

GROWTH OF HUMAN DIPLOID CELLS (STRAIN MRC-5) IN DEFINED MEDIUM; REPLACEMENT OF SERUM BY A FRACTION OF SERUM ULTRAFILTRATE

KATHLEEN LAMBERT* AND S. J. PIRT

*Microbiology Department, Queen Elizabeth College
(University of London), Campden Hill, London, W. 8, England*

SUMMARY

A calf serum ultrafiltrate fraction permitted growth for at least 3.5 generations, including one subculture, of MRC-5 cells in defined medium in the absence of whole serum. The active material has a molecular weight of 10000 Daltons or less. This suggests that there may be no requirement for a large macromolecular component of serum. The ultrafiltrate was assayed by maximum cell yield from a serum-limited inoculum in a defined medium containing non-limiting amounts of vitamins, amino acids, glucose, a 68-component supplement, iron and methylcellulose. The levels of vitamins, amino acids and glucose were based on quantitative measurements of uptake and the levels of the other components by minimum amount required for maximum yield in defined medium without ultrafiltrate or serum. With excess ultrafiltrate maximum cell yield was limited by the defined part of the medium, probably the supplement. The cell doubling time in defined medium with ultrafiltrate fractions was 70 h compared with 27 h in the medium with serum. Excess ultrafiltrate did not inhibit growth. The lowered growth rate is attributed to a nutritional deficiency in the supplement.

INTRODUCTION

Replacement of serum by defined media for cell growth is essential to obtain full reproducibility of the cell's environment, reduce costs and avoid toxins or infections from serum. Identification of nutrients specific to serum must be preceded by definition of the *known* nutrient requirements, to ensure that these do not limit growth when serum is reduced. We have previously reported (Lambert & Pirt, 1975) the quantitative requirements of MRC-5 human diploid cells for amino acids, B group vitamins, glucose, inositol and choline. More recently (Lambert & Pirt, 1977) we determined the requirements for methylcellulose, iron and a chemically defined supplement. This work resulted in a defined medium allowing significant growth (1.5 generations) of MRC-5 cells without added serum. However, growth then became limited by unknown serum growth factors. We have found maximum cell yield (cells/ml medium) produced by test fractions when they are growth limiting to be the most appropriate (Pirt & Lambert, 1977) means of assaying the growth factor. McKeehan & Ham (1977), using a different assay method (clonal growth of WI-38 human diploid cells)

* Present address: Department of Experimental Pathology, Charing Cross Hospital Medical School, Fulham Palace Road, London, W. 6, England.

found that despite comprehensive optimization of the defined nutrients and culture conditions in their system (McKeehan, McKeehan, Hammond & Ham, 1977), only limited division of WI-38 cells occurred in the absence of serum protein. Unknown serum factors then limited growth.

In recent attempts to purify the growth factor, we found that growth factor activity was gradually lost on prolonged dialysis of serum but that activity could be recovered, particularly in fraction IV, in fractions of serum prepared by the cold ethanol method of Cohn *et al.* (1946). The diffusible nature of the growth factor and its association with the α -globulins seemed consistent with the properties of somatomedin (Van Wyk, 1975) or NSILA (Rinderknecht & Humbel, 1976). We have applied the NSILA purification method to calf serum to separate molecules of more than 10000 Daltons from those of less than 10000 Daltons and tested the fractions on MRC-5 cells. Here we report (a) growth factor activity in the < 10000 Daltons fraction in the absence of serum, (b) significant growth (3.5 generations including one subculture) of MRC-5 cells in serum ultrafiltrate fraction in the absence of serum, and (c) limitation of growth by the defined part of our medium in the presence of excess ultrafiltrate.

MATERIALS AND METHODS

Details of the cell line, stock culture, preparation of phosphate-buffered saline (PBS) and measurement of growth rate have been given elsewhere (Lambert & Pirt, 1975).

Culture media

The composition of the improved experimental culture medium, MEME, is shown in Table 1. The medium is prepared and stored as a series of sterile stock solutions as described for MEMA and MEMB (Lambert & Pirt, 1975) or MEMC and MEMD (Lambert & Pirt, 1977). The modifications from MEMD are the increase in inositol concentration from 1.0 to 2.0 mg/l. and the inclusion of selenium as a 5 mg/l. ($\times 1000$) stock solution of sodium selenite sterilized by membrane filtration and stored at 4 °C.

Preparation of inocula

Three-day-old stock cultures, with the serum content of the medium reduced to 5% (v/v) to cause limitation of the inoculum by serum factors, were used as inoculum. Monolayers were washed twice with PBS and the cells removed by treatment with 0.5% trypsin (1/250 Difco) in citrate buffer, pH 7.6. After resuspension in PBS containing 10% serum, cells were separated by centrifugation at 150g for 5 min, washed in PBS, and resuspended in the appropriate experimental medium. This procedure was found to eliminate detectable protein carry-over in the medium (Lambert & Pirt, 1977). Cells were separated by aspiration with a 5-ml pipette, counted and inoculated to give an initial population density attached to the culture surface of $5-7 \times 10^4$ cells/ml ($1-1.4 \times 10^4$ cells/cm²).

To prepare inocula from cells grown in serum-free medium, trypsin treatment was avoided and the cells were removed from the monolayer as follows. Monolayers were washed twice with PBS; 1.0 ml of the fresh experimental medium was added and the cells were scraped from the surface into the medium using a Pasteur pipette covered with silicone-rubber tubing. Growth medium was added and the cells separated by aspiration. Cells were then pooled, counted and inoculated into fresh vessels to give an initial population density attached to the culture surface of ca. 3×10^4 cells/ml (0.6×10^4 cells/cm²).

Cultural procedures for MRC-5 cells

Stock cultures were grown in sealed 75-cm² (250-ml) Nunc plastic flasks containing 15 ml of medium. For experimental cultures 5-ml volumes of medium in 25-cm² (30-ml) Nunc plastic flasks were used. To obtain a complete growth curve and estimate of maximum cell yield (maximum cell population in numbers minus initial population attached) 11 or more such replicates were used.

The air above the medium was replaced by 5% CO₂ in air and all cultures were incubated at 37 °C.

Table 1. *Experimental culture medium MEME*

Inorganic salts	mg/l.	Amino acids	mg/l.
NaCl	6530	L-Arginine HCl	126.4
KCl	400	L-Cystine	48.0
MgCl ₂ .6H ₂ O	200	L-Glutamine	584.0
CaCl ₂ .2H ₂ O	264	L-Histidine HCl	38.3
NaH ₂ PO ₄ .2H ₂ O	150	L-Isoleucine	52.5
NaHCO ₃	2200	L-Leucine	52.5
		L-Lycine HCl	73.1
		L-Methionine	14.9
		L-Phenylalanine	33.0
Vitamins		L-Threonine	47.6
D-Biotin	0.5	L-Tryptophan	10.2
Choline chloride	2.0	L-Tyrosine	36.2
Folic acid	0.5	L-Valine	46.9
i-Inositol	2.0		
Nicotinamide	0.5	Trace elements and polymer	
Calcium-D-pantothenate	0.5	Na ₂ SeO ₃	0.005
Pyridoxine hydrochloride	0.5	FeSO ₄ .7H ₂ O*	1.0
Riboflavin	0.10	Sodium carboxy-	1450.0
Thiamine hydrochloride	0.5	methyl-cellulose*	
Sugar		Additional components	
Glucose	2000†	Penicillin	100000
			units/l.
		Medium supple-	15% (v/v)
		ment‡	

* These amounts are in addition to those in the medium supplement.

† One half added after 72 h of incubation.

‡ As described in Lambert & Pirt (1975, 1977).

Cell counts

Cell suspensions prepared from monolayers by trypsinization were diluted in PBS and cell counts made in a Fuchs-Rosenthal counting chamber using trypan blue exclusion to distinguish viable cells. Cells stained with trypan blue were not counted. For cells grown without serum, cell attachment was less firm and 3-s treatment at 37 °C (as opposed to 30 s for cells grown with serum) was sufficient to detach the monolayer.

Determination of protein

The protein content of assay media and fractions was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Elution of protein from columns was followed by absorbance at 280 nm (Pye Unicam SP1800 spectrophotometer).

Fractionation of serum

Calf serum (Gibco Biocult L262501) was used.

Acid treatment. 25-ml aliquots of serum were made 0.5 M with acetic acid by dropwise addition of glacial acetic acid over 5 min with constant stirring, then stirred for 1 h at room temperature (18–22 °C). The acidified serum was then filtered through a 0.45- μ m Millipore filter before gel filtration or ultrafiltration.

G-75 Sephadex chromatography. 10 ml of acid-treated serum were applied on a Pharmacia K50/100 column of Sephadex G-75 (bed volume 1600 ml), equilibrated in 0.5 M acetic acid at room temperature. Columns were poured at room temperature at a flow rate of 250 ml/h and run at 200 ml/h. Elution of protein was followed by E_{280} absorbance and the columns were calibrated using Blue Dextran 2000 (Pharmacia Fine Chemicals) and bovine insulin (Sigma Chemical Co.). One fraction (> 10000 Daltons) comprising the eluate from 27–55 % of the total bed volume and a second (< 10000 Daltons) comprising 56–98 % of the total bed volume were collected and lyophilized. The dried samples were redissolved in glass-distilled water and lyophilized. This procedure was repeated once more prior to the growth factor assay to remove acetic acid.

Ultrafiltration. 15-ml aliquots of acid-treated serum were ultrafiltered through immersible molecular separator membranes (Millipore) with a nominal cut off limit of 10000 Daltons. Ultrafiltration was continued until a volume of 2.0 ml of non-filterable material remained on the protein side of the membrane, a process taking 26–28 h at room temperature. The volumes of ultrafiltrate (< 10000 Daltons) and non-filterable protein (> 10000 Daltons) were recorded and the fractions were suspended in distilled water and lyophilized as for the Sephadex G-75 fractions.

Desalting chromatography of ultrafiltrate. Recovery of ultrafiltrate was estimated from the recovery of E_{280} and E_{260} absorbance from the columns. The flow rate for all separations was 20 ml/h. The void volume of columns was determined using Blue Dextran 2000 and the salts fraction detected from conductivity measurements using an electrolytic conductivity measuring bridge (Electronic Switchgear (London) Ltd). For fractions A, B and C, ultrafiltrate from 105 ml acidified serum was resuspended in 10.0 ml distilled water, filtered through a 0.45- μ m membrane filter and applied to a Pharmacia K16/40 column containing Sephadex G-25, bed volume 68 ml, equilibrated in distilled water at room temperature. The E_{280} -absorbing eluate not containing salts was lyophilized as 2 fractions, A and C. The central portion of the eluate containing salts, was lyophilized, resuspended in 10.0 ml distilled water and loaded onto a Pharmacia K26/40 column containing Biogel P-2, bed volume 106 ml, equilibrated in distilled water at room temperature. The E_{280} -absorbing eluate not containing salts was lyophilized as fraction B.

For fractions D, E and F, ultrafiltrate from 190 ml acidified serum was resuspended in 6.0 ml distilled water, filtered through a 0.45- μ m membrane filter and applied to a Pharmacia K26/40 column containing Biogel P-2, bed volume 178 ml, equilibrated in distilled water at room temperature. The E_{280} -absorbing eluate not containing salts was lyophilized as 2 fractions D and F. The central portion of the eluate, containing salts, was lyophilized, resuspended in 7.5 ml distilled water and rechromatographed on the Sephadex G-25 column, (bed volume 68 ml). The E_{280} -absorbing eluate not containing salts was lyophilized as fraction E.

Preparations of fractions for growth factor assay

Lyophilized fractions from chromatography were resuspended in PBS. Ultrafiltrate and non-filterable protein from ultrafiltrate were resuspended in distilled water to allow for the tonicity of the serum salts. (Isotonic distribution on each side of the membrane at the end of ultrafiltration assumed.) All test fractions were adjusted to pH 7.2 and membrane-filtered before storage at 4 °C. Amounts of fractions to be added to test media were calculated as volumes of original serum per unit volume taking into account dilutions and mechanical losses throughout the fractionation procedure.

Growth factor assay of fractions and serum

All assays included a 5% serum control and a blank containing basal medium with no serum or fraction. Maximum cell yield from a serum-limited inoculum was determined for each test sample and the blank value subtracted. The unit of growth factor activity was the amount which gave a cell yield of 10^5 cells. The units attributable to each fraction were compared to serum within each assay and thus related to a single eternal standard serum, which had a growth factor activity of 37 units/ml.

RESULTS

Growth factor activity of Sephadex G-75 and ultrafiltration fractions

The 2 fractions of acid-treated serum from Sephadex G-75 and ultrafiltration were assayed for growth factor activity at a concentration equivalent to 5% original serum. The > 10000 Daltons fraction from each fractionation promoted cell attachment and

Table 2. *Characterization of active serum fractions*

Fraction	Recovered activity,* %	Activity, units†/mg protein	t_d ,‡ h
Serum	100	0.4	27
Ultrafiltrate	18	6	70
Ultrafiltrate + non-filterable residue	28	0.2	42
A + B + C§	31	11	70
E	17	9	70

* Corrected for mechanical losses.

† 1 unit of growth factor activity produces 10^5 cells.

‡ Population doubling time.

§ A, B and C were fractions prepared by chromatography of serum ultrafiltrate on Sephadex G-25 followed by Biogel P-2 (Fig. 1).

|| E was one fraction prepared by chromatography of serum ultrafiltrate on Biogel P-2 followed by Sephadex G-25 (Fig. 2).

division but cells detached from the culture surface after 120 h. A similar effect was observed with whole serum acid treated for 30 h without ultrafiltration. Acid treatment accounted for the largest proportion of the loss of growth factor activity during fractionation (49% lost after 28 h acid treatment and 38% lost after 4 h acid treatment). The loss of growth factor activity on lyophilization was 14% of the total and for ultrafiltration itself 9% of the total. It seems likely that acid treatment converted some serum protein into a toxic form.

The < 10000 Daltons fraction contained half the growth factor activity remaining after acid treatment. Cells were less firmly attached to the culture surface than with whole serum, and trypsinization treatment for counting had to be reduced from 30 to 3 s to avoid loss of cells. Although activity was found in the < 10000 Daltons fraction prepared both by G-75 and ultrafiltration, the latter procedure, being less laborious, was adopted as a routine method for preparing larger amounts of the serum growth

factor. Table 2 compares growth factor activity of ultrafiltrate with or without non-filterable residue to that of untreated serum, tested at 5% (v/v) medium concentration; 18% of the growth factor activity of serum was recovered in the ultrafiltrate, with a 15-fold purification based on protein content. The growth rate with ultrafiltrate was slower (t_d 70 h) than that with serum (t_d 27 h). The non-filterable residue had a lower specific activity than serum but addition of the non-filterable residue to the ultrafiltrate increased the growth rate. Thus extraction of growth factor activity in our fractionation was not complete. This could mean either that our method of

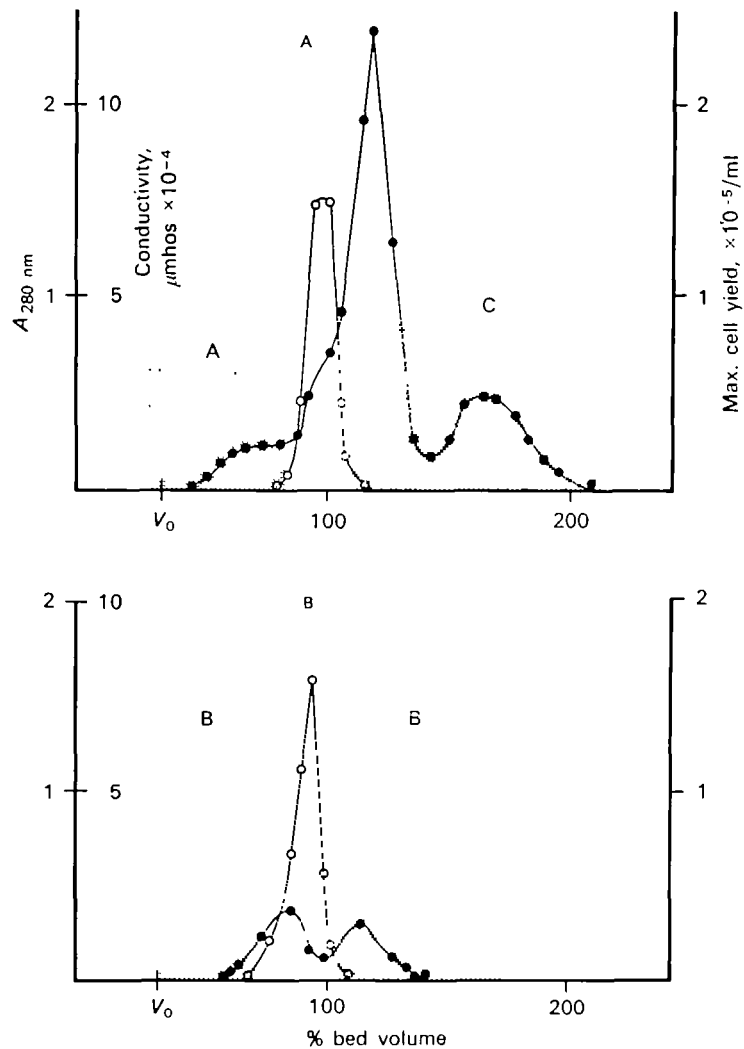


Fig. 1. A, serum ultrafiltrate chromatographed on Sephadex G-25 in distilled water at pH 6.7. B, 79–109% bed volume (salts fraction) from A rechromatographed on Biogel P-2 in distilled water at pH 6.7. Three fractions, A, B (both portions combined) and C were collected. The height of the histograms indicates the maximum cell yield obtained for these fractions tested in MEME at concentrations of 20% (A and C) and 10% (B) by volume. ●, absorbance at 280 nm; ○, conductivity.

fractionation caused loss of the growth factor or that more than one growth factor was supplied by the serum.

The maximum cell yield obtained with ultrafiltrate at 5% (v/v) serum equivalent was $0.4 \pm 0.1 \times 10^5$ cells/ml, compared to 1.8×10^5 cells/ml with untreated serum. When the ultrafiltrate was tested at 10–20% (v/v) serum equivalent, no increase in cell yield was obtained. This was not due to breakdown of ultrafiltrate in the medium: adding the ultrafiltrate in 2 parts, one initially and one after 72 h, did not produce a

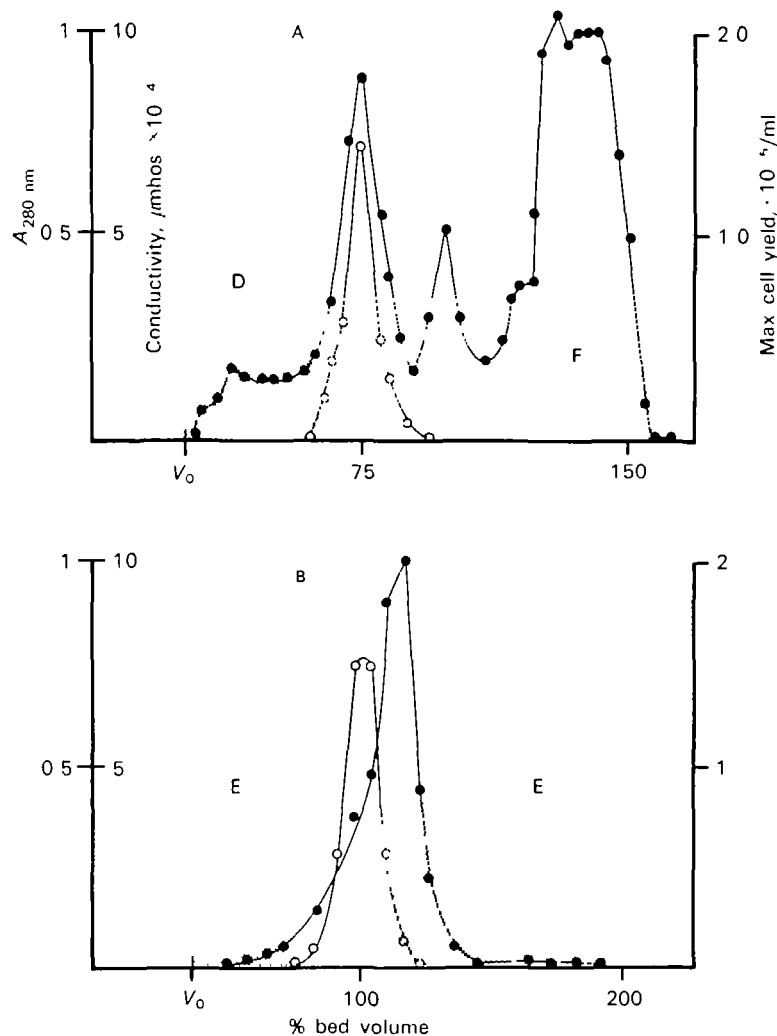


Fig. 2. A, serum ultrafiltrate chromatographed on Biogel P-2 in distilled water at pH 6.7. B, 65–88% bed volume (salts fraction) from A rechromatographed on Sephadex G-25 in distilled water at pH 6.7. Three fractions, D, E (both portions combined) and F were collected. The *height* of the histograms indicates the maximum cell yield obtained for these fractions tested in MEME at concentrations of 20 or 40% (D and F) and 15 or 30% (E) by volume. The maximum cell yield for E at 5% by volume was 0.3×10^5 /ml. ●, absorbance at 280 nm; ○, conductivity.

larger cell yield than that obtained when all the ultrafiltrate was included at the start. The simplest explanation is that at ultrafiltrate concentrations greater than 5% (v/v) serum equivalent, cell yield was limited by a nutrient other than ultrafiltrate. The presence of an inhibitor seemed unlikely since increase in the concentration of ultrafiltrate had no effect on growth rate (Pirt, 1975). Before testing the ultrafiltrate at higher concentrations, however, the possibility of suboptimal tonicity due to unsuspected salts in the ultrafiltrate was excluded by desalting the ultrafiltrate.

Characterization of growth factor activity of desalted serum ultrafiltrate

The ultrafiltrate was desalted by chromatography on Sephadex G-25 followed by Biogel P-2 (Fig. 1) or Biogel P-2 followed by Sephadex G-25 (Fig. 2). A surprising result was that material absorbing at 280 nm (E_{280} absorbance followed a very similar

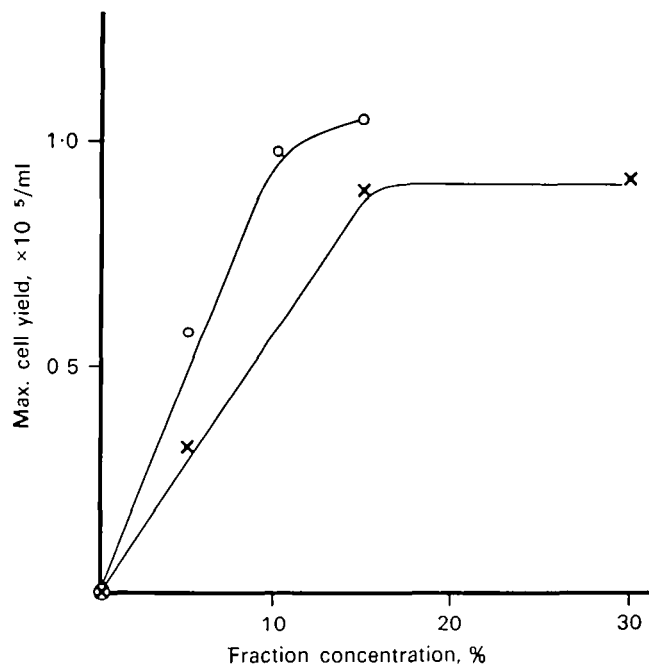


Fig. 3. Effect of serum ultrafiltrate fraction concentration (% by volume) on maximum cell yield of MRC-5 cells grown in defined medium MEME. \times , fraction E; \circ , fractions A + B + C.

pattern) was eluted well beyond the bed volume of the column, that is retarded by some factor other than molecular size. (The fractionation ranges for Biogel P-2 and Sephadex G-25 are 100–1800 and 1000–5000 Daltons respectively.) In both preparations (Figs. 1, 2), 5% of the ultrafiltrate applied to the column could not be separated from the salts and was not tested. The rest of the column filtrate was in each case combined into 3 fractions, A, B, C (Fig. 1) or D, E, F (Fig. 2). These fractions were all found to have some growth factor activity (height of histograms in Figs. 1 and 2) when tested at 5–40% (v/v) serum equivalent. The growth factor activity was more

concentrated in the fractions immediately preceding and following the salts. Fraction E had 17% of the activity of serum (Table 2) with a 22.5-fold purification. For pooled fractions A+B+C (Table 2) 31% of the activity of serum was obtained with a 27.5-fold purification. The purification factor for ultrafiltrate was 15-fold only, suggesting that desalting removed an inhibitor or a toxic substance. Growth rate in

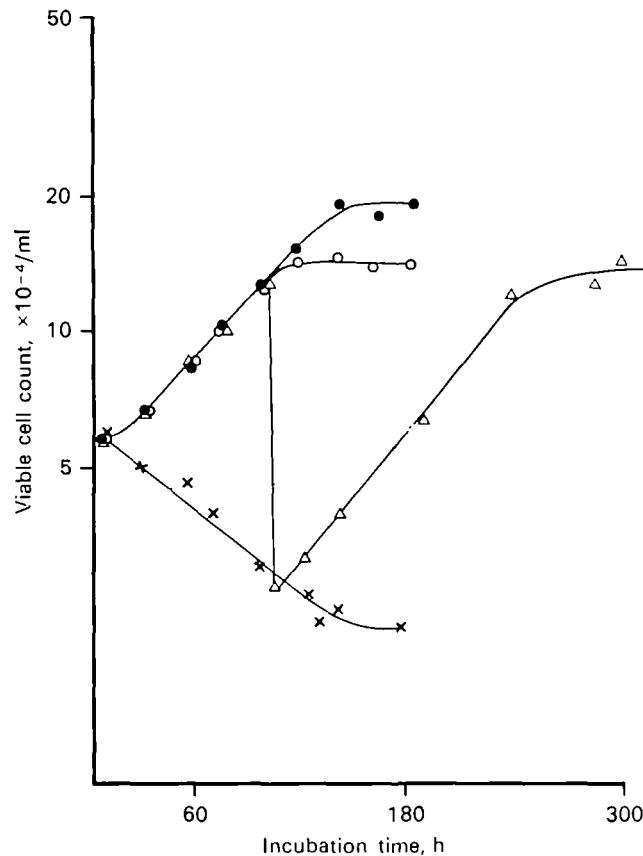


Fig. 4. Growth of MRC-5 cells in monolayer culture without serum. ●, in MEME + 15% by volume ultrafiltrate fractions A+B+C; ○, in MEME + 5% by volume ultrafiltrate fractions A+B+C; Δ, in MEME + 15% by volume ultrafiltrate fractions A+B+C subcultured by scraping, after 103 h, into fresh medium; ×, in MEM (Eagle, 1959) with no additions.

purified fractions was not improved, although cell yield was increased; the low growth rate in purified fractions suggests that serum contains enriching substances which increase growth rate but have no effect on cell yield (maximum population).

Maximum cell yield obtained with reconstituted fraction E or A, B and C combined, increased when their concentration was raised from 5 to 15% (v/v) medium concentration. Above 15% serum equivalent, no further increase in cell yield was obtained (Fig. 3). Similarly, no difference in cell yield occurred when fractions D and F were tested at 20 or 40% (Fig. 2). This was not due to instability of the material in the

medium since supplementation with further fraction, 72 h after inoculation, into the same medium did not affect cell yield. Hence it is concluded that some factor other than ultrafiltrate became growth limiting. To test whether there was a requirement for unknown serum factors at this point, cells at their maximum yield in defined medium with 15% combined fractions A+B+C were subcultured by scraping from the culture surface into fresh defined medium. Further division, to a total of 3.5 doublings occurred (Fig. 4). A similar result was obtained when cells were subcultured by scraping from defined medium with 20% fraction D. This growth on subculture we interpret to mean that the material limiting growth immediately before subculture was not a serum growth factor but rather the defined part of our medium, probably the supplement, since limitation of the maximum population by the Eagle's medium amino acids, vitamins and glucose could be eliminated from knowledge of the quantitative requirements (Lambert & Pirt, 1975).

The population doubling time for MRC-5 cells in defined medium with ultrafiltrate growth factor was limited to 70 h compared to 27 h with serum (Table 2). The increase in ultrafiltrate fraction from 5 to 15%, which affected cell yield (Fig. 4) had no effect on growth rate. The fact that increase in the amount of ultrafiltrate in the medium failed to increase the growth rate is attributed to a second nutritional deficiency in the medium which affects growth rate but not cell yield.

DISCUSSION

Few workers seem to have appreciated the necessity for defining quantitative requirements for known nutrients before attempting to reduce or replace serum in growth media for normal human diploid cells. McKeehan & Ham (1977) and McKeehan *et al.* (1977) have, however, demonstrated how systematic qualitative and quantitative modification of the medium and culture conditions can reduce the amount of dialysed foetal bovine serum protein required for clonal growth of WI-38 cells to 25 µg/ml (0.05% (v/v) whole serum). Unfortunately McKeehan & Ham do not give their results in terms of cell yield per unit weight of nutrient utilized, a limitation on the application of their results to other systems. Factors limiting growth of WI-38 colonies may not be identical to those limiting division in the more crowded conditions of routine cell culture where nutrient depletion and accumulation of cell-derived products become important. Results from our growth factor assay system (cell yield produced from a relatively large serum-limited inoculum) have the advantage of direct application to routine cell culture of human diploid cells.

Our results show that calf serum ultrafiltrate contains an unknown factor, of molecular weight 10000 Daltons or less, limiting cell yield of MRC-5 cells. Further purification of the ultrafiltrate yielded no discrete active fraction, suggesting that the growth factor, rather than a single peptide hormone, could be a mixture of factors. These could include characterized peptide growth factors such as NSILA, somatomedins, EGF or FGF (Rechler & Nissley, 1977), although to date none of these has been shown to completely replace serum for growth of serum-limited human fibroblasts (Gospodarowicz & Moran, 1976; Shields, 1977). Cell yield could also be limited

by lack of a trace element. Our medium contains 11 trace elements but lacks vanadium and molybdenum which McKeehan *et al.* (1977) found to be most effective for clonal growth at 5×10^{-9} and 7×10^{-9} M, respectively. However, they also found considerable background growth in the absence of added amounts of these salts: it thus seems likely that we have non-limiting levels of these components derived as contaminants from other medium constituents.

Specific growth rate of MRC-5 cells in defined medium with high or low levels of ultrafiltrate was much lower than in defined medium with serum, suggesting a second nutritional deficiency (in addition to the ultrafiltrate factor) in our medium. The specific growth rate limitation is reminiscent of that obtained with iron deficiency in the defined medium for LS cells (Birch & Pirt, 1970) and this, together with association of the growth factor activity with the salts fraction of desalted ultrafiltrate suggests that the second factor may be iron, or a deficiency in the medium supplement. Chelation of iron (1 mol. of EDTA per mol. of Fe) in our medium may have been ineffective.

The 3.5 MRC-5 cell doublings obtained with ultrafiltrate in the absence of whole serum indicate that there may be no requirement for a macromolecular component of serum or that the requirement does not become apparent until after 3.5 generations. The only serum factor specifically shown to stimulate growth rate of human diploid cells (Houck & Cheng, 1973) may be a macromolecule operating as a carrier for a smaller molecule with a much greater activity. Extension of our assay system beyond 3.5 generations for MRC-5 cells requires the solution of a technical problem in the making of cell suspensions without serum to inhibit trypsin; scraping cells from the culture surface by physical means is an inefficient method of obtaining a rapid viable suspension. Extension to a multiple subculture assay is important, as exemplified by the insulin requirement of HeLa cells, which only became apparent after 13.5 generations in defined medium (Blaker, Birch & Pirt, 1971).

In conclusion, our progress towards growth of human diploid cells without serum is summarized in Fig. 4, which compares growth of MRC-5 cells in MEM (Eagle, 1959) without serum, to growth in our defined medium with the active fraction of serum ultrafiltrate. Any further development of the defined medium for MRC-5 cells should aim at identifying both the factor limiting maximum cell population and that limiting the growth rate and preferably extend the maximum yield assay to several subcultures.

We gratefully acknowledge a grant in aid from the Medical Research Council. We thank Mr J. P. Jacobs of the N.I.M.R. Laboratories, Hampstead, for the supply of MRC-5 cells, Dr C. F. Thurston for advice on protein fractionation and Mr G. Wilkie for technical assistance.

REFERENCES

- BIRCH, J. R. & PIRT, S. J. (1970). Improvements in a chemically defined medium for the growth of mouse cells (strain LS) in suspension. *J. Cell Sci.* **7**, 661-760.
- BLAKER, G. J., BIRCH, J. R. & PIRT, S. J. (1971). The glucose, insulin and glutamine requirements of suspension cultures of HeLa cells in a defined culture medium. *J. Cell Sci.* **9**, 529-537.

- COHN, E. J., STRONG, L. E., HUGHES, W. L., MULFORD, D. J., ASHWORTH, J. N., MELIN, M. & TAYLOR, H. J. (1956). Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids. *J. Am. chem. Soc.* **68**, 459-475.
- EAGLE, H. (1959). Amino acid metabolism in mammalian cells in culture. *Science, N.Y.* **130**, 432-437.
- GOSPODAROWICZ, D. & MORAN, J. S. (1976). Growth factors in mammalian cell cultures. *A. Rev. Biochem.* **45**, 531-558.
- HOUCK, J. C. & CHENG, R. F. (1973). Isolation, purification and chemical characterization of the serum mitogen for diploid human fibroblasts. *J. cell. Physiol.* **81**, 257-270.
- LAMBERT, K. & PIRT, S. J. (1975). The quantitative requirements of human diploid cells (strain MRC-5) for amino acids, vitamins and serum. *J. Cell Sci.* **17**, 397-411.
- LAMBERT, K. & PIRT, S. J. (1977). The nutrient requirements of MRC-5 human diploid cells. *Devl biol. Standard.* **37**, 67-70.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the folin phenol reagent. *J. biol. Chem.* **193**, 265-275.
- MCKEEHAN, W. L. & HAM, R. G. (1977). Methods for reducing the serum requirement for growth *in vitro* of nontransformed diploid fibroblasts. *Devl biol. Standard.* **37**, 97-108.
- MCKEEHAN, W. L., MCKEEHAN, K. A., HAMMOND, S. L. & HAM, R. G. (1977). Improved medium for clonal growth of human diploid fibroblasts at low concentrations of serum protein. *In Vitro* **13**, 399-416.
- PIRT, S. J. (1975). *Principles of Microbe and Cell Cultivation*. Oxford: Blackwell.
- PIRT, S. J. & LAMBERT, K. (1977). Towards a chemically defined medium for the growth of normal human diploid cells. *Devl biol. Standard.* **37**, 63-66.
- RECHLER, M. M. & NISSLEY, S. P. (1977). Somatomedins and related growth factors. *Nature, Lond.* **270**, 665-666.
- RINDERKNECHT, E. & HUMBEL, R. E. (1976). Polypeptides with nonsuppressible insulin-like and cell-growth promoting activities in human serum: isolation, chemical characterization, and some biological properties of forms I and II. *Proc. natn. Acad. Sci. U.S.A.* **73**, 2365-2369.
- SHIELDS, R. (1977). Growth hormones and serum factors. *Nature, Lond.* **267**, 308-310.
- VAN WYK, J. J. (1975). Postsession discussion of papers. In *Advances in Metabolic Disorders*, vol. 8 (ed. R. Luft & K. Hall), p. 393. New York & London: Academic Press.

(Received 29 June 1978)