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Modern Approaches to the Study of Mammalian Cells in Culture*

By H. MOSER**

The development of powerful new culture techniques has opened up novel approaches to the study of mammalian cells *in vitro*. These modern techniques permit many of the quantitative experimental approaches which are possible with many micro-organisms to be applied now to biochemical and genetical studies of single mammalian cells *in vitro*. It is the purpose of this short article to introduce the reader to some aspects of modern cell culture technology and to summarize some of the studies on mammalian cells carried out by the author and other research workers.

Modern Cell Culture Techniques

1. Chemical Dissociation of Mammalian Cells. The liberation of large numbers of single mammalian cells from tissue fragments or tissue cultures by chemical means without appreciable loss of viability has been a significant achievement[1–5]. This technique involves the brief incubation of solid tissue or of tissue cultures in balanced salt solutions deficient in Ca^{++}, Mg^{++}, and $H_2PO_4^-$ at 37°C, in the presence of trypsin, mixtures of tryptic enzymes (pancreatine) or in the presence of a chelating agent (Versene). Final dissociation is achieved mechanically and may yield cell suspensions composed of up to 90% single cells.

2. Partially defined and defined growth media for mammalian cells. The conventional tissue and cell culture media are prepared by combining electrolyte solutions ('balanced' salt solutions) with complex and uncharacterized ingredients such as lymph, plasma, and tissue extracts (notably chick embryo extract) or ultrafiltrates of the latter. These complex, undefined nutrients, do not permit exact biochemical and physiological studies. Moreover, since they introduce into the nutrient 'cytotoxic' compounds along with essential growth factors, they are suboptimal growth media for mammalian cells. Thus the formulation of *chemically defined background media* composed of electrolytes, carbohydrates, amino acids, vitamins, and other purified compounds (ROSENBERG's S 27[6], FISHER's V 614[7], EAGLE's Basal Mixture[8,9], WHITE's medium[10,11], MORGAN's 199 and M 150[12,13], HEALEY's 703[14], MORGAN's M 416[15], HEALEY's 858[16], EVANS' NCTC-107[17] and -109) made possible many new approaches to physiological, biochemical, and genetic studies of mammalian cells *in vitro*.

The *growth media* (complete media) of today can be classified as four groups[18]:

a) Serum supplemented growth media, i.e. chemically defined background media supplemented with 10% to 20% whole adult or fetal mammalian serum. These media are defined except for the molecular (dialyzable) and the macromolecular (non-dialyzable) portion of the serum supplements.

b) Serum protein supplemented growth media, i.e. chemically defined background media supplemented with 5%–10% dialyzed adult or fetal mammalian serum. These growth media are defined except for the

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macromolecular portion of serum (serum protein complex). In these nutrient media the omission of a single essential growth factor produces a nutritional deficiency and early death of the culture.

c) Chemically defined, protein containing, growth media, i.e. chemically defined background media supplemented with certain purified proteins isolated from mammalian sera (protein fractions with ultracentrifugal and electrophoretical homogeneity of at least 97%[19]).

d) Protein-free chemically defined growth media, for example growth medium NCTC-107 of EVANS *et al.* for L-929 mouse cells in monolayer culture.

The trend in this area of technology is to provide minimized optimal media, that is media which omit unessential and cytotoxic molecular or macromolecular species and permit the sustained growth of single (widely separated) cells *in vitro*.

3. The control of hydrogen-ion concentration in cell cultures. The rate of mammalian-cell reproduction is a function of the pH of the culture fluid. Maximum reproduction rate, as a function of hydrogen-ion concentration, is attained at pH = 7 or at a pH very close to this value[20]. At pH = 7.6 non-malignant (normal) mammalian cells stop dividing but remain viable (maintenance state)[20]. Since the sustained growth of mammalian cells *in vitro* is very susceptible to small changes in pH, modern cell culture employs refined methods for maintaining the pH within the desired limits or at a fixed value.

Since bicarbonate is the major buffer system of the growth media for mammalian cell cultures, and since actively growing somatic cells produce lactic acid, the pH, at a given temperature and a given atmospheric pressure, is a function of the bicarbonate concentration in the medium, of the partial pressure of CO_2 in the surrounding gas phase, and of the glycolytic activity of the growing cells. At low population densities in stationary cultures or even also at high population densities in perfused agitated cultures the acid production of the organism is negligible. Under such conditions the pH of the culture approximates

$$pH = pK_a + \log ((HCO_3^-)/(CO_2))$$

(Henderson-Hasselbach equation) where pK_a is a constant, (CO_2) the molar concentration of CO_2, (HCO_3^-) the molar concentration of bicarbonate ion in the culture fluid. Thus since the pK_a of the bicarbonate buffer system is 6.322 at 37°C pH values close to 7 can be maintained by adjusting the partial pressure of CO_2 in the gas phase and the bicarbonate concentration in the culture fluid.

Classic cell culture which employed anaerobic culture devices (tightly stoppered culture vessels) did not permit accurate gas and therefore pH control. Modern cell culture devices are of the aerobic type which are either placed in a humidified atmosphere containing 5% to 10% CO_2 (CO_2 incubator) or which are individually flushed with the gas mixture.

4. Monolayer cell culture. This stationary culture technique[2, 21] omits complex supporting structures (plasma clot, fibrinogen clot) in which cells or tissue explants are embedded. Dissociated cells, in Ca^{++} containing nutrient, at pH = 7 and a temperature close to 37°C adhere directly to the glass surface of the culture vessels (flasks, tubes, Petri-dishes etc.). As they begin to grow they stretch out horizontally on the glass surface and finally form confluent cell sheets consisting of only one layer of cells. In static monolayer culture two distinct morphological cell types are most frequently observed: Fibroblast-like cells, i.e. elongated spindle-shaped cells with needle-like extensions (Fig. 1), and epithelial-like cells, i.e. polygonal-shaped cells (Fig. 2). Prior to mitosis cells of both types assume a round form.

Monolayer-cell cultures prove to be very susceptible to infection by virus. This property has been exploited by DULBECCO and VOGT[3, 22] in the development of their ingenious plaque assay technique for animal viruses.

5. Fluid Suspension Culture Method. The ability to grow dispersed mammalian cells in fluid suspension analogous to the growth of unicellular microorganisms in liquid growth media has been a major achievement. The method involves the inoculation of a dense cell suspension into a growth tube with an agitating mechanism and filled with growth medium deficient or poor in Ca^{++}. Under appropriate conditions of agitation, a pH close to 7, a temperature of 37°C and sustained nutrient supply, the cells begin to multiply suspended mainly as single cells. Throughout the logarithmic growth phase they sustain their rounded form (see Fig. 3). Various types of agitated systems have been suggested as satisfactory for continuous growth of mammalian cells in fluid suspension[22, 23–28].

Undoubtedly the most advanced system is the cytogenerator of GRAFF and MCCARTY[20] (see Fig. 4). The cytogenerator is an agitated culture system with perfusion and gas control. The cells produced are retained within a U-shaped growth tube. Fresh nutrient is delivered to the growth tube by perfusion through a sep-

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Fig. 1. Fibroblast-like cells derived from adult human skin (normal-diploid strain M-1).

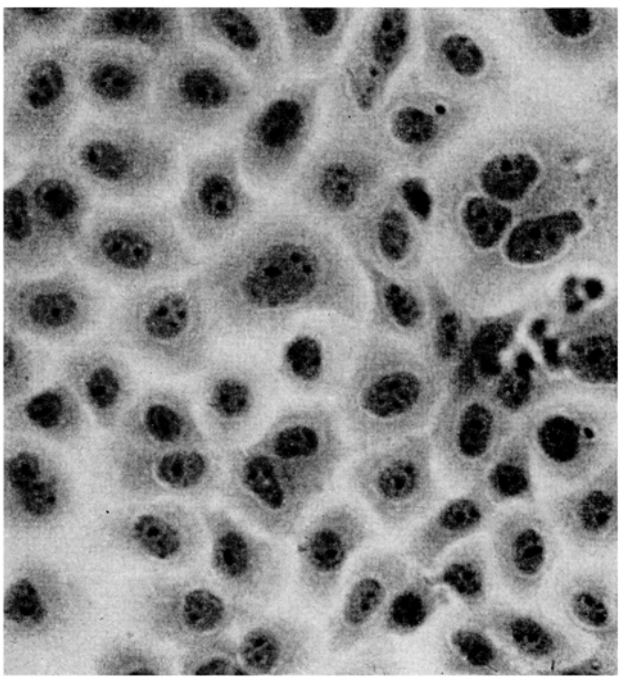


Fig. 2. Epithelial-like cells derived from adult sternal human marrow (altered heteroploid strain Detroit-98C6b).

tum (sintered glass filter), and the spent medium which includes accumulated cell metabolites is removed in a similar fashion. Accurate, matched variable pumps are used to keep outflow rate and inflow rate closely equal in order to maintain the volume of the culture fairly constant. The composition of the gas phase (CO_2-air) is controlled and can be regulated to adjust the pH of the culture – which contains sodium bicarbonate as the buffer – within the desired value. Alternate gas pressure pulses provide sufficient agitation to retain the cells in suspension. Because of its features this system is particularly suited for the quantitative study of cell metabolism and for mass production of mammalian cells and viruses *in vitro*.

Cells of several altered (hyperdiploid) cell strains, notably of the now famous mouse L-fibroblast strain and the Mouse Ascites strain have been successfully grown in the cytogenerator[20]. Figure 5 illustrates the growth of L-929 mouse cells in this system and in a medium consisting of Ca^{++} – free Eagle's Basal Mixture and of 10% adult horse serum. If the pH is kept close to pH = 7, and if the concentration of glucose in the growth tube is maintained above a certain value

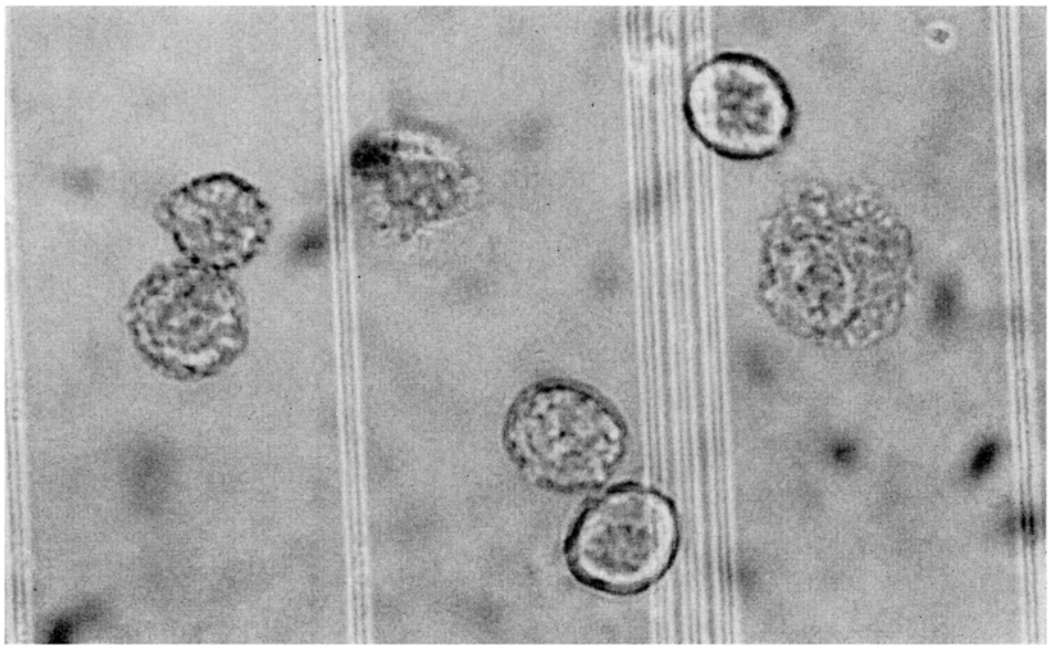


Fig. 3. L929 mouse cells in suspension culture.

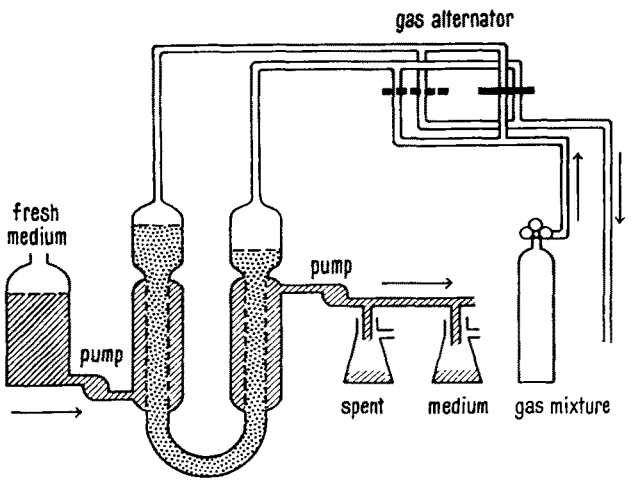


Fig. 4. Schema of the cytogenerator [reproduced with the permission of the author from Trans. N. Y. Acad. Sci. *21*, 505 (1959).

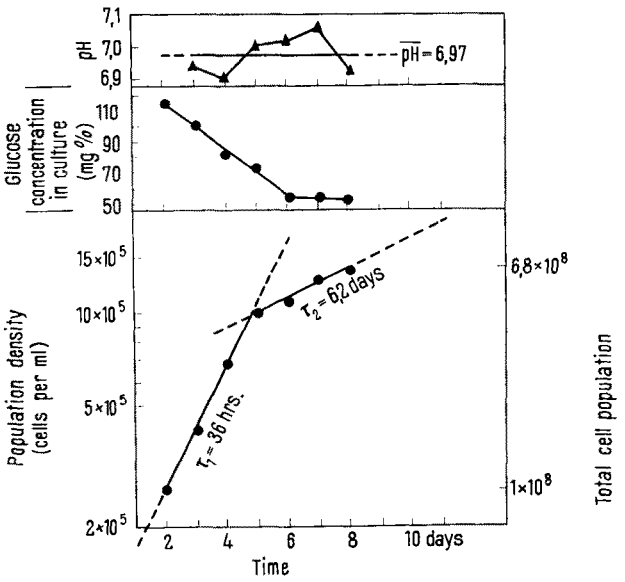


Fig. 5. Growth of L929 mouse cells in the cytogenerator. (Temperature 37°C).

(limiting concentration)[29] the L-929 cells reproduce logarithmically at maximum rate (i.e. at a doubling time approximating 24 h) at 37°C. Attempts are being made now by the author in collaboraton with Dr. S. Graff to propagate unaltered (diploid) human cells in this fashion.

6. The clonal culture method. Plating procedures for single cells derived from man and other mammals analogous to the quantitative plating of bacteria on agar media have been developed by Puck *et al.*[30, 31]. By this method a determined number of carefully trypsinized cells from predominantly mono-disperse cell suspensions are inoculated into Petri-dishes containing liquid growth medium and evenly distributed over the glass surface. When the Petri-dishes are incubated in hori-

[29] This is achieved either by regulating the rate of glucose input (perfusion rate) or regulating the glucose consumption (population density, pH).

[30] T. T. Puck, P. I. Marcus, and S. J. Cieciura, J. exp. Med. *103*, 273 (1956).

[31] T. T. Puck, S. J. Cieciura, and H. Fisher, J. exp. Med. *106*, 145 (1957).

zontal position at 37°C in a humidified atmosphere of CO_2 and air, and the pH adjusted to 7.0 or 7.1, the widely separated single cells attach to the glass and begin to multiply, forming macroscopic two-dimensional colonies upon incubation for 10 to 20 days. Both hyperdiploid (altered) cells of epithelial-like morphology and diploid (unaltered) cells of fibroblast-like morphology can be grown by this method into macroscopic clones. The plating efficiencies obtained with diploid-fibroblast-like cells are generally lower (5% to 40%) than those obtained with epithelial-like hyperdiploid altered epithelial cells[32,33] (Tab. I), and a more complex growth medium is required for the sustained growth *in vitro* of single cells of the unaltered fibroblast-like variety[34]. The epithelial cells form tightly packed colonies with well-defined edges. In sharp contrast to this the fibroblast-like cells form loosely packed colonies with large surface area and not well-defined contours[35] (see Fig. 6).

Although this method of clonal growth has many limitations and shortcomings, it provides undoubtedly an excellent tool for the screening of large numbers of mammalian cells under controlled conditions for physiological and genetic studies, and a tool for rapid cloning of heterogenous cell cultures.

7. Cloning techniques. The isolation of phenotypic (functional or morphologic) variants such as tumor or hormone producing cells[36] or of genetic variants (mutants) from heterogenous cell cultures is achieved by methods which involve (a) the transfer of an isolated cell to a small volume of growth medium which permits sustained multiplication, or (b) by the isolation of colonies produced by single cells in Petri-dishes or in culture tubes.

EARLE *et al.*[37] developed a very exacting technique for the production of clonal cell strains. This technique involves the sealing of individual cells in capillary tubes where diffusible metabolic products essential for multiplication remain in association with the cells. Another technique developed by VOGT and DULBECCO[38] involves the isolation and transfer of an individual cell into a small drop of growth medium deposited under paraffin oil on a Petri-dish. The clones which are formed in the individual-drop cultures can be trypsinized and subcultured for the production of mass cultures.

The method of PUCK, MARCUS, and CIECIURA consists in the direct isolation of well-separated colonies formed by single cells on Petri-dishes[30]. By this method each of the isolated subcultures must be passed through several single-cell isolation procedures in order to insure genetic purity.

A technique of clone isolation developed and tested by the author[18] is based on the distribution of single cells over a large number of tubes inoculated with equal amounts of a monodisperse cell suspension. If 1 ml aliquots of a monodisperse cell suspension containing in the average μ ($\mu \sim 1$) viable cells per unit volume are introduced into a large number of culture tubes with a flat bottom (Leightontype tubes), the fraction of tubes yielding x macroscopic colonies approximates $P(x) = (\mu^x/x!) \exp(-\mu)$ (Poisson distribution, $x = 1, 2, 3 \ldots, \infty$). The fraction, $P(1) = \mu \exp(-\mu) \sim 0.37$, of the tubes contain only a single colony. Thus clones can be isolated by selecting in such an experiment the tubes in which only a single colony appears. In practice, the tubes inoculated with 1 ml aliquots are incubated in horizontal position in the CO_2 incubator, so that there is enough space for the development of several isolated macroscopic colonies. The tube cultures with a single colony are trypsinized and subcultured, and the procedure repeated to insure genetic purity of the isolate.

8. Karyotype analysis in mammalian cell culture. The study of chromosomal constitution of mammalian cells in culture by the chromosome spreading techniques developed by HSU and POMERAT[39] and others not only permits the convenient determination of the chromosomal constitution of an individual but also provides an exact tool for determining alterations of cells *in vitro* (polyploidy, aneuploidy, chromosome aberrations). The method involves pretreatment of actively growing cultures with colchicine for 'accumu-

Tab. I. Plating efficiencies of heteroploid epithelial-type cell strains in EAGLE's Basal Mixture supplemented with 10% adult horse serum.

Human strain	Experiment, number	Incubation time, days	Number of cells plated	Colony counts	Mean colony count	Plating efficiency (in %)
Detroit-98	1	14	219	106 119	113	51.6
Detroit-98C6b	1	15	209	100 135	118	56.6
Detroit-98C6b	2	16	369	277 225 193 185 248	226	61.3
Detroit-98C6b	3	15	242	125 152	139	57.5
D-189	1	16	142	122 87 82	97	68.3
Human Amnion (Hayflick-Fernandez)	1	16	265	120 90 80	97	36.6

[32] H. MOSER and K. TOMIZAWA, Annual Report of the Biological Laboratory, Long Island Biological Association, Cold Spring Harbor, L. I., N. Y. 68th year (1958).

[33] R. Z. LOCKHART, JR. and H. EAGLE, Science *129*, 252 (1959).

[34] T. T. PUCK, S. J. CIECIURA, and A. ROBINSON, J. exp. Med. *108*, 945 (1958).

[35] T. T. PUCK, S. J. CIECIURA, and H. W. FISHER, J. exp. Med. *106*, 145 (1957).

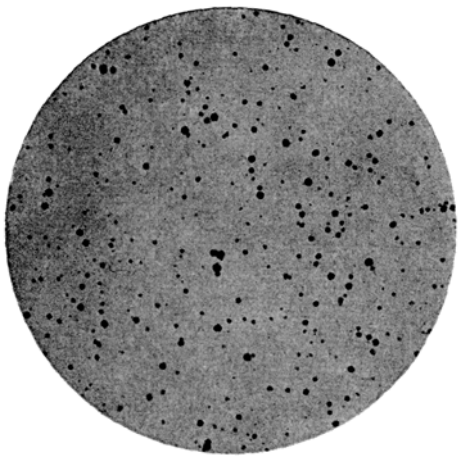
[36] K. W. THOMPSON, M. M. VINCENT, F. C. JENSEN, R. T. PRICE, and E. SCHAPIRO, Proc. Soc. exp. Biol. Med., N. Y. *102*, 403 (1959).

[37] W. R. EARLE, Methods med. Res. *4*, 217 (1951).

[38] M. VOGT and R. DULBECCO, Virology *5*, 425 (1958).

[39] T. C. HSU and C. M. POMERAT, J. Heredity *44*, 23 (1953).

lation' of cells in metaphase, and with hypotonic salt solution for spreading the chromosomes. This method yields preparations where the 46 chromosomes of man can be clearly counted and identified in numerous metaphase plates (see Fig. 10).



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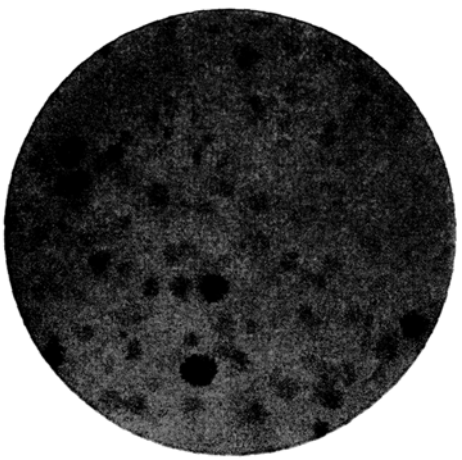

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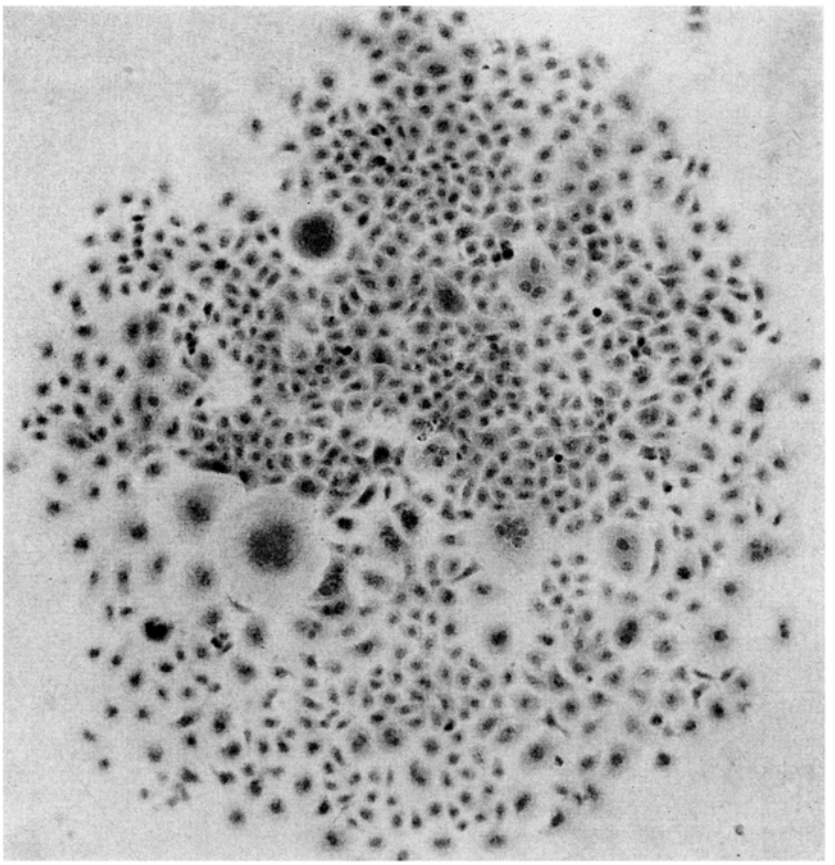

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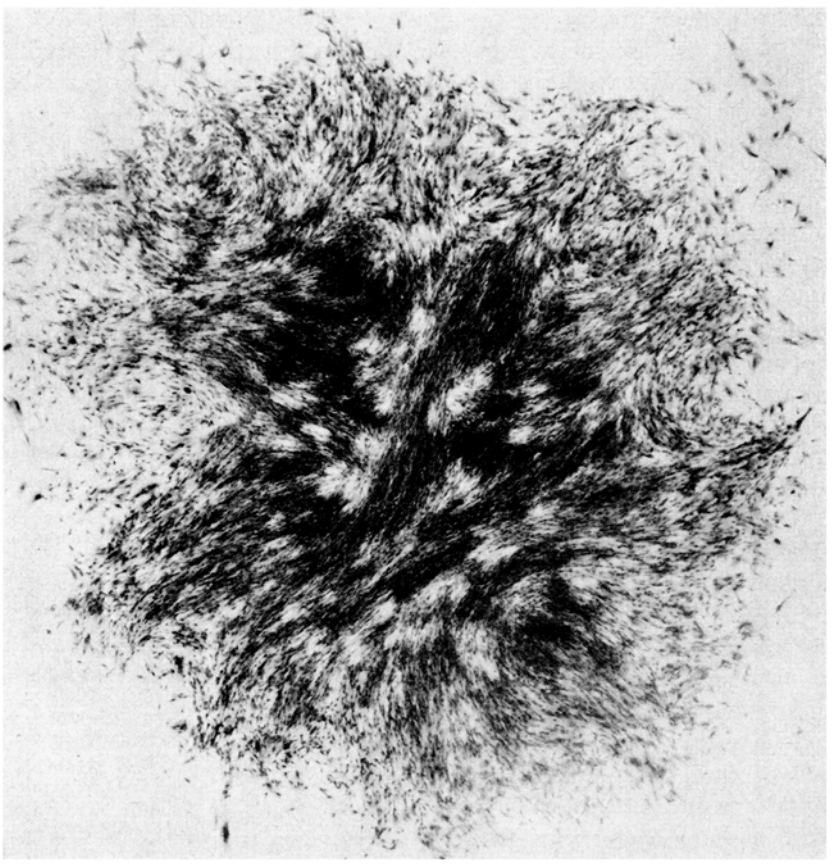

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Fig. 6. a) Clonal growth of epithelial-like cells (altered-heteroploid strain Detroit-98C6b). b) Clonal growth of fibroblast-like cells (normal-diploid strain M-1). c) A colony formed by an epithelial-like cell (Detroit-98C6b). d) A colony formed by a fibroblast-like cell (M-1).

The Evolution of Mammalian Cell Cultures

1. Adaptation and selection in primary cultures. Practically every tissue of the mammalian organism yields cells which are able to multiply *in vitro* under appropriate conditions of culture. In the past the initiation of cell cultures from biopsy materials has often failed as a result of suboptimal growth media and adverse culture conditions. The introduction of media with minimum cytotoxicity such as PUCK's fibroblast medium[34] has made it possible to initiate cell cultures with excellent regularity from almost any body tissue, individual, and many mammalian species.

When cells are dissociated from tissue material, profound adaptive processes occur during an initial period (*lag period*), in which certain cells of the explant make the transition from a possibly non-multiplying state (*maintenance state*) in the body to a state of very high reproduction rate *in vitro* (*adaptation*). Often only a small fraction of the explanted cells are capable of initiating reproductive growth in the new *in vitro* environment. As indicated in a case of explanted human skin studied by the author, this fraction may be as small as 10^{-3} to 10^{-4}. In this case approximately 2×10^5 trypsin-dispersed cells extracted from normal skin[40] were cultured with PUCK's fibroblast medium in Petri-dishes. Ten days after the seeding of the plates a few fibroblasts appeared as pairs and quadruplets in several isolated areas on the plates. Four weeks later these foci of active growth yielded wellseparated macroscopic colonies. An average of 10 colonies per plate, i.e. per 2×10^5 plated cells, was counted (see Fig. 7). If we assume that every colony originated from a single cell (as is indicated in this case), we obtain for the plating efficiency of these explant-cells a value of about 5×10^{-4}. The majority of cells which remained in lag, did not detach from the glass and could be indentified after many weeks (see Fig. 8). However, a certain portion of the cells which did not divide, stretched out on the glass surface and grew into mononucleate giant cells (see Fig. 9). Upon transplantation the primary colonies of survivors yielded a fast growing cell strain with diploid karyotype which has been maintained now for twelve months. In contrast to this in many other cases reproductive growth is initiated in a large fraction of the explanted cells after a short lag period, and foci of active growth appear in numerous areas after only a few days.

Undoubtedly *selection* processes play a role in many instances once growth is initiated in primary cultures. Since primary cell cultures are heterogenous, selection may operate because of differential reproduction rates, survival rates or migratory capacities of the different phenotypic (morphologic and functional) or genetic (chromosomal) variants which compose primary cell populations.

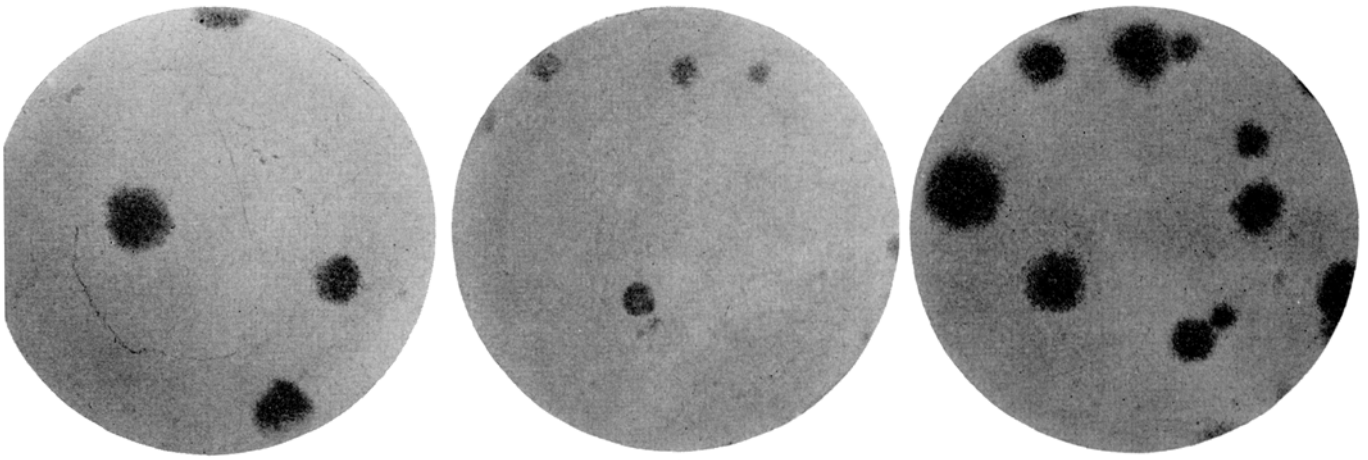


Fig. 7. Primary colonies formed by skin fibroblasts. (Primary culture of trypsinized adult human skin).

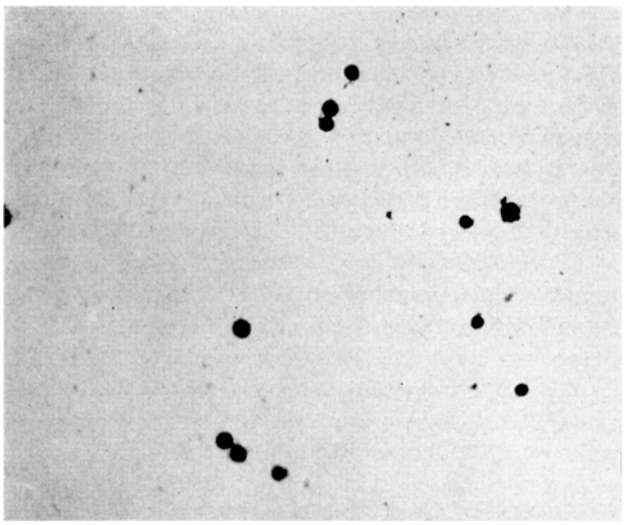


Fig. 8. Cells remaining in dormant state (lag) in primary culture of trypsinized adult human skin.

[40] Split thickness skin from an adult human female (caucasian).

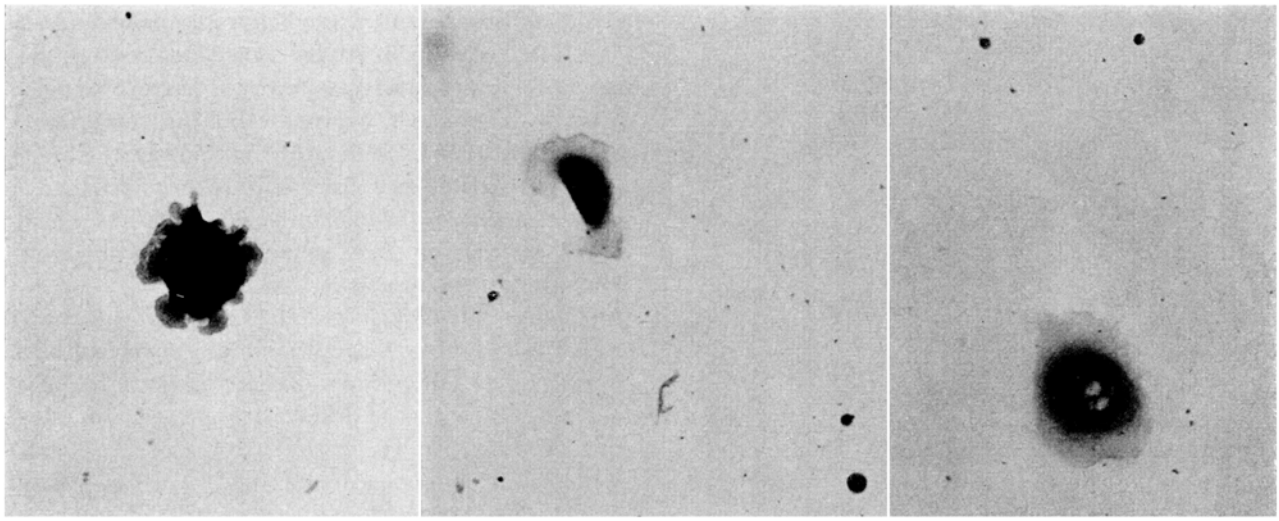


Fig. 9. Giant cells formed in primary culture of trypsinized adult human skin.

Heteroploid-cell transformation. When initiated and subcultured in conventional nutrients (growth media containing adult human, horse or bovine serum supplements), primary cell cultures with diploid karyotype, after several passages (culture transplants), undergo a drastic transformation from euploidy to heteroploidy[41,42]. During this process alterations of the chromosomes, both in number and morphology, occur spontaneously concurrent usually with a temporary refractory state (non-multiplying phase) of cell reproduction. Shortly before the refractory state, aneuploid cells begin to accumulate in the culture and to replace the original diploid cells. Finally a characteristic heteroploid karyotype dominates in the cell culture which has now attained an unlimited capacity to multiply *in vitro* in the same growth medium as the original (primary) cell culture. In the newly formed cell line (*established heteroploid-cell strains*) cells with normal-diploid karyotype are completely missing and cannot be re-established in these cultures. Thus during the process of transformation the normal diploid karyotype is irreversibly lost. In general heteroploid cell transformation is initiated after about seven passages, i.e. after about two months of culture *in vitro*.

Cultures with stable euploid karyotype. Long-term culture of normal mammalian cells with stable diploid chromosome complement has been accomplished[34] to a large extent in the case of mammalian cells with fibroblast-like morphology mainly by the use of pretested[34] fetal bovine serum instead of adult mammalian serum for incorporation into a defined molecular background medium. In such a growth medium the usual pattern of chromosomal heteroploidy and growth inhibition fails to occur if frequent media changes keep cell nutrition optimal, and if control of pH (7.0–7.1) and temperature (37° C) is strict. The diploid fibroblast-like cells reproduce under these conditions with a generation time of about 16–24 h as do established cell strains, and chromosome analysis of the human cell lines uniformly yield, for 6 to 9 months and probably longer, the characteristic complement of $2n = 46$ (for normal diploid human cells) (see Fig. 10). The incidence of polyploidy and aneuploidy in these long-term cultures is close to or equal to that observed in primary human cultures (2% to 3%).

Evidence obtained by PUCK *et al.* indicates that heteroploid-cell transformation and concurrent non-multiplying episodes occurring in cells grown in conventional growth media are artificially induced *in vitro* by mitotic inhibitors present in adult mammalian sera. Adult mammalian sera contain a large amount of γ-globulin while fetal bovine serum is deficient in this protein fraction (see Fig. 11). Preliminary experiments[34] indicate that the γ-globulin fraction of adult serum proteins contains factors toxic to cell growth *in vitro*. These growth inhibitors present in adult sera (but not in fetal bovine serum) may be the major cause for the induction in basically diploid cell populations of multipolar spindel formation and other alterations of the normal course of mitosis which give rise to numerous polyploid and unbalanced aneuploid karyotypes (refractory state of growth), and lead to the final overgrowth of a heteroploid cell type with superior selective advantage (established altered cell strain). Thus experimental evidence indicates that heteroploid cell transformation *in vitro* is an artefact induced in the conventional suboptimal culture environment, and that normal (diploid) cells have basically a stable chromosomal constitution[43].

[41] T. C. HSU, C. M. POMERAT, and P. S. MOORHEAD, J. nat. Cancer Inst. *19*, 867 (1957).

[42] P. B. MEDAWAR, Biol. Rev. *22*, 360 (1947).

[43] Tumor cells may not have such stable properties.

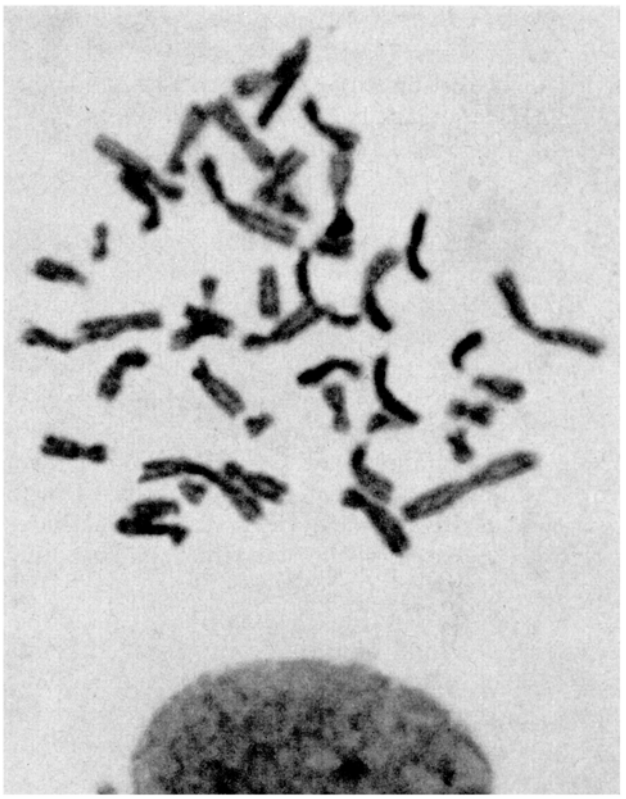


Fig. 10. Normal-diploid human chromosome complement (female), $2n = 46$, of fibroblast-like cells maintained for 6 months in culture. (Strain M-1).

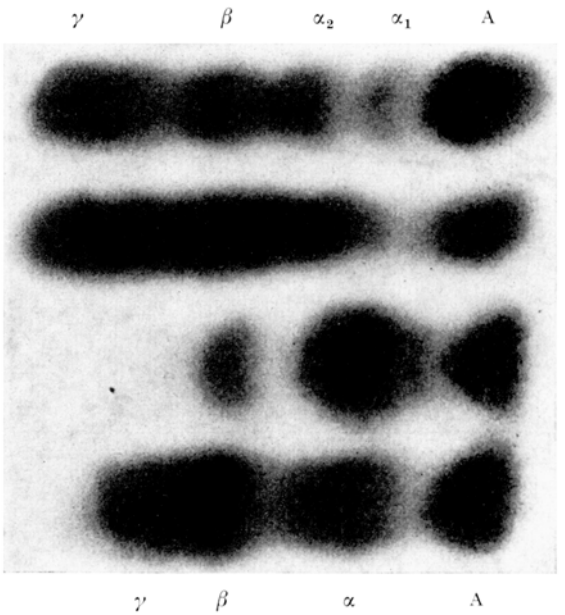


Fig. 11. Paper electropherograms of mammalian sera.
I: adult human serum. II: adult horse serum. III: fetal bovine serum. IV: adult bovine serum, i.e. calf serum. A: Albumin. α: α-globulin. β: β-globulin. γ: γ-globulin.

The possibility to maintain in long term culture mammalian cell strains with normal and stable chromosome constitution represents a major achievement which permits new approaches to fundamental problems of somatic cell variation and new uses of cell cultures for practical purposes. The elimination of the chromosomal alterations and the concurrent refractory state of growth permits systematic studies in somatic cell genetics or of the growth and metabolism of normal and malignant cells. Moreover, it may prove to be one of the keys to the establishment of functional-cell banks for therapeutic applications (bone marrow-cell banks, skin-cell banks) and for other uses (*in vitro* production of antibodies, hormones, and viruses).

The Requirements for Growth of Single Human Cells in vitro

The studies of EAGLE[8,9] and of others show clearly that both altered (heteroploid) and normal (diploid) human cells in serially propagated monolayer culture require for growth a minimum of 12 amino acids (L-arginine, L-cystine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, β-phenyl-L-alanine, L-threonine, L-tryptophane, L-tyrosine, L-valine), one half amide, L-glutamine, 9 vitamins (choline, folic acid, nicotinamide, pantothenic acid, pyridoxal, riboflavin, thiamine, biotin, i-inositol), five electrolyte ions (Na^+, K^+, Ca^{++}, Mg^{++}, $H_2PO_4^-$) and glucose (*minimum essential molecular growth factors*). In a minimal medium composed of these essential molecules and supplemented with a small amount of dialyzed mammalian serum (EAGLE's *basal growth medium*) a variety of human cell strains can be propagated indefinitely and with a generation time in the logarithmic growth phase approximating 20 to 24 h. In this minimal medium the omission of any single molecular growth factor leads to early death of the whole cell culture.

Additional nutritional requirements for growth, however, manifest themselves not only in different human cell strains but also under modified conditions of culture. Thus normal (diploid) human cells with fibroblast-like morphology have additional not yet clearly defined molecular requirements for sustained growth in dense monolayer cultures[34,35], on the other hand altered epithelial-like cells which multiply vigorously in heavily inoculated monolayer cultures and in basal growth medium fail to grow in the same minimal medium when the cultures are initiated with a small number of single cells.

The specific growth factors for single human cells have been determined in various altered human cell strains. Studies by the author are in progress to determine these factors in normal (diploid) human cells of epithelial-like morphology.

1. The effect of dialyzed serum on single Detroit-98C6b[44] *cells.* Heavily seeded static monolayer cultures of this cell line proliferate with a doubling time of about 24 h in the basal mixture of EAGLE supplemented with 10% of serum which has been dialyzed for 24 h against running saline at 4°C (basal growth medium). Attempts, however, to clone single cells according to the method of PUCK *et al.*[32,33,45] in the same growth medium, fail. In basal growth medium the rate and extent of cell multiplication decreases in proportion to the number of cells plated ('Dilution'-effect[32]). In the presence of dialized serum the cells attach to the glass surface but the majority of cells undergo only a limited number of divisions, forming abortive colonies, when plated out in small numbers (10^2 to 10^3 cells/10 ml medium).

Dialysis removes from serum the specific molecules essential for sustained growth of widely separated single cells but not essential for closely packed cells. Figure 12 illustrates the activity of serum as a function of time of dialysis. In these experiments fixed volumes of serum were dialyzed against 20 vol each of saline (6.8 g NaCl/l) at 7°C with agitation. Under these conditions the activity of serum decreases by approximately 70–80% during the first 30 min of dialysis, indicating that small or medium-sized molecules are composing the active molecular fraction of serum.

2. The effect of serum ultra-filtrate on single Detroit-98C6b cells. Heat treatment of adult horse serum ultrafiltrate results in the formation of a precipitate. Addition of the supernatant (protein free) ultrafiltrate fraction to basal growth medium restores the plating efficiency of single Detroit-98C6b cells to the value obtained in medium containing whole serum. Thus the active factors of serum are heat-stable molecules.

3. The 'feeder' effects of dense Detroit-98C6b cultures on single cells. Feeder-layer experiments carried out in basal growth medium demonstrate that actively growing cells in dense monolayer culture synthesize and release into the supernatant minimal nutrient the specific molecules necessary for sustained growth of widely separated cells in the same minimal medium[45].

4. The effects of defined molecules involved in the tricarboxylic acid cycle on single Detroit-98C6b cells. Pyruvate is an important metabolite involved in the TCA cycle and in the anaerobic conversion of glucose to lactate. Supplementation of the basal growth medium with sodium pyruvate at a concentration of 1 *mM*/l increases the plating efficiency in the average to about one-half of the standard value. In the presence of ATP (1 mg/l) or vitamin B_{12} (0.1 mg/l) the effect of pyruvate is reduced. Further supplementation with citric acid (30 mg/l), succinic acid (2 mg/l), lactic acid (100 mg/l), fumaric acid (2 mg/l) does not increase the effect of sodium pyruvate (1 *mM*/l) on the plating efficiency. Oxalacetic acid, another metabolite of the TCA cycle, does not show any activity in the presence of pyruvate.

5. The effects of nonessential amino acids and of pyruvate on single Detroit-98C6b cells. In cell strain HeLa and HeLa-S3 supplementation of the minimal medium (containing dialyzed serum) with the non-essential amino acid L-serine suffices to augment the plating efficiency of single cells to its standard value in medium containing whole serum[33]. In contrast to HeLa cells the plating efficiency of Detroit-98C6b cells is increased only to about one third of the standard value when the basal growth medium is supplemented with the seven non-essential amino acids L-alanine, L-aspartic acid, L-asparagine, L-glycine, L-glutamic acid, L-proline, and L-serine at concentrations of 0.2 *mM*/l. However, the addition to the basal growth medium of sodium pyruvate (1 *mM*/l) in combination with the seven non-essential amino acids or with L-serine alone at 0.2 *mM*/l yields plating efficiencies close to or in excess of the standard value and fully developed macroscopic clones (see Fig. 13 and 14).

6. Conclusions. Human cell strains have a basic and natural requirement for growth consisting of 12 amino acids, 1 half amide, 9 vitamins, 5 electrolytes, and glucose. These are the *minimum essential molecular growth factors.* They initiate, in the presence of serum protein, sustained growth of a variety of cell strains in heavily inoculated monolayer cultures. Widely separated single cells in small inocula of these cell strains have additional molecular requirements for sustained growth *in vitro* such as non-essential amino acids or pyruvate, depending upon the cell strain. These 'anomalous' requirements of small inocula—*which we define as quasi-essential growth factors*—are either (a) metabolites easily lost from the intracellular pool; (b) compounds which facilitate the biosynthesis of certain

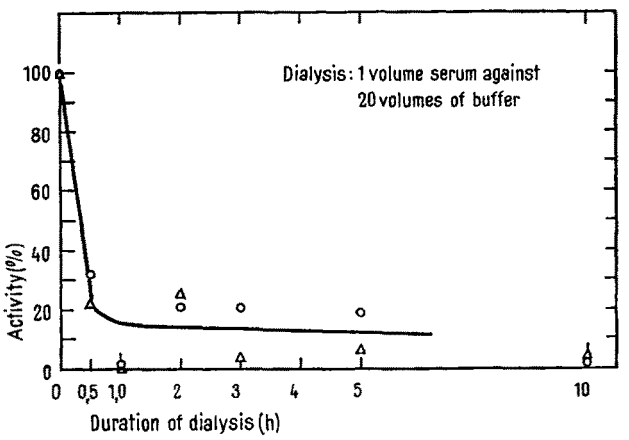
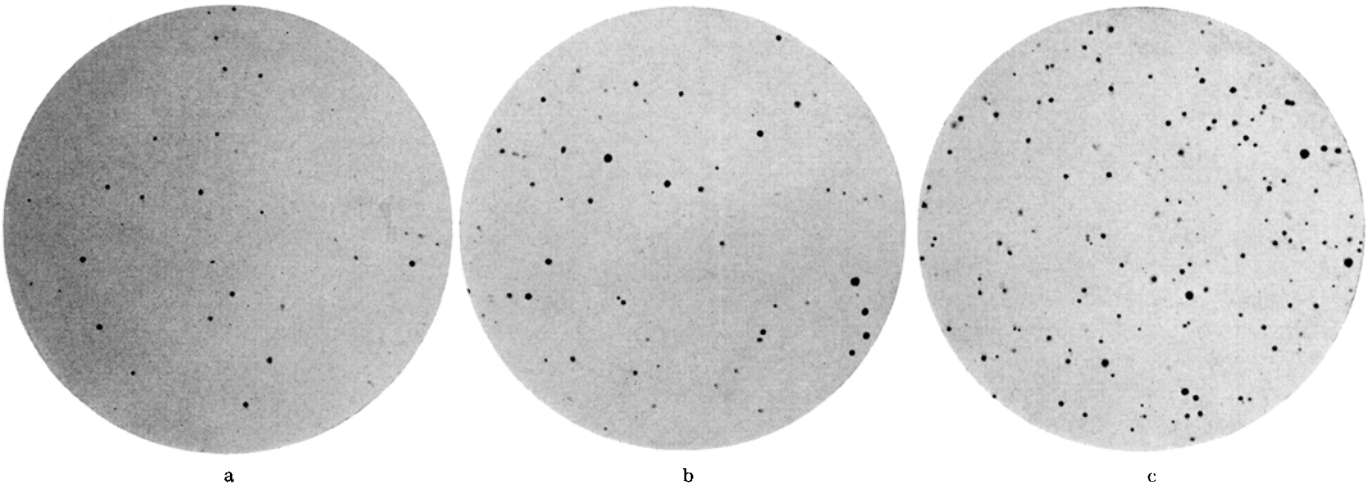


Fig. 12. Activity of dialyzed serum in promoting growth of single Detroit-98C6b cells as a function of duration of serum dialysis.

[44] A clonal derivative of the Detroit-98 strain (modified human sternal marrow).

[45] H. MOSER and C. MADDEN Annual Report of the Biological Laboratory, Long Island Biological Association, Cold Spring Harbor, L. I., N. Y., 69th year (1959).



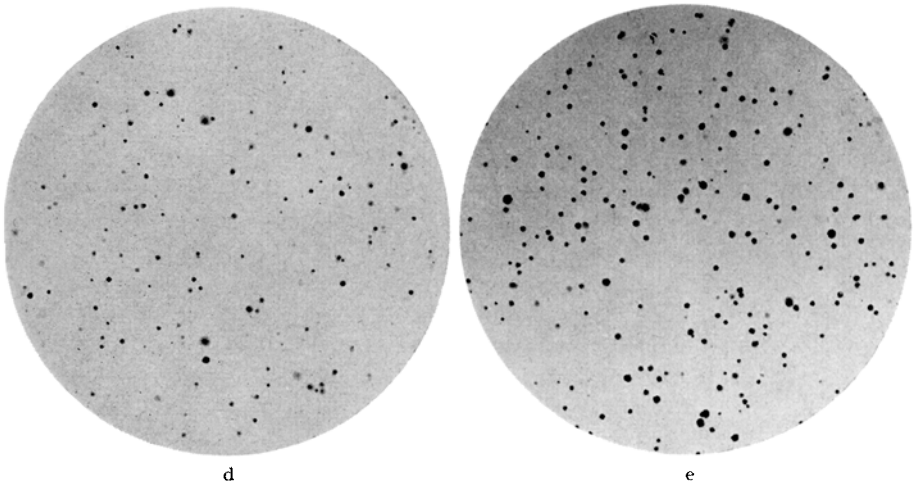


Fig. 13. The effect of defined molecules on the clonal growth of human cells (strain Detroit-98C6b) in basal growth medium (Eagle's basal mixture plus 15% dialyzed serum).

a) control (basal growth medium). b) nonessential amino acids. c) sodium pyruvate. d) nonessential amino acids plus pyruvate plus oxalacetic acid. e) nonessential amino acids plus whole molecular portion of serum.

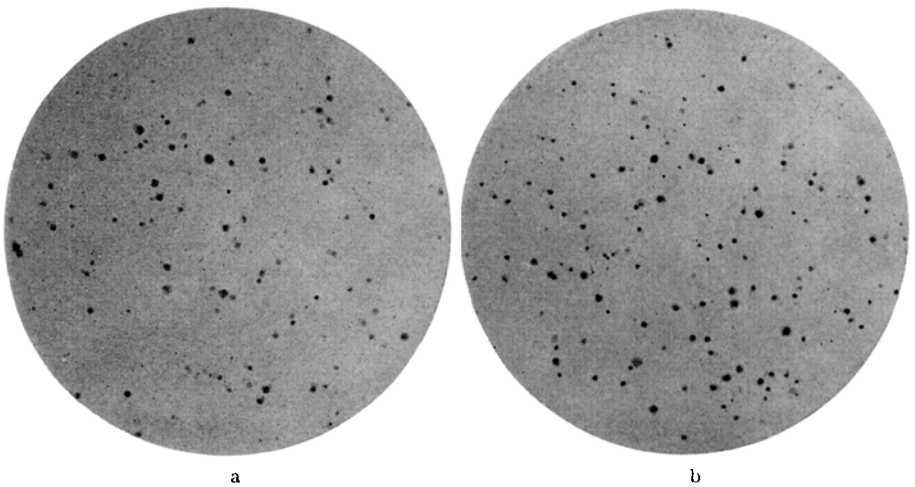


Fig. 14. The effect of defined molecules on the clonal growth of human cells (strain Detroit-98C6b) in basal growth medium.

a) control (standard cloning medium, i. e. Eagle's basal mixture plus nonessential amino acids plus 15% whole serum). b) Eagle's basal mixture plus 15% dialyzed serum plus L-serine plus sodium pyruvate.

metabolites; (c) compounds which increase the retention of metabolites by the cell, or (d) compounds which inhibit cytotoxic materials (such as proteolytic enzymes) excreted by widely isolated cells. These molecular quasi-essential growth factors or their active substitutes are provided by complete serum and lost upon serum dialysis.

The nature of the supplements to the basal growth medium which are active in promoting sustained growth of single somatic cells suggests that they are metabolites, and they or their precursors are either easily lost from single cells or have a small biosynthetic rate. Let us assume (see Fig. 15) that B is a compound which is synthesized from an essential amino acid (sup-

plied externally), A, at rate k_1, and is converted into C, a building block of proteins and of cell structure, at rate k_3. If B leaks from the cell into the external medium at rate k_2, synthesis of C and therefore of cell growth and multiplication is accomplished only if the biosynthetic rate k_1 exceeds the rate of removal of B from the intracellular pool into the external medium. The rate of leakage of B (per cell) is obviously a function of the surface area (per cell) exposed to the medium, and of the rate of accumulation of B in the external medium. Because the free surface area per cell in monolayer cultures decreases with increasing cell density—due to cell reaggregation and packing of cells—and because the accumulation rate of B in the media increases with increasing cell density, the rate of leakage increases with decreasing cell density in the inocula in a medium lacking B ('dilution' effect). However, if B is supplied with the nutrient fluid, leakage of B is reduced or suppressed because of concentration effects, and the intracellular B pool maintains a level adequate for continued synthesis of C and therefore for sustained growth of isolated single cells.

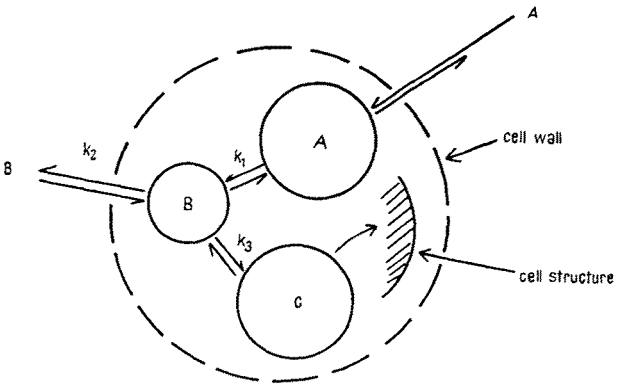


Fig. 15. Model of isolated mammalian cell.

Studies of the 'anomalous' nutritional requirements lead to the formulation of *minimal media for single mammalian cells*. The development of such growth media is stimulated particularly by their possible use for rapid selection of rare biochemical mutants in large cell populations ('selective media' for mammalian cells).

The Lethal and Semi-Lethal Effects of Radiations on Human Cells in Culture

The methods for rapid growth of single cells into well separated macroscopic colonies developed by PUCK *et al.* made possible a quantitative assessment of the lethal and semi-lethal effects of radiation on human cells under controlled environmental conditions. The effects of both ionizing radiation[46-48] and of ultraviolet light[32,48,49], have been studied in this fashion in a variety of human somatic cell strains.

1. The fate of cells irradiated with X-rays or ultraviolet light. The irradiation of plates seeded with singel mammalian cells destroys, in a fraction of the cell population (specific survival fraction) the capacity of a single cell to grow into a macroscopic colony (composed of from 10^3 to 10^4 cells). The fate of irradiated cells that fail to form visible colonies is the following: An irradiated cell may form an *abortive colony*, that is, it may undergo a limited number of cell divisions to form a self-sustaining microcolony of less than 50 cells. An irradiated cell may fail to divide at all, simply disintegrate without leaving a trace on the stained plate, or grow into a single *giant cell* (see Fig. 16). The fraction of giant cells increases with increasing radiation dose. Exposure of a plate seeded with about 10^4 HeLa cells to an X-ray dose of 1000–5000 r will, after incubation for two weeks yield a population consisting entirely of giant cells[46]. Giant cells may survive for long periods without regaining their capacity to divide even in regularly renewed nutrient, and are highly susceptible to infection and destruction by virus[46]. Observations by the author indicate that X-rays induce a higher proportion of giant cells and abortive colonies than does a corresponding dose[50] of ultraviolet radiation.

2. Ultraviolet survival curves. By application of the plating methods described earlier, and with further modification of those techniques MOSER and TOMIZAWA[32] determined the survival curves of ultraviolet treated cells of the following hyperploid epithelial-type cell strains: Detroit-98 (derived from human adult sternal marrow), Detroit 98C6b (clonal derivative of Detroit-98), D-189 (malignant human foreskin of Leighton), Hayflick-Fernandez strain derived from human amnion. Figures 17, 18, 19 represent curves in which the specific survival fraction[51], S_D, is plotted on a logarithmic scale against the incident dose of ultraviolet light, measured in mW-sec cm^{-2}. (1 mW-sec cm^{-2} = 100 ergs mm^{-2}).

When plotted in this fashion, the UV-survival curves of single heteroploid somatic cells exhibit two distinct sections, a log-linear portion with an initial shoulder, and a non-log-linear section manifesting itself at high UV doses. The existence of an initial shoulder in all the UV-survival curves indicates a multiple-hit-killing mechanism of UV irradiation in heteroploid human somatic cells. The first portion of the survival curves fits the equation $S_D = 1 - (1\text{-exp}\ (-\ kD))^n$ where D is the

[46] T. T. PUCK and P. I. MARCUS, J. exp. Med. *103*, 653 (1956).
[47] T. T. PUCK, D. MORKOVIN, P. I. MARCUS, and S. J. CIECIURA, J. exp. Med. *106*, 485 (1957).
[48] T. T. PUCK, Amer. Nat. *94*, 95 (1960).
[49] H. H. LEE and T. T. PUCK, Radiation Res. *9*, 142 (1958).
[50] I. e. a dose that produces an equal fraction of colonyforming survivors.
[51] If $S(0)$ stands for the survival fraction at dose $D = 0$ (controls), $S(D)$ for the survival fraction at dose D, the specific survival fraction for D is defined as $S_D = S(D)/S(0)$.

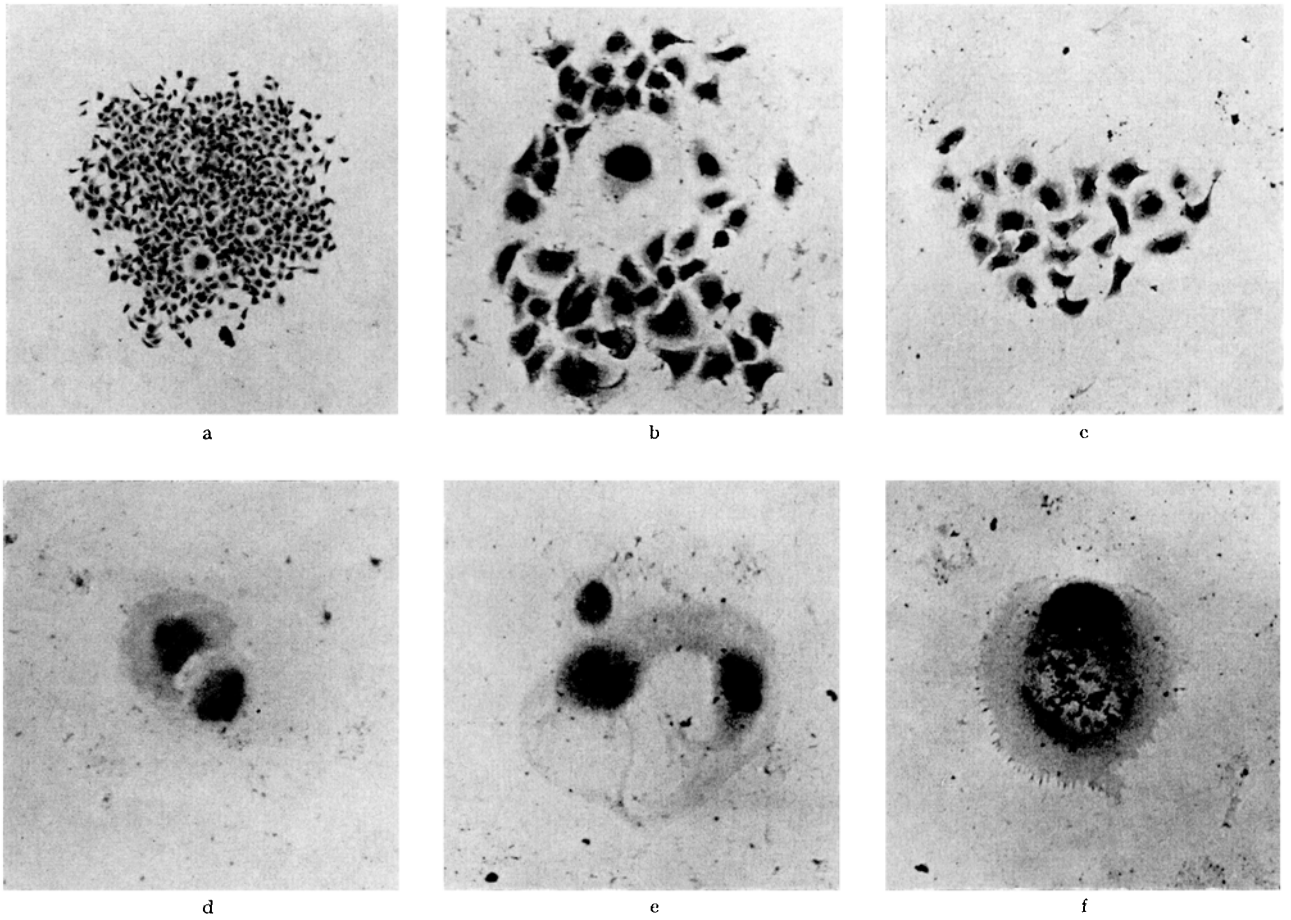


Fig. 16. Fates of ultraviolet irradiated epithelial-like human cells (altered-heteroploid strain Detroit-98C6b).

a) macroscopic colony formed by UV survivor. b) abortive colony with single giant cell. c) abortive colony without giant cell. d, e) multinucleate giant cells. f, g) mononucleate giant cells.

Tab. II. Parameters of UV Survival Curves.

Strain	n	D_0 (mW-sec cm^{-2})	D_x (mW-sec cm^{-2})	S_x
D-189	2.5	0.775	5.94	1.0×10^{-3}
Human Amnion	1.5	1.070	4.95	1.4×10^{-2}
Detroit-98	8.5	0.684	5.40	3.0×10^{-3}
Detroit-98C6b (clonal strain)	3.0	1.000	4.95	2.0×10^{-2}

UV dose, k a constant measuring the radiation sensitivity, and n the hit number. The hit number is determined by extrapolation of the straight line of the log-linear portion of the curve to its intercept of the S_D axis, $S_0^* = n$. The value of k is determined from the slope of the log-linear portion of the curve. The constant k is related to the mean lethal dose, D_0, i.e. the dose which reduces, in the long-linear portion of the curve, the survival fraction to e^{-1} (or 37%).

Our analysis of the UV survival curves in hyperploid human cells yields hit numbers varying from 1.5 to 8.8 and mean lethal doses, D_0, varying from 78 to 107 ergs mm^{-2} (Table II). At a critical UV dose, D_x, which corresponds to a specific survival fraction, S_x, the radiation sensitivity begins to change as a function of UV dose. The dose at which radiation resistance 'develops' in UV-irradiated heteroploid somatic cells depends on the cell strain tested, and varies in our case from 500 to 600 ergs mm^{-2}.

Although the survival curves as such lend themselves only to a limited understanding of the UV-killing mechanism, the strain-specifity of their parameter values indicates the existence of differences in the genetic apparatus of the cells belonging to different heteroploid

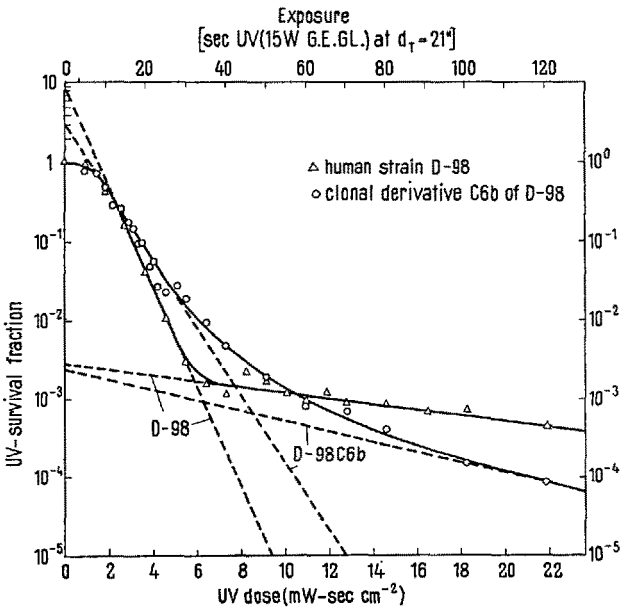


Fig. 17. Ultraviolet survival curves of strains Detroit-98 and of clonal derivative Detroit-98C6b.

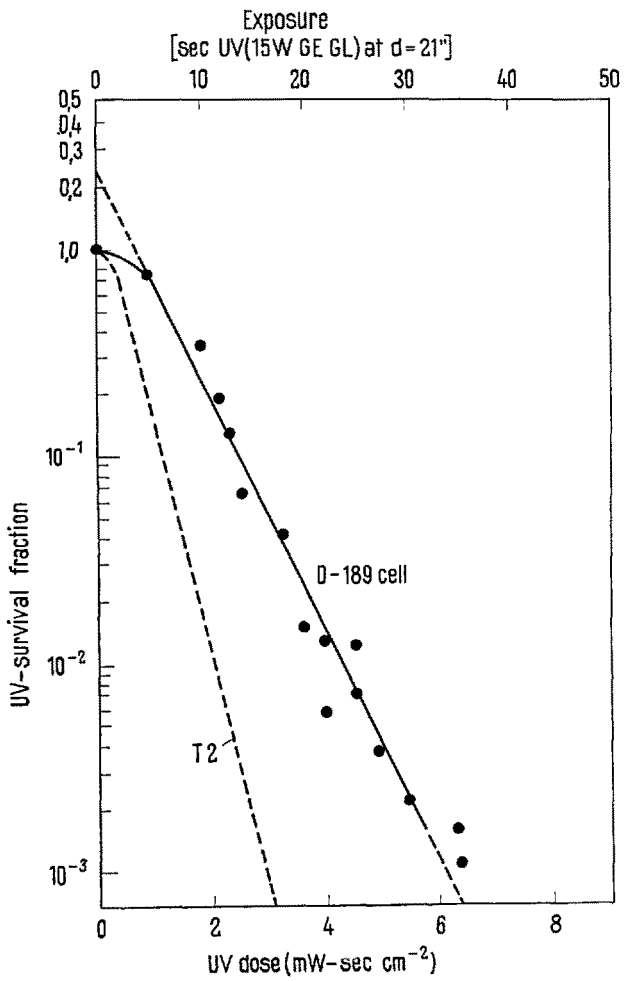


Fig. 18. Ultraviolet survival curve of human strain D-189 and of bacteriophage T2.

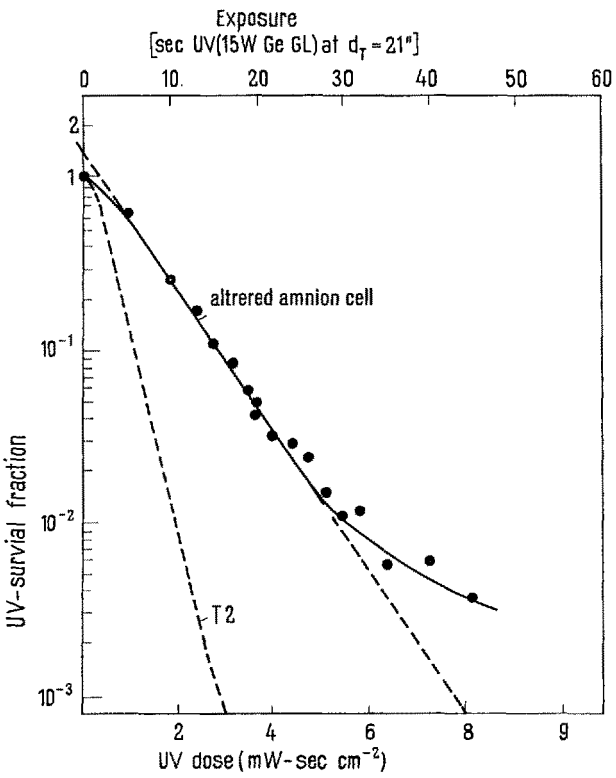


Fig. 19. Ultraviolet survival curve of human amnion-cell strain Hayflick-Fernandez and of bacteriophage T2.

strains. Cytological observations support this conclusion.

X-ray survival curves. The studies of PUCK *et al.*[46–48] demonstrate that also X-ray treated human cells *in vitro* display multi-hit type survival curves. While the hyperploid cells yield hit numbers close to 2 the diploid-fibroblast-like cells have a hit number close to one. The mean lethal dose which is about 100 r for the altered heteroploid cells and only about 50 r for the diploid cells indicates the 'protective' effect of supernumery chromosomes on the reproductive death of human cells which have been exposed to ionizing radiation.

Acknowledgments. The author is indebted to Dr. S. GRAFF, Professor of Biochemistry, for critical review of the manuscript, and to Mrs. ADA GRAFF and her staff for the preparation of the paper electropherograms presented in this publication. For the photographic work the author wishes to thank Mr. E. R. HAJJAR of Columbia University, Mr. H. JONES of the Carnegie Institution of Washington (Fig. 16) and Dr. M. K. DUTT of the Carnegie Institution of Washington (Fig. 10).

Zusammenfassung

Der Verfasser gibt eine kurze Übersicht über die neueren Methoden der *in vitro*-Kultur von Säugerzellen. Eingehend wird die Entwicklung von stabilen Kulturen diploider Zellen und Untersuchungen über die spezifischen Wachstumsfaktoren isolierter menschlicher Zellen besprochen. Auch die Wirkung der Ultravioletttstrahlung auf solche Zellen wird diskutiert.