimulatory to cell cultures were tested to determine whether they could induce an effect similar to that of the alpha-one protein. Insulin (8, 23), linoleic acid (11), putrescine (11), and carbamyl phosphate (23) were tested. None of these materials induced the immediate growth of unadapted conjunctiva cells (Fig. 9). It was noted that insulin improved the appearance of the cells. These materials were not examined for their effect on the period of adaptation.

DISCUSSION

The biologically active protein, which has been subject of the above experiments, has been designated as an alpha-one protein, a name assigned in conformity with the customary designation of proteins appearing in electrophoretic patterns as bands in the area of albumin. The marked trailing of this material on electrophoretic migrations appears to be characteristic. At least to date, all efforts to eliminate a trailing component have failed. This, coupled with the reduction of trailing in the presence of cholic acid and followed by the reappearance of trailing on elimination of the cholic acid, suggests that trailing is indeed characteristic and that present preparations are probably homogeneous.

The association of this alpha-one protein with beta lipoprotein in the glass bead column and in the solution, infranatant to the fractions of density up to 1.22 obtained by density gradient centrifugation, suggest that interaction between these two high molecular weight species is characteristic in the native state. When deprived of the beta lipoprotein, the alpha-one protein was shown to hold small molecular weight ninhydrin-positive substances very tenaciously. These observations suggest that the binding power of this protein for certain substances is considerable. In view of these demonstrable adsorptive properties of the alpha-one protein, it is reasonable to consider that it has its effect on the growth and spreading of cells through attachment to the external cell wall surface (12, 9). However, even granting this possibility, the mechanism by which it exerts its effect is unknown.

The method for preparation of the alpha-one protein from human serum cannot be successfully applied to the preparation of fetuin. Indeed, electrophoretic analyses show that the alpha-one protein and fetuin are different. The only similarity observed between alpha-one protein and fetuin was in carbohydrate moieties and in the biological response of cells. One commercially available fetuin gave a low biological response relative to that of the alpha-one protein, and two others were quite inactive. If fetuin preparations of Fisher, Puck and Sato, (7) contained trace amounts of alpha-one protein, it could not be detected by electrophoretic analysis. From these results it may be concluded that the alpha-one protein derived from human serum is not identical with fetuin. It is also unlikely that the activity of fetuin is due to contamination with the alpha-one protein. The inability to prepare active alpha-one protein from adult bovine, or equine serum does not prove the absence of an alpha-one protein from embryonic calf serum at a particular stage of development.

That the alpha-one protein is not separated from either adult bovine or equine sera may be because these sera lack lipid-bearing material with adsorptive properties comparable to those of the human beta lipoprotein fraction. It has been shown elsewhere (15) that in the bovine species a large alpha type lipoprotein, absent at birth, appears during the first 7 days after birth. Thus when bovine or equine sera are processed for biologically active material, which may not be the same as the human alpha-one protein, it is not adsorbed but discharged from the microbead column in the first few fractions, along with other serum proteins including this large lipoprotein fraction.

Lieberman and Ove (18) by using a DEAE cellulose column found that they could prepare a growth-stimulating substance from bovine,

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equine, and human sera. Whereas this substance was initially effective at a concentration of 1 μg per ml of medium, it did not support growth indefinitely. Such would be the expected result either if the substance they isolated were not identical with the alpha-one protein or if the defined base medium were inadequate (14). Their report lacks any data on starch or acrylamide gel electrophoresis by which to compare the identity and purity of the two substances.

None of the small molecular weight compounds used by Ham (11) or Michl (23) initiated the immediate growth of unadapted cells, and thus did not duplicate the effects produced by the alpha-one protein. Michl (23) has degraded his calf serum fractions, producing a smaller molecular weight substance with cell flattening ability in which 10 different amino acids were identified. As of now, the alpha-one protein derived from human serum has not been successfully degraded without losing the biological activity defined in this report.

The rate of growth of "unadapted" conjunctiva cells in the presence of the alpha protein, as compared to their rate of growth in the chemically defined medium, is significant. Apparently the process of adaptation involves an alteration that does not occur if alpha protein is present in the medium. That the effect induced by adding the alpha protein is not permanently impressed on the cell is concluded from the observation that these particular cultures stop growing if the alpha protein is removed.

Conjunctiva cells that have been fully "adapted" to the chemically defined medium, though their rate of growth is less noticeably stimulated, spread to form a sheet in the presence of the alpha protein. The stimulation of the growth rate of "adapted" conjunctiva by serum and serum from which the classical lipoproteins (3) have been removed shows that there are other growth promoting substances in serum in addition to the alpha protein. There is ample evidence in the literature that such substances exist (18, 14, 32, 13).

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