SERUM FRACTIONATION AND THE EFFECTS OF BOVINE SERUM FRACTIONS ON HUMAN CELLS GROWN IN A CHEMICALLY DEFINED MEDIUM

RICHARD HOLMES, Ph.D., and SONYA W. WOLFE

From the Alfred I. du Pont Institute of the Nemours Foundation, Wilmington, Delaware

ABSTRACT

Serum has been fractionated by curtain electrophoresis using carboxymethyl cellulose dissolved in sodium bicarbonate electrolyte. Various fractions were produced from bovine serum and added to replicate cultures of Chang's endoepithelial cells and HeLa cells grown in a chemically defined medium. The effects of each of the various fractions on the appearance of the cultures and on cell multiplication were studied. Three different fractions were obtained and two were subjected to further purification. One fraction associated with albumin promoted survival, attachment, and flattening as well as cell multiplication. A second fraction associated with the alpha globulins promoted survival and multiplication of some cells. A third fraction caused cells to aggregate and form free floating clumps. An adequate chemically defined medium for continuous growth of human cells was used throughout the study. The response of cells to alterations in their environment which simulated some of the effects produced by serum fractions is described.

INTRODUCTION

Dialyzed serum is known to contain substances which promote attachment and flattening of tissue cells on glass surfaces (1–3), and which increase the rate of multiplication of cells grown *in vitro* (4–6). To study these reactions, recent developments in curtain electrophoresis for fractionating serum (7) were used in conjunction with a chemically defined medium for growing human cells in continuous culture (8). Fractions were prepared from bovine serum by paper curtain electrophoresis, using suitable electrolytes containing carboxymethyl cellulose. These serum fractions were then examined for their effects on human tissue cells cultured in a chemically defined medium.

Attachment to glass has been attributed to a correct relationship between the surface charge of the cells and that of the glass (9, 10). Treatment of the glass surface (9) or the addition of serum (10) or serum fractions (1–3) has been shown to produce a suitable relationship. Cell multiplication was promoted under these conditions. Under slightly different conditions of culture, cell multiplication was achieved by adding digests either of serum (11) or of other proteins (8, 12, 13). Stimulation of multiplication by any specific peptide, with the possible exception of insulin (14), has not been demonstrated. Multiplication invoked by peptide-like substances does not require that the cells become attached to a glass surface (15). However, some cells multiplying under chemically defined conditions attach themselves to glass in time, and this time may be shortened by adding peptide-like substances (8).

Of the several chemically defined media (16) employed in past studies of the effects of protein or protein digests on cells, none have been reported to be adequate for continuous multiplication of

any line of human cells. The first chemically defined medium fulfilling these requirements for Chang's endoepithelial-like cells from human liver was described by one of us (8). Currently, Bakken *et al.* have reported on the establishment of a line of human epithelial cells in their defined medium (17). Rappaport has also found that if glass surfaces were appropriately treated, continuous multiplication of HeLa cells was achieved using a relatively simple chemically defined medium (9).

METHODS

Use of Terms: "Adapted" refers to cells which only after a considerable lapse of time were able to grow in chemically defined medium A or A2 at a rate approaching that attained in the presence of serum. In previous experiments this period of time varied between 90 and 120 days (8). Changes in the appearance of cultures that can occur during this period have been described by Bakken *et al.* (17).

"Unadapted" describes cultures of cells which possess the potential of being able to "adapt" to defined medium A or A2 but which at the time of their use, immediately after removal of serum supplements, would multiply either not at all or only very slowly, and would survive if maintained through the adaptation period.

"Spreading." Individual cells initially in the form of spheres may be attracted to glass surfaces and become flattened, as has been illustrated by Weiss (10). The area of glass covered by individual spreading cells would seem to depend on the size of the cell as well as on the forces of attraction at the interface. The degree of spreading (see Results) depends on the characteristics of the cell line and its response to the environment. Whether this reaction influences formation of a confluent sheet composed of a larger number of cells is not considered.

"Clumping" refers to cells which in response to their environment assume a spherical shape and then adhere together to form aggregates composed of a variable number of cells. The effects of "clumping" on cell multiplication are not considered. The possibility of a relationship between the forces involved in "spreading" and in "clumping" is not considered.

Preparation of Medium A2: The composition of medium A2 is shown in Table I. A series of solutions numbered 1 to 12, corresponding to the numbers of the groups shown in the table, were prepared. For convenience, the solutions were divided into portions sufficient for 100 ml of medium. Solutions 1, 3, 4, and 7 to 9 were frozen prior to storage; later all the solutions were combined with the materials of group 13 as required to form the complete medium. The final volume was adjusted, as indicated by the experiment, to 100 ml or less with water and sterilized by cold filtration through Morton fritted glass filters. Conductivity grade water was used throughout.

Further details for preparation of the various solutions may be obtained from the literature (18–21).

Handling of Cultures: Monolayer cultures of strains I and II of Chang's human liver epithelial cells (7), HeLa cells, and human intestinal cells were carried in cotton-plugged T30 flasks. Uniformity of the gaseous atmosphere in any test series was assured by placing the culture flasks in desiccators filled with air containing 3 per cent by volume of carbon dioxide. For some experiments, cells of the parent culture which had not previously been conditioned to grow in defined medium A2 were submitted to one or two consecutive subcultures in medium A2 to remove serum. Usually after two subcultures the rate of multiplication was reduced (see Results). Cells in monolayer cultures were suspended with the aid of a silicone rubber scraper. The number of cells in an inoculum was determined 2 hours after it was placed in a T30 flask by counting four different fields under the microscope. Final comparison of the number and appearance of cells was by means of photographs of the replicate culture series prepared from the same cell suspension. Replicate cultures produced under these conditions did not vary more than ±10 per cent in numbers, provided cells were counted before they formed confluent sheets and the experimental conditions did not cause excessive clumping. Despite these precautions, conditions were selected wherever possible that would produce a ten-fold or greater increase in cell numbers over that of the controls.

Serum Fractionation, Analyses, and Preparation of Fractions: Curtain or paper strip electrophoresis with or without use of carboxymethyl cellulose 70L[1] was carried out in a manner previously described (6). Horizontal starch gel analyses were carried out by inserting the samples into slots in the gel in a manner recommended by Smithies (22).

Dialyses were carried out at 4°C in glass-stoppered graduated cylinders which were rocked at a rate of three oscillations per minute. The volume ratio outside the sac to that inside the sac was 10 to 1. Unless otherwise stated, outside solutions were changed three times over a period of 24 hours. Cellulose dialyzer tubing (Arthur H. Thomas Co., Philadelphia, cat. no. 4465-A2) was pre-washed in running distilled water for 8 hours. No toxic effect on cells was observed from the use of washed dialyzer tubing.

[1] 70L designates grade of carboxymethyl cellulose (supplied by Hercules Powder Co., Wilmington, Delaware) of 0.7 degree of carboxymethyl substitution per anhydroglucose unit, 3.0 being theoretical maximum; L designates low viscosity with average molecular weight 35,000.

TABLE I

Composition of Medium A2

	mg/liter		*mg/liter*
Group 1		*Group 5*	
NaCl	6000.0	Hypoxanthine	1.0
KCl	400.0	Xanthine	1.0
$MgSO_4 \cdot 7H_2O$	200.0	*Group 6*	
$CaCl_2 \cdot 2H_2O$	140.0	Niacin	0.025
Na_2HPO_4	60.0	Niacinamide	1.0
KH_2PO_4	60.0	Pyridoxine·HCl	0.025
Na acetate·$3H_2O$	83.0	Pyridoxal	1.0
Phenol red	20.0	Thiamine·HCl	1.0
Glycine	100.0	Riboflavine	1.0
L-Arginine·HCl	140.0	D-Pantothenate (Ca)	0.01
L-Histidine·HCl·H_2O	40.0	*i*-Inositol	11.0
L-Lysine·HCl	140.0	*P*-Amino-benzoic acid	0.05
L-Tryptophan	20.0	Choline chloride	2.0
L-Phenylalanine	50.0	α-Tocopherol phosphate (Na)	0.01
L-Methionine	30.0	Folic acid	1.0
L-Serine	50.0	Biotin	1.0
L-Threonine	60.0	Thioctic acid	1.5
L-Leucine	120.0	Adenosine-5 -phosphate	0.02
L-Isoleucine	40.0	D-Ribose	0.2
L-Valine	50.0	D-2-Deoxyribose	0.2
L-Glutamic acid·H_2O	150.0	*Group 7*	
L-Aspartic acid	60.0	Diphosphopyridine nucleotide	7.0
L-Alanine	50.0	Triphosphopyridine nucleotide	1.0
L-Proline	80.0	Coenzyme A	2.5
L-Hydroxyproline	20.0	Cocarboxylase	1.0
Deoxycytidine·HCl	10.0	Flavine-adenine dinucleotide	1.0
Deoxyadenosine	10.0	Uridine triphosphate (Na)	1.0
Deoxyguanosine	10.0	Vitamin B12	2.5
Thymidine	10.0	*Group 8*	
Group 2		Adenosine triphosphate (Na)	1.08
L-Tyrosine	40.0	*Group 9*	
L-Cystine	20.0	Cholesterol	0.2
Group 3		Menadione	0.01
L-Cysteine·HCl	75.0	Calciferol	0.1
Glutathione (reduced)	15.0	Vitamin A	0.1
Ascorbic acid	17.5	β-Estradiol	0.1
Group 4		Tween 80	5.0
L-α-Amino-*n*-butyric acid	2.5	*Group 10*	
L-Asparagine	4.1	Cholic acid	20.0
L-Ornithine·HCl	3.5	*Group 11*	
L-Taurine	2.0	Carboxymethyl cellulose 70L	250.0
L-Homocystine	1.0	*Group 12*	
L-Ergothionine·HCl·H_2O	1.0	$FeNH_4(SO_4)_2 \cdot 12H_2O$	0.86
D-Glucosamine·HCl	3.2	*Group 13*	
Glucuronolactone	1.0	$NaHCO_3$	1400.0
Na glucuronate	1.0	Dextrose	1000.0
Glucose-1-phosphate (K)	1.0	Streptomycin (or dihydro) (SO_4)	100.0
Citric acid	1.0	Penicillin G (Na)	5.0
5-Methyl-deoxycytidylic acid	0.2	L-Glutamine	400.0
		Fructose-1,6-phosphate (Mg)	100.0

Fractions produced by curtain electrophoresis of 100 ml of dialyzed serum were dialyzed against water to remove excess amounts of electrolyte. The volume of each fraction remaining within the dialysis sac was adjusted to 25 ml either by pervaporization at 4°C or by the addition of water. The fractions were cold-sterilized by filtration. For testing, 1 volume of each fraction was added to 4 volumes of medium A2 which had been made up to only 80 per cent of its final volume. In the controls, water replaced the curtain fraction. All cultures were inoculated with 0.5 ml of cells suspended in medium A2.

RESULTS

Medium A and Its Modification to Give Medium A2: Commencing with medium 858 as described by Healy, Fisher, and Parker (19), medium A was developed to grow human cells under chemically defined conditions. It was not until medium A was completed that two strains were obtained from cultures of Chang's human liver endoepithelial-like cells (8). These two strains have been in culture for over two years. No serious attempts have made to remove non-essential components from the medium. Furthermore, a chemically defined medium suitable for growth of a number of cell lines may be expected to contain some ingredients which are not essential for certain individual cell lines (see below and reference 23).

Medium A incorporates L-homocystine, ergothionine HCl, glucose-1-phosphate, citric acid, thioctic acid, β-estradiol, and cholic acid. The effect of these substances was tested over a range of concentrations. For this purpose "unadapted" Chang's cells were subcultured once in a modified version of medium A which incorporated all improvements except the materials listed above. Replicate cultures were prepared from these cells using each of the materials in different concentrations. That concentration in which either the cells adhered better to the glass or the multiplication rate was improved over that of the controls was selected for incorporation into the medium. When strain II was established in medium A (8), the effect of increasing the concentration of all ingredients listed above at the same time, exclusive of β-estradiol and cholic acid, was retested. The multiplication rate of strain II cells was not increased. However, removal of these materials over a period of two subcultures caused a progressive decrease in multiplication rate.

Beta-estradiol was incorporated in medium A on the basis of an observation made by Talalay *et al.* (24). A suitable concentration was selected in a manner similar to that already described.

Cholic acid was added in the belief that it might aid the transport of lipid-like materials (25). When cholic acid was removed from medium A, the multiplication rate of strain II cells was reduced. The interval between subcultures was increased from 12 to 30 days. Increasing the cholic acid concentration by more than a factor of 2 was deleterious to the cells. Desoxycholic acid would not replace cholic acid.

The beneficial action on cells of adding carboxymethyl cellulose to the medium has been reported (26). Some of its properties have been described (7).

To extend the usefulness of medium A in the present study, some work was done on the effect on cells of altering the concentration of some of the other components. For example, the multiplication rate of HeLa cells in medium A remained quite slow and the cells were poorly attached to the glass. It was found that by doubling the concentration of the amino acids and by increasing the amounts of some of the vitamins, as suggested by Eagle (4), within 90 days HeLa cells grew well in medium A2. These alterations in the medium also permitted strain II cells to be subcultured every 8 days instead of every 12 days. It has been shown since, through the kindness of Dr. Paul B. Hamilton of the Alfred I. du Pont Institute, who conducted the analyses, that cultures of strains I and II and HeLa cells used certain amino acids at different rates. The concentrations of arginine, glutamine, and serine in the medium were rapidly lowered in all three cases. Strains I and II caused a decrease of aspartic acid, whereas the growth of HeLa cells was accompanied by an increase in aspartic acid. Further changes in the total amino acid content of the medium as opposed to altering the concentration of individual amino acids, did not improve the appearance of the cultures. The appearance of cultures of HeLa, Strain I and II cells grown in medium A_2 is illustrated in Figs. 2 A, C, and D.

Characteristics of Growth of Cells in Medium A2 and Effects of Added Supplements: The multiplication rate of HeLa cells "adapted" to grow in medium A2 was unaffected, within the limits of experimental error (±10 per cent), by the addition of 20 per cent adult bovine serum, provided comparisons were made on cultures started with

not less than 10^4 cells per ml. Fig. 1 compares the multiplication rates obtained under these conditions. Cultures started with less than 10^4 cells per ml did not multiply as rapidly in the absence of serum. In either case individual cells from cultures containing serum covered a larger area of the glass surface of the culture flask. Differences in the appearance of these cells due to the presence of serum are shown in Fig. 2 A and B. The cells represented in Fig. 2 A were from replicate cultures containing no serum; Fig. 2 B represents cells from cultures containing 20 per cent adult bovine serum. It can be seen that in the absence of serum HeLa cells stick and spread to a limited extent on the glass surface, whereas the addition of serum greatly accentuates the spreading of individual cells. This spreading effect caused by serum has been reported by other investigators. It was concluded from these results that factors in serum which promoted spreading of HeLa cells were not absolutely essential for multiplication of "adapted" cells. These factors, nevertheless, promoted cell survival and multiplication if the number of cells present in the inoculum was less than 10^4 per ml.

The addition of protamines or histones to cultures of "adapted" HeLa cells grown in medium A2 induced somewhat greater spreading of individual cells on glass surfaces. In early stages of culture these cells appeared to adhere to one another with less tendency to separate. Either salmine sulfate or calf thymus histone (Nutritional Biochemicals Corp., Cleveland) in low concentrations promoted this reaction. With increased amounts of either material the cells ceased to adhere and spread on glass, but combined to form free floating clumps of rounded cells. The appearance of 48-hour-old cultures of HeLa cells grown in medium A2 containing 0.0, 0.0001, 0.001, and 0.01 per cent salmine sulfate are respectively illustrated in Figs. 3 A, B, C, and D. Both salmine sulfate and calf thymus histone, known to be complex materials, upon electrophoresis either on starch or paper at pH 8.4 or 8.6 showed the presence of numerous components which remained at the origin or moved toward the negative electrode. Comparison of Fig. 2 B and Fig. 3 C shows that while HeLa cells adhered and spread in the presence of 0.001 per cent salmine sulfate, they still did not spread as well as in serum.

Strain I of Chang's cells grown continuously in

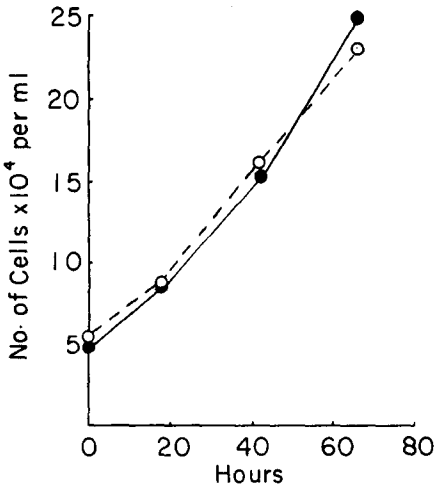

FIGURE 1

Comparison of the rate of multiplication of cultures of "adapted" HeLa cells inoculated with 5×10^4 cells per ml. Solid line represents rate of multiplication in medium A2; dashed line, that in medium A2 to which 20 per cent bovine serum has been added.

medium A2 containing 0.0001 per cent disodium versenate became very sensitive to the presence of factors in serum which cause cells to spread. Strain II or HeLa cells appeared to be unaffected by 0.0001 per cent versenate. To prevent the latter cells from becoming attached to glass surfaces required between 0.001 and 0.01 per cent versenate. Continuous culture of strain I, as described, produced cells which were round and mostly free floating. Though most of the cells were free floating, they continued to multiply and were subcultured every 3 to 4 days provided a high density of cells was maintained in the inoculum (greater than 10^5 cells per ml). Care was observed upon renewal of the medium to avoid removal of cells from the culture. The characteristic appearance of these cells is shown in Fig. 8 A.

Effect of Bovine and Calf Serum Fractions Prepared by Electrophoresis at pH 8.4: To obtain more information on the nature of the serum components that promote survival and multiplication and affect the appearance of tissue cells, various sera were fractionated by paper curtain electrophoresis using sodium bicarbonate electrolyte (see Methods). Bovine sera produced from a cow and her male calf which materially differed in composition have been described (7). Chang's endo-

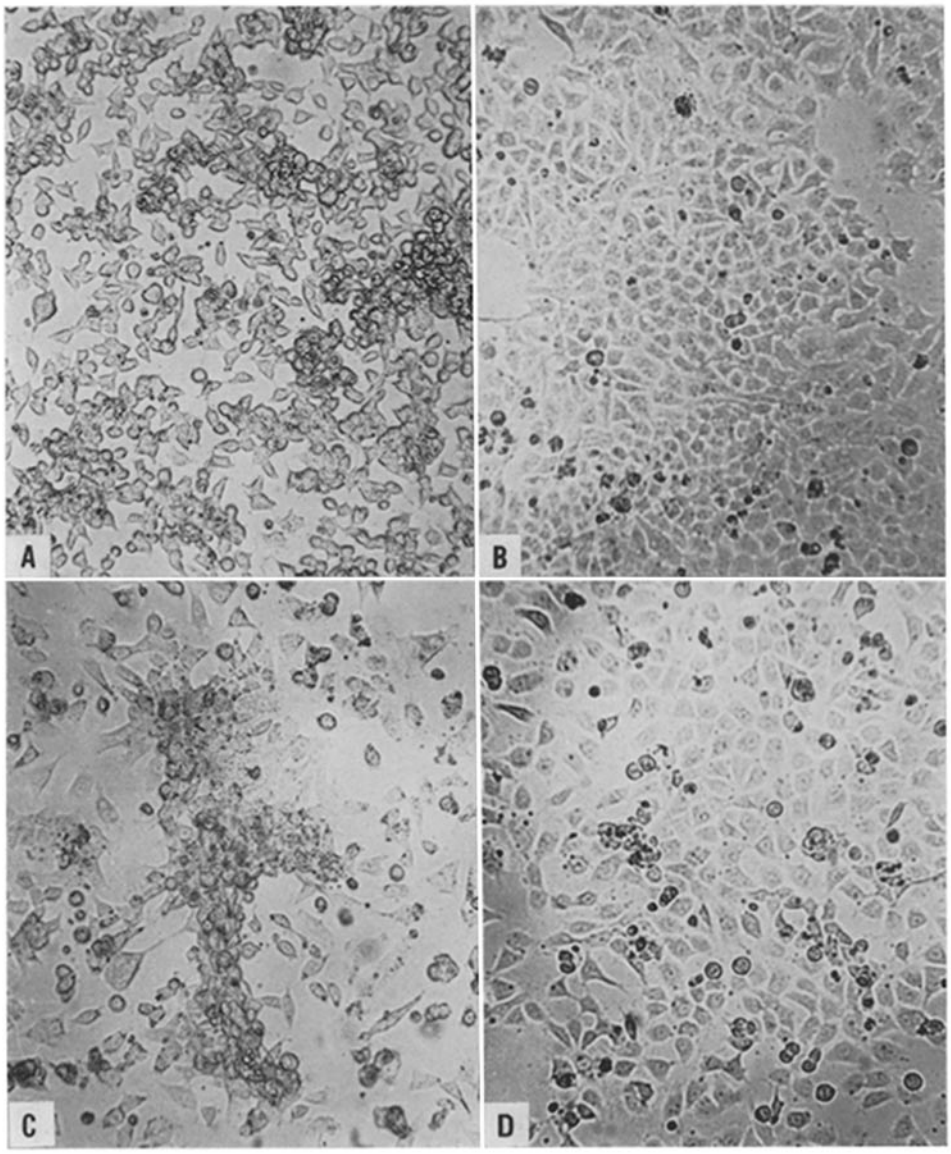

FIGURE 2

A, a 72-hour-old culture of "adapted" HeLa cells grown in medium A2. B, a replicate culture to which 20 per cent bovine serum has been added. C, a 20-hour-old culture of strain I of "adapted" Chang's cells grown in medium A2 (at time of writing in 52nd subculture). D, a 20-hour-old culture of strain II of "adapted" Chang's cells grown in medium A2 (presently in 77th subculture). × 110.

epithelial cells which had not been adapted to medium A were selected for these experiments. It was expected on the basis of previous observations (8) that after two subcultures in medium A the ability of these cells to survive subculturing would be poor and that their multiplication rate in the defined medium would be slow. It was hoped that in order to survive and multiply immediately these cells would require some portion of the dialyzed serum. Replicate cultures of these cells along with each of the fractions obtained by curtain electrophoresis of the two different types of sera were prepared. The cells in each culture were counted (see Methods) at the end of 48

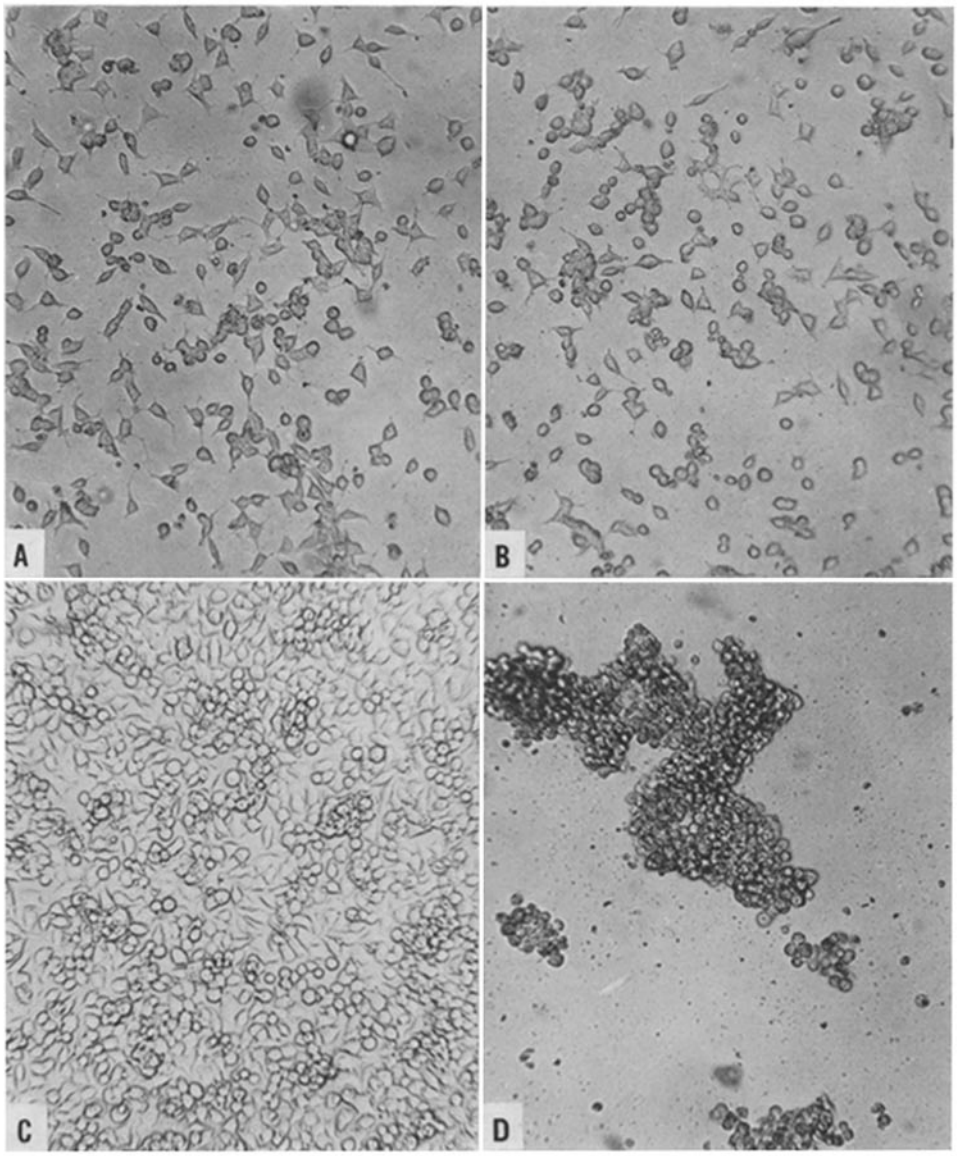

FIGURE 3

Illustration of the appearance of 24-hour-old replicate cultures of "adapted" HeLa cells containing different amounts of protamine: A, 0.0 per cent salmine sulphate; B, 0.0001 per cent; C, 0.001 per cent; D, 0.01 per cent. × 110.

hours. The plots of the results obtained with corresponding fractions are compared in Fig. 4. These experiments were repeated with two different sets of test animals and the results were similar. We concluded that within each replicate set differences in the activity of each component, as presented in Fig. 4, had significance. However, since serum components were adsorbed on paper and might also be dissociated during electrophoresis, greater significance was attached to the observation that electrophoresis separated either type of serum into two components. The first peak (on the left in Fig. 4) represents a fraction that promoted survival and growth and extensive

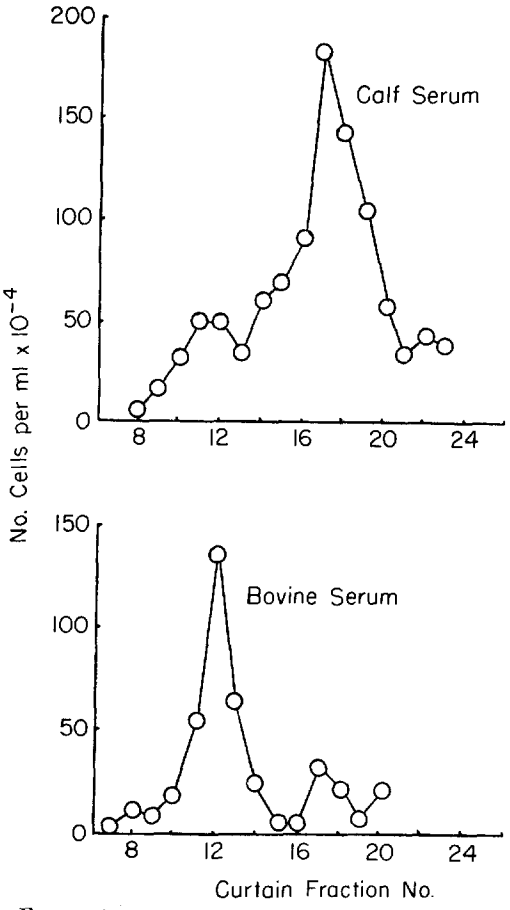

FIGURE 4

A comparative plot of the number of cells present 48 hours after the addition of bovine or calf serum fractions to replicate cultures inoculated with "unadapted" Chang's cells. The two different sera compared were fractionated by curtain electrophoretic separations conducted under identical conditions.

spreading of individual Chang's cells. The second peak represents a fraction which promoted cell survival and growth but only limited spreading of individual cells. Response of the cells to the first fraction, as shown by their attachment and spreading on glass, was quite rapid. In certain instances (see below) it occurred within 2 hours. Response of the cells to the second fraction was much slower. Effects on growth produced by the latter were observed within 24 hours but were more pronounced after 48 hours of incubation. The first fraction, characterized by its ability to promote rapid spreading of individual cells, will be referred to as component 1. The second fraction, characterized by its ability to promote cell multiplication with limited spreading of individual cells, will be referred to as component 2.

Precipitation and Recovery of Components 1 and 2 from Serum: Substances which produced a response in "unadapted" HeLa cells similar to the response produced by component 2 were found in precipitates obtained from adult bovine sera adjusted to pH 5.0 with acetic acid. The amount of precipitate was increased when the adjusted sera were dialyzed against water. The precipitated material which was soluble either in 0.1 M acetic acid adjusted to pH 4.0 with sodium hydroxide or in 0.1 per cent sodium bicarbonate solution continued to promote cell multiplication after the precipitation had been repeated three times. This material, when added to medium A2 as a supplement, promoted the multiplication of "unadapted" HeLa cells for five consecutive subcultures without difficulty, during which time control cultures had not multiplied sufficiently to permit one subculture. Some precipitates were more effective than others, and for this reason further purification was attempted. The final precipitate, dissolved in sodium bicarbonate solution, when subjected to paper electrophoresis was strongly adsorbed on the paper. This adsorption was prevented by adding carboxymethyl cellulose to the buffer (7). Three consecutive curtain separations were carried out using 0.026 M sodium bicarbonate adjusted to pH 8.4 with sodium hydroxide and containing 0.01 per cent carboxymethyl cellulose. The course of purification of material similar in action to component 2 is illustrated in Fig. 5 by separations 1, 2, and 3. The three curtains shown in Fig. 5 were obtained at the end of each separation. Each curtain was dried and developed at 100°C for 5 minutes after the paper had been wetted with a 0.1 per cent solution of ninhydrin in acetone containing 2 per cent acetic acid. Portions of the various fractions produced by each separation were tested for their effects on replicate cultures of "unadapted" HeLa cells. The fraction most effective in promoting cell multiplication was separated further on the next curtain. The final product from the third curtain (Fig. 5, separation 3) continued to be strongly adsorbed on paper not treated with carboxymethyl cellulose. Its electrophoretic mobility was the same as that of component 2. It was effective at a concentration of 0.006 per cent.

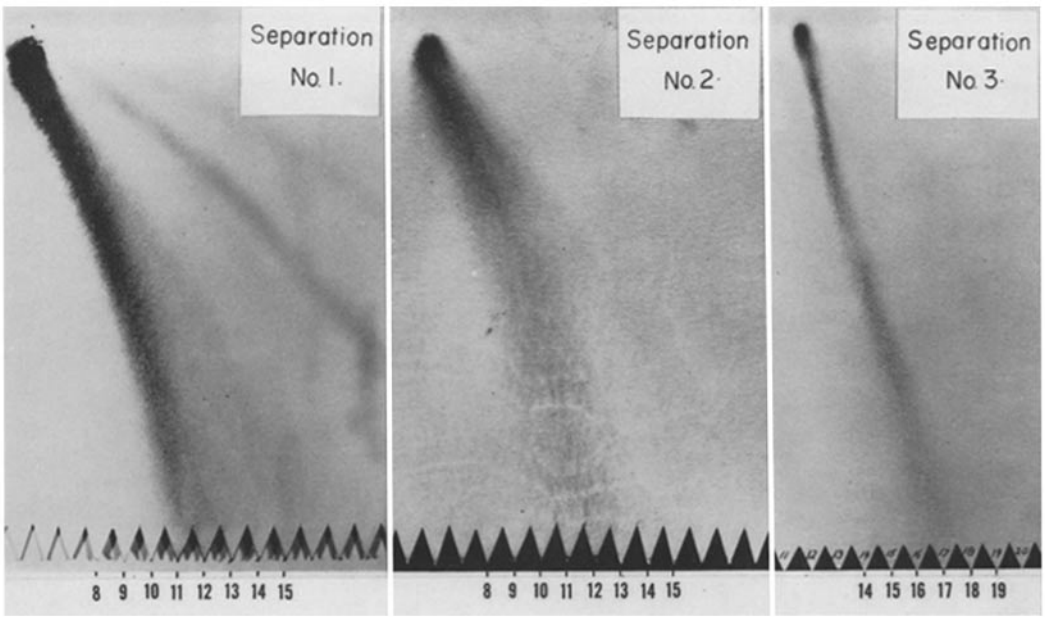

FIGURE 5

Illustration of the progressive separation of component 2 (see text) from dissolved serum precipitate by curtain electrophoresis. The curtain separation procedures utilized 0.01 per cent carboxymethyl cellulose 70L dissolved in 0.026 M sodium bicarbonate electrolyte adjusted to pH 8.4. Numbers which appear at the bottom of each separation are used to identify samples obtained from respective drip points of a 32 point curtain.

No material with properties of component 1 was recovered by this procedure.

The material from serum produced by repeated precipitation at pH 5.0, as described above, contained a number of substances which were separated by electrophoresis (Fig. 5, separation 1). These substances, other than component 2, which was obtained from this curtain at drip point 11, were of significant interest, and the replicate culture series evaluating them is reproduced in Fig. 6. Fig. 6 shows that material obtained from drip points 14 and 15 caused cell clumping. This clumping reaction outwardly appeared to be similar to the response invoked by an excess of protamine sulfate, illustrated in Fig. 3 D. Whether this material would promote cell attachment when used at a lower concentration was not determined.

As a result of information obtained from the above fractionation procedure, a simplified separation was devised which produced good yields of two substances with properties of either component 1 or 2.

Raising the pH of the electrolyte used in the curtain separations improved definition of the separating fractions without causing deterioration in the response of cells to the fractions. Either sodium bicarbonate 0.026 M or boric acid 0.05 M, both adjusted to pH 9.0 with sodium hydroxide and supplemented with the addition of 0.01 per cent carboxymethyl cellulose, was used to separate adult bovine sera. Prior to separation, the sera were equilibrated by dialysis against the chosen electrolyte. Fractions produced by curtains operated with sodium bicarbonate were very active in producing cellular responses of the type already described. Fractions from curtains operated with sodium borate were almost as satisfactory. While it was our practice to remove the excess of electrolyte from all fractions by dialysis prior to their incorporation in the test medium (see Methods), this was more necessary in the case of borate than of bicarbonate. A determination of the effects on tissue cultures of borate ions showed increased toxicity above 10^{-4} M. Paper strip analyses, using barbital buffer pH 8.6 con-

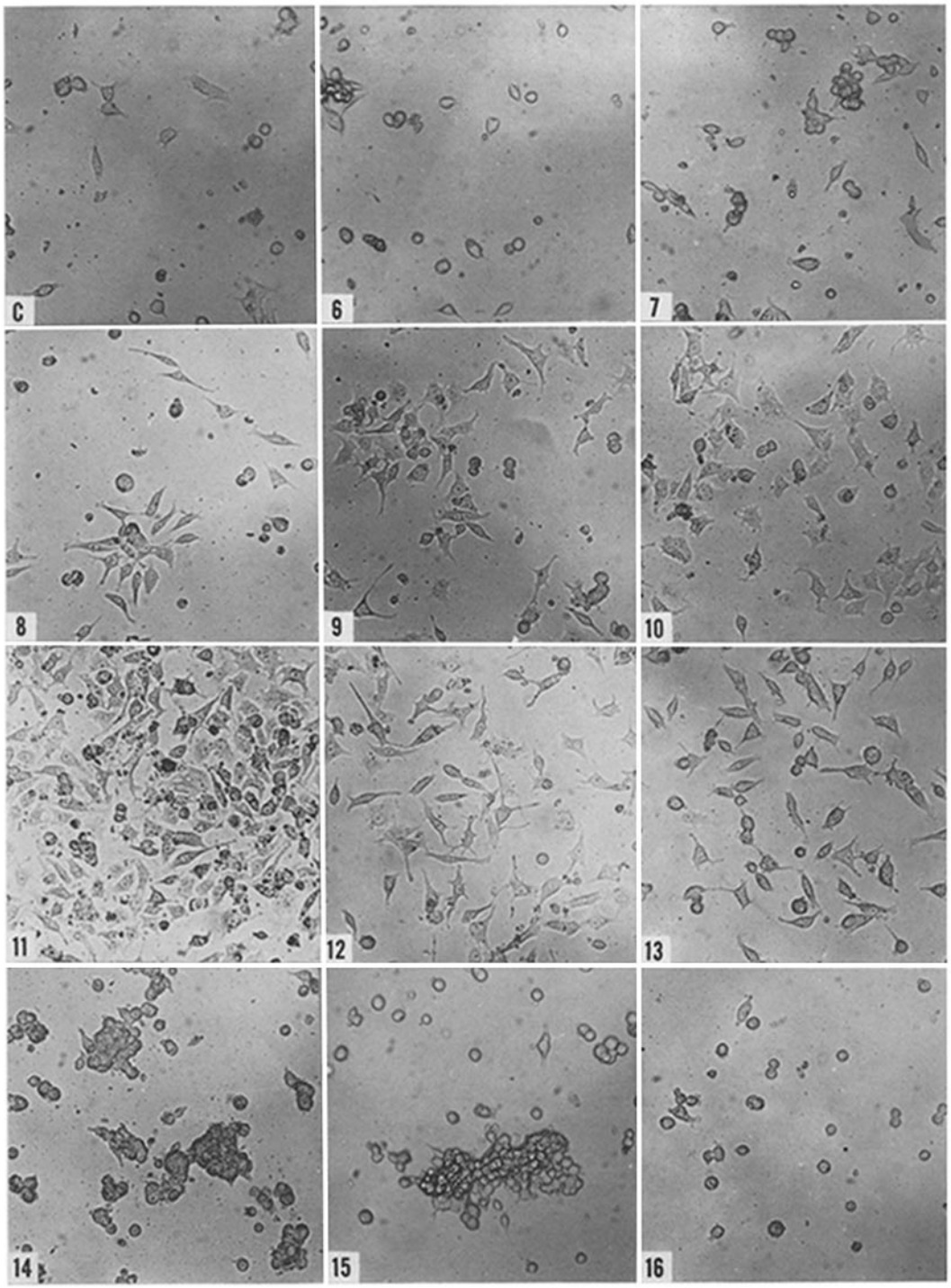

FIGURE 6

Illustration of the appearance of each of the 24-hour-old replicate cultures of "unadapted" HeLa cells prepared with serum fractions obtained from the first curtain separation shown in Fig. 5. C, control culture. Nos. 6–16 inclusive correspond to the numbers shown at the bottom of separation 1 with the addition of samples from drip points 6, 7, and 16 from the same separation. × 110.

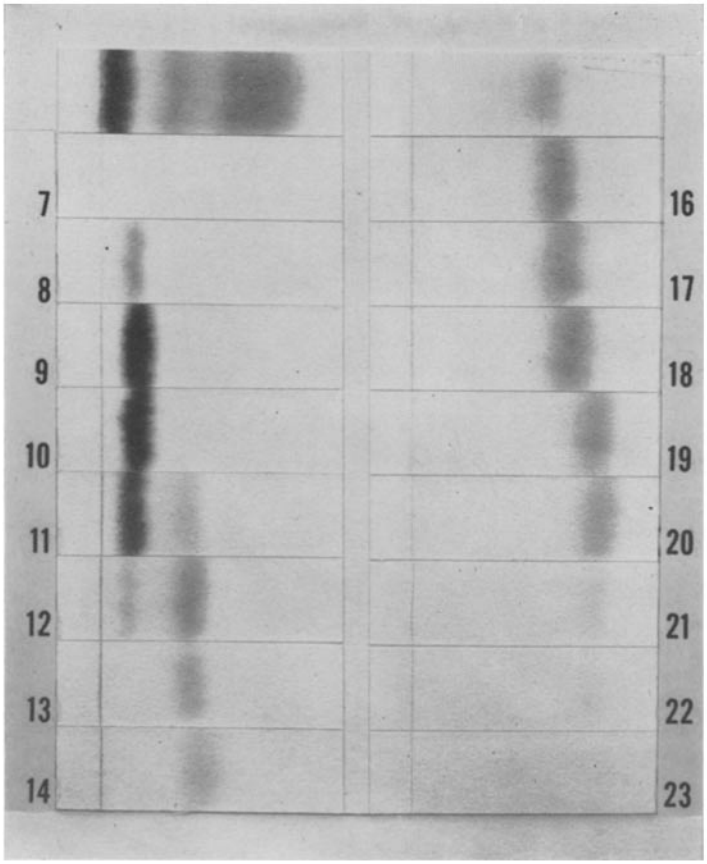

FIGURE 7

Comparison of paper electrophoretic patterns of individual fractions of bovine serum obtained by a curtain separation using sodium bicarbonate electrolyte and carboxymethyl cellulose. The analyses were carried out with pH 8.6 sodium barbiturate and 0.05 per cent carboxymethyl cellulose. The left top pattern is a reference pattern prepared from whole serum. In this pattern the heavy band at the left, close to the origin, contains material such as albumin. The band at the extreme right contains gamma globulin. Component 1 was identified in fraction 10, component 2 in fractions 14 and 15.

taining 0.05 per cent carboxymethyl cellulose, of a sodium bicarbonate separation are shown in Fig. 7. Fraction 10, Fig. 7, contained the bulk of material with component 1 properties, and fractions 14 and 15 that with component 2 properties. Fraction 10 also contained a large portion of albumin, while fractions 14 and 15 contained no albumin that could be detected by electrophoresis. The use of borate electrolyte had the advantage of producing a somewhat sharper curtain separation. On the basis of the response of cells to the fractions, sodium bicarbonate was the electrolyte of choice.

The effects of component 1 on strain I grown in the presence of 0.001 per cent sodium versenate are illustrated in Fig. 8. This material was produced by direct precipitation at pH 5.0 of fraction 10 illustrated in Fig. 7. Fraction 10, which was produced by curtain electrophoresis of 100 ml of serum, using sodium bicarbonate electrolyte containing carboxymethyl cellulose, was adjusted to pH 5.0 with acetic acid and dialyzed against three changes of water. The precipitate removed by centrifugation was dissolved in 50 ml of 0.1 per cent sodium bicarbonate solution and reprecipitated at pH 4.8 with dilute acetic acid. The yellow colored precipitate dissolved in 100 ml of 0.025 per cent sodium bicarbonate solution was added to the

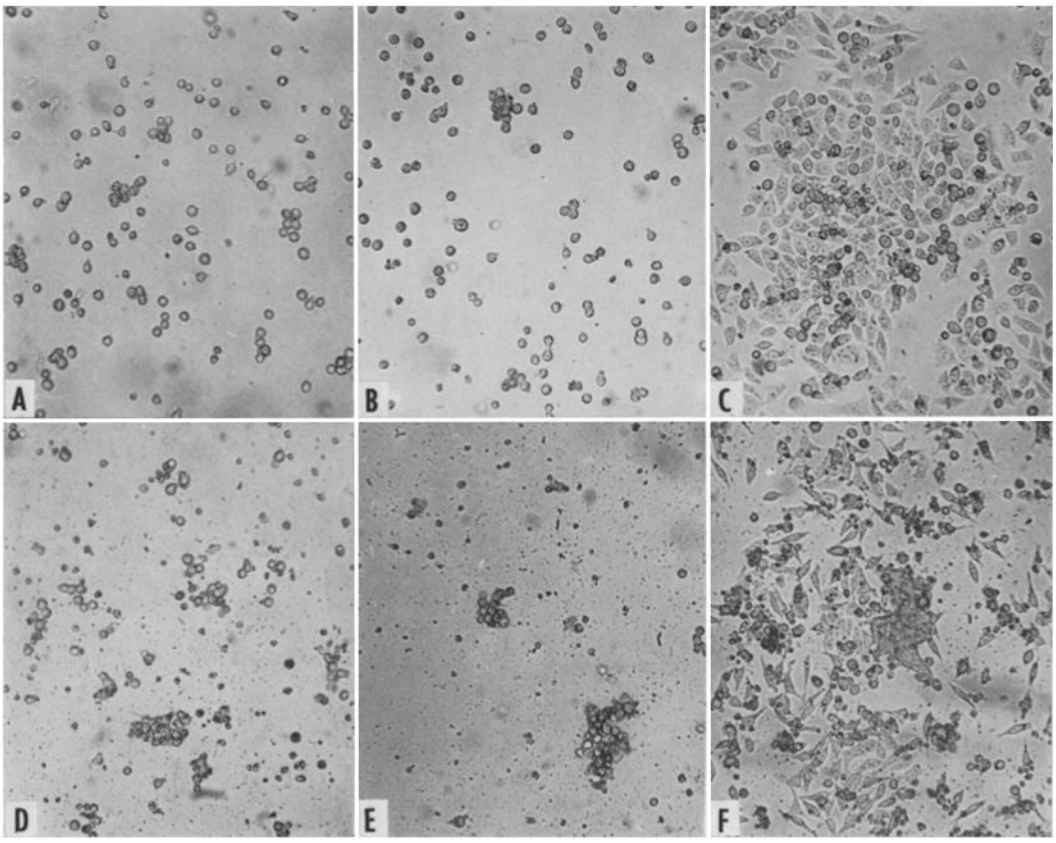

FIGURE 8

Illustration of the appearance of 24-hour-old cultures to which component 1 was added. Cultures A, B, and C were inoculated with "adapted" strain I of Chang's cells grown in the presence of 0.0001 per cent sodium versenate. D, E, and F were inoculated with "unadapted" cells derived from human intestine. A and D were placed in medium A2; B and E were placed in medium A2 supplemented with 0.1 per cent albumin (Armour); C and F were placed in medium A2 supplemented with component 1. × 80.

test culture at a concentration of 20 per cent by volume. Fig. 8 A shows the appearance of control cultures of strain I; Fig. 8 B shows that adding 0.1 per cent albumin (Armour) produced no further response; Fig. 8 C shows the pronounced spreading effect caused by the addition of component 1. The cultures illustrated in Fig. 8 were 24 hours old; however, the spreading effect produced by the addition of component 1 was apparent within 2 hours.

A line of human intestinal cells carried in medium A2 supplemented with 5 per cent adult bovine serum was transferred to unsupplemented medium A2. After 10 days the cells were removed with a scraper and one third of the cells transferred to medium A2, another third to medium A2 supplemented with 0.1 per cent albumin, and the remaining third to medium A2 containing component 1 in the concentration described above. After 24 hours' incubation no cells survived either in medium A2 or in the culture containing albumin. With the addition of component 1, cells which were initially in very poor condition were well on their way to recovery. The appearance of these cells is illustrated in Figs. 8 D, E, and F, respectively.

DISCUSSION

Components 1 and 2 were similar to each other in some respects, dissimilar in others. Both com-

ponents were able to promote survival and multiplication of a number of cell lines, but only component 1 possessed the property of causing cells to attach and spread on glass within 2 hours. Component 1 invokes a response similar to that described by other investigators for the attachment factor (1, 2, 14). Component 2, though it was not lost through the dialysis sac during preparation, promoted a response similar to that of serum digests (11, 27) which would pass through the sac. Component 2 which survived a relatively rigorous separation was a sufficient supplement for multiplication of "unadapted" Chang's and HeLa cells. However, the fact that both of these cell lines can be grown in a completely defined medium suggests that component 2 is an intermediate whose rate of synthesis is limited in "unadapted" cells. A similar situation has been demonstrated with the case of mesoinositol (23). An alternate explanation, based on other reports (9, 10), is that both components alter the charge on the surface of the cell, which in turn may affect permeability. The latter explanation would seem more applicable to effects caused by component 1. Other explanations such as detoxification of the glass surface (17) are not excluded. In cell lines which respond to component 2, cell spreading promoted by component 1 is not essential for survival and multiplication. Whether these varied responses produced by either component 1 or 2 are exerted *in vivo* cannot be determined from these experiments. It appears likely that in the presence of either component some cells will grow in a less complex medium than medium A2.

The substance obtained from serum which caused cell "clumping" has not been studied sufficiently to warrant any extensive conclusions.

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