Extracellular Regulation of Fibroblast Multiplication: A Direct Kinetic Approach to Analysis of Role of Low Molecular Weight Nutrients and Serum Growth Factors

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The principles of enzyme kinetic analysis were applied to quantitate the relationships among serum-derived growth factors, nutrients, and the rate of survival and multiplication of human fibroblasts in culture. The survival or multiplication rate of a population of cells plotted against an increasing concentration of a growth factor or nutrient in the medium exhibited a hyperbolic pattern that is characteristic of a dissociable, saturable interaction between cells and the ligands. Parameters equivalent to the K_m and V_{max} of enzyme kinetics were assigned to nutrients and growth factors. When all nutrient concentrations were optimized and in steady state, serum factors accelerated the rate of multiplication of a normal cell population. The same set of nutrients that supported a maximal rate of multiplication in the presence of serum factors supported the maintenance of non-proliferating cells in the absence of serum factors. Therefore, under this condition, serum factors are required for cell division and play a purely regulatory role in multiplication of the cell population. The quantitative requirement for 18 nutrients of 29 that were examined was significantly higher (P < 0.001) for cell multiplication in the presence of serum factors than for cell maintenance in the absence of serum factors. This indicated specific nutrients that may be quantitatively important in cell division processes as well as in cell maintenance. The quantitative requirement for Ca^{2+}, Mg^{2+}, K^+, Pi, and 2-oxocarboxylic acid for cell multiplication was modified by serum factors and other purified growth factors. The requirement for over 30 other nutrients could not clearly be related to the level of serum factors in the medium. Serum factors also determined the Ca^{2+}, K^+, and 2-oxocarboxylic acid requirement for maintenance of non-proliferating cells. Therefore, when either Ca^{2+}, K^+, or 2-oxocarboxylic acid concentration was limiting, factors in serum played a role as cell "survival or maintenance" factors in addition to

their role in cell division as "growth regulatory" factors. However, with equivalent levels of serum factors in the medium, the requirement for Ca^{2+}, K^+, and 2-oxocarboxylic acids was still much higher for multiplication than for maintenance. Kinetic analysis revealed that the concentrations of individual nutrients modify the quantitative requirement for others for cell multiplication in a specific pattern. Thus, specific quantitative relationships among different nutrients in the medium are important in the control of the multiplication rate of the cell population. When all nutrient concentrations were optimal for multiplication of normal cells, the multiplication response of SV40-virus-transformed cells to serum factors was similar to that of normal cells. When serum factors were held constant, transformed cells required significantly less (P < 0.001) of 12 of the 26 nutrients examined. Therefore, the transformed cells only have a growth advantage when the external concentration of specific nutrients limits the multiplication rate of normal cells. Taken together, the results suggest that the control of cell multiplication is intimately related to external concentrations of nutrients. Specific growth regulatory factors may stimulate cell proliferation by modification of the response of normal cells to nutrients. Transforming agents may confer a selective growth advantage on cells by a constitutive alteration of their response to extracellular nutrients.

Key words: cell growth, nutrients, growth factors, transformation, cloning, kinetic analysis

The behavior of most normal cells in culture is controlled by nutrients, non-nutritive, diffusible regulatory factors (polypeptides and steroids), and the extracellular substrate or matrix [for recent reviews, see references 1–7]. A full understanding of the interaction of cells with factors within each domain is essential to understand the mechanisms that control cell growth and function. Single nutrients, non-nutritive growth factors, and matrix components that are required by a number of cell types have been identified. Because of the relative ease of isolation and characterization, the identity of individual nutrient requirements is most complete [6]. More recently, a number of non-nutritive regulatory factors that affect cells in culture have been identified [2, 7]. Studies on the identification of the extracellular matrix components that affect the behavior of cells in culture are in their early stages [8–10]. Despite progress in the qualitative identity of individual factors in each domain of the environment, quantitation of the behavior of cells in response to individual factors has received little consideration.

In this paper we review application of the principles that are followed in the analysis of enzyme kinetics to analyze the interplay between environmental factors and the behavior of cells in culture. We demonstrate the usefulness of the approach in quantitating the requirements for nutrients and regulatory factors in cell maintenance and proliferation, in studying the relationships between nutrients and regulatory factors, and in describing differences between normal and transformed cells in their response to both regulatory factors and nutrients.

MATERIALS AND METHODS

Cells

Cell strains WI-38 (ATCC CCL75), MRC-5 (ATCC CCL171), and FLOW 2000 (lot 32103, Flow Laboratories, Rockville, MD) were used as representative normal human lung fibroblasts. Stock cultures were used at passage doubling levels 15 to 20. Throughout the text, these strains are referred to as N-HLF. SV40 virus-transformed WI-38 (subline 2RA,

ATCC CCL75.1) was used as a transformed cell prototype and is referred to as SV-HLF in the text. A strain of normal human foreskin fibroblasts (ERAL-7) was used to study the multiplication response to epidermal growth factor (EGF). N-HLF were refractory to EGF under our culture conditions. Mycoplasma contamination and karyotype were monitored by standard methods.

Assay Media and Culture Conditions

All tissue culture methods, the development and preparation of optimal test media of the MCDB 100 series, and the macromolecular fraction of fetal bovine serum (FBSP) have been described elsewhere [11–16]. FBSP was used as a convenient and complete source of factors that increase the rate of cell multiplication above that supported by an optimal set of low molecular weight nutrients and defined culture conditions [11, 15, 17].

Cell Multiplication Assay

The assay was the clonal multiplication assay that was developed specifically for human diploid fibroblasts by McKeehan et al [14]. One hundred cells harvested at 4°C [13] from a stock monolayer culture were inoculated into 60-mm polylysine-coated plastic Petri dishes [12] containing 5.0 ml of the medium indicated in the text. Plates were incubated in a humidified atmosphere of 5% carbon dioxide and 95% air when medium MCDB 104 was used and in 2% carbon dioxide and 98% air when media MCDB 105 to 107 were used. Incubations were carried out at 37°C for the times indicated in the text. The medium was then removed and the cells were fixed and stained [14] or harvested by trypsinization for counting.

Cell Maintenance Assay

Cell maintenance was assessed as follows: 100 cells were inoculated into Petri dishes containing the maintenance test medium. After 4 to 7 days of incubation, the test medium was removed and replaced with standard growth medium (ie, medium MCDB 104 to 107 containing 500 μg FBSP per ml). After 10 days of additional incubation, the cells were fixed and stained [14]. The total number of colonies were counted. Each colony represents a single cell and its progeny, if any, that remained attached and viable during the first incubation and capable of proliferation in the second incubation. The requirement for maintenance was defined as the extracellular concentration ($S_{0.50}$) of the component under test in the first incubation that was required to support the attachment and viability of 50% of the maximal number of clone-forming cells that resulted in the second incubation when the test component was saturating in the first incubation. The data (number of clone-forming survivors versus substrate concentration) were treated by the Henri-Michaelis-Menten and Lineweaver-Burk methods as described below for the multiplication assay.

Calculation of Kinetic Parameters

Colony size in photocell units was converted to multiplication rate by the formula

$$r = \frac{3.32 \,(\log_{10} C_t - \log_{10} C_o)}{t}$$

where r = cell generations per day; 3.32 = reciprocal $\log_{10} 2$; C_t = colony size in photocell units at the end of t days; C_o = colony size in photocell units when t = 0 and t = time of

incubation in days. C_0 was determined as described in the Appendix. For N-HLF and ERAL-7 cells, C_0 = 0.080 photocell units and for SV-HLF, C_0 = 0.11 photocell units.

Multiplication rate, r, at various concentrations of the substrate under consideration was substituted for V (velocity) and S (substrate) in the Henri-Michaelis-Menten formula used in enzyme kinetic analysis. Experimental multiplication rates for various concentrations of substrate were fitted to a right rectangular hyperbola described by the Henri-Michaelis-Menten equation. Kinetic parameters, $S_{0.50}$ and R_{max}, for each extracellular condition were estimated from the Lineweaver-Burk equation or other methods of linearization of the Michaelis-Menten equation. In a typical experiment, multiplication rate, r, was determined from the mean colony size in triplicate dishes for a series of 6 different substrate concentrations. A "least squares" straight-line equation was fitted to the $1/r$ versus $1/[S]$ data or other forms of the data for different linearization methods. $S_{0.50}$ and R_{max} were determined from the $1/r$ and $-(1/[S])$ intercepts, respectively. The standard error of $S_{0.50}$ and R_{max} was estimated by Gaussian analysis of variance due to the linear regression analysis and deviations from linearity of the $1/r$ versus $1/[S]$ data.

Definition of Terms

Throughout the text, the requirement of a substrate for *cell multiplication* refers to the concentration (the $S_{0.50}$ value) required to support a half-maximal *rate of cell multiplication*. *Cell multiplication rate*, r, in cell generations per day describes the doubling rate of the test population of cells. Cell multiplication rate reflects the average interdivision time per cell that divides during the assay and the rate of cell death and detachment from the culture dish.

Maintenance refers to support of attachment of cells to the culture substrate and the support of cells in a viable state where they can undergo multiple cycles of division under the appropriate conditions.

A *regulatory* role of an extracellular component in cell multiplication was inferred when the requirement for cell multiplication was significantly different from the requirement for maintenance. If the $S_{0.50}$ value for multiplication was identical to the $S_{0.50}$ value for maintenance, then it was assumed that the requirement for cell multiplication reflected the requirement for maintenance of attachment and viability of single cells. That component under that condition does not play a regulatory role in cell multiplication.

A *zero order response* of cells to the addition of an extracellular factor means that increasing levels of the factor in the medium had no detectable stimulatory effect on the rate of maintenance or multiplication. A zero order response indicated that cells did not require the component or that background levels in the medium, intracellular stores or cellular synthesis, were adequate to support the maximal rate of maintenance of multiplication under the test condition.

Nutrient refers to inorganic salts that originate from the environment and low molecular weight biogenic substances that originate from the diet or cells in the organism. *Growth factor* or *growth regulatory factor* refer to steroid or polypeptide hormone-like factors or other yet undefined, biologically derived, non-nutritive factors that are in body fluids.

RESULTS AND DISCUSSION

Application of the Principles of Enzyme Kinetic Analysis to Cell Growth in Culture

To reproducibly quantitate the multiplication response of cells to extracellular variables, we designed an experimental system that fits the assumptions that are common to

enzyme kinetic analysis. Two other laboratories have also applied this approach to deline-
ate interrelationships among culture variables that affect cell proliferation. Ellem and
Gierthy [18] applied the principles to study the effect of serum concentration and cell
density on DNA synthesis in "quiescent" dense cultures of human diploid fibroblasts. In
model systems similar to those used in our studies, Lechner and Kaighn [19–21] used the
approach to study interactions among serum-derived and purified mitogens, Ca^{2+}, phorbol-
esters, and proliferation of normal and tumorigenic prostatic epithelial cells. The approach
is documented and discussed in the Appendix.

Kinetic Analysis of the Quantitative Requirement for Serum Factors for Multiplication

Multiplication of a population of normal diploid human lung fibroblasts (N-HLF)
requires nutrients and macromolecular factors that are commonly in serum. By systematic
replacement of the direct nutritive roles of crude serum with a set of defined nutrients, the
requirement for serum-derived factors for multiplication was reduced to a small fraction
of the nondialyzable macromolecules (FBSP) in it [15, 17]. The set of defined nutrients
that was optimal for multiplication in the presence of FBSP also supported the maintenance
of cells in the absence of FBSP or other growth factors [15, 17]. Thus, under this condi-
tion, the role of factors in FBSP in multiplication can be considered "regulatory." The
active factors at play in the FBSP affect cellular processes that are involved specifically in
cell division relative to processes involved in basic maintenance of viable cells. A kinetic
analysis showed that the multiplication response of cells to growth factors in FBSP is
clearly biphasic (Fig. 1). The two phases of the response are so well separated that each
hyperbolic region of the response was analyzed independently, and two sets of kinetic
parameters were assigned to FBSP (Fig. 2). The biphasic response may indicate 1) two
classes of active factors for which the cellular requirement is different; 2) two classes of
cells in the test population that have different $S_{0.50}$ values for the same factor(s); 3) two
cellular processes that affect multiplication rate and whose quantitative requirements for
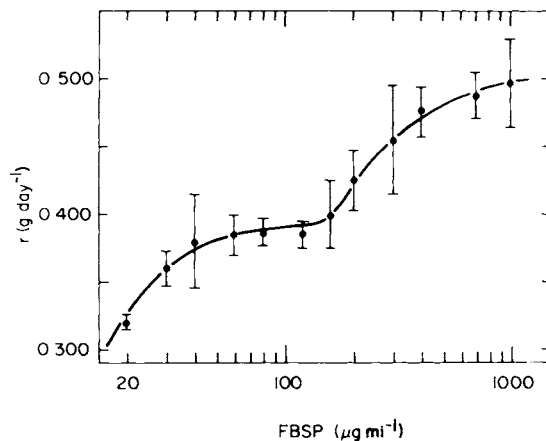

FBSP $(\mu g\,ml^{-1})$

Fig. 1. Effect of FBSP on rate of multiplication of N-HLF. The indicated concentrations of FBSP
were added to medium MCDB 105 containing 100 N-HLF (PDL 15 to 20). After 14 days of incuba-
tion, colony size in photocell units was measured and converted to multiplication rate, r, in cell genera-
tions (g) per day as described in Materials and Methods. Data from four individual experiments were
combined. Each point is the mean ±SE of triplicate dishes. A right rectangular hyperbola was fitted
to data from 20 to 120 μg per ml and another to the data for 175 to 1,000 μg FBSP per ml.
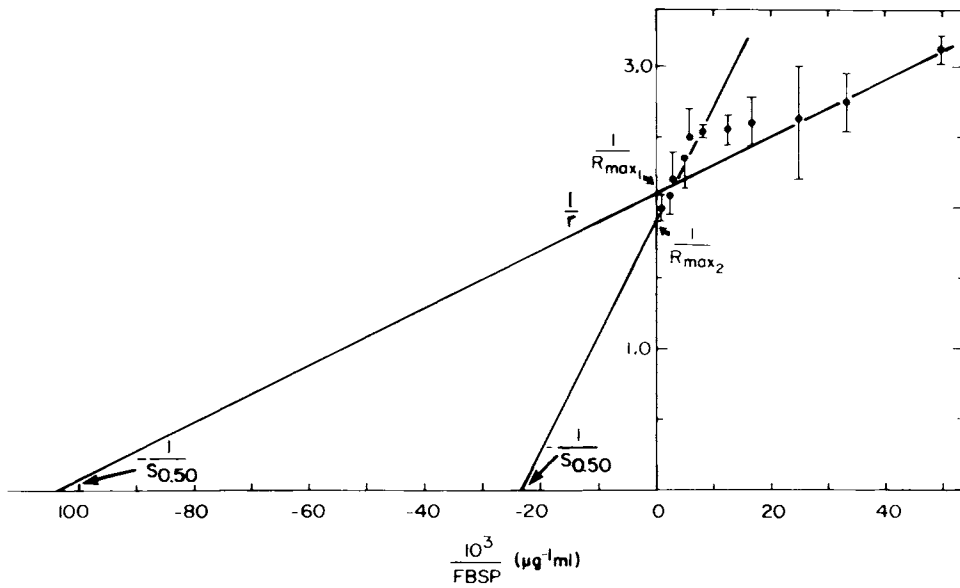
Fig. 2. Double-reciprocal (Lineweaver-Burk) transformation of multiplication rate of N-HLF versus FBSP concentration. The data from Figure 1 were transformed to $1/r$ and 10^3 ml μg^{-1}/FBSP. Since the data in Figure 1 are clearly biphasic, two linear plots were fitted to the data by "least squares" estimate. Regression of both plots yielded two sets of kinetic parameters. The ordinate intercepts yielded the maximal growth rate (R_{max}) possible under the condition. The abscissa intercepts yielded the FBSP concentration ($S_{0.50}$) where multiplication rate was half-maximal. The kinetic parameters for the data were $S_{0.50} = 9.7$ μg ml^{-1}, $R_{max} = 0.475$ g day^{-1} for the first-phase response and $S_{0.50} = 43.1$ μg ml^{-1}, $R_{max} = 0.520$ g day^{-1} for the second phase of the response.

growth factor(s) are different; or 4) a "negative cooperative" interaction between multiple sites of action of factor(s) [22]. Since FBSP is a crude mixture of growth regulatory factors, the biphasic response may simply indicate two classes of factors for which the cellular requirement for multiplication is markedly different (postulate 1 above). The first phase may indicate primarily the cellular response to a factor with the lowest $S_{0.50}$ value. As the concentration of FBSP increases, a factor with a higher $S_{0.50}$ value comes into play to further stimulate multiplication rate. However, Figure 3 shows that the response to purified epidermal growth factor (EGF) in the ng per ml range was similar in nature to that exhibited by the response to crude FBSP in the μg per ml range. The two regions of the multiplication response of human foreskin fibroblasts to EGF were so marked that an effect of EGF on multiplication would be overlooked if the factor were added to the medium only between 1 and 20 ng per ml. Since the manufacturer demonstrated a high degree of purity for the EGF preparation [24], it was unlikely that the biphasic response represents the activity of two chemically different growth factors. Furthermore, since the test population of cells was relatively pure, we favor possibilities 3 or 4 above to account for the biphasic multiplication response to growth factors. The biphasic behavior in cell multiplication rate may be related to the negative cooperative interaction among receptor sites for EGF and other growth factors that has been observed in isolated factor-receptor binding studies [25–28]. However, the observed phenomena may reflect cooperative interactions between cellular processes that affect multiplication rate but are far removed
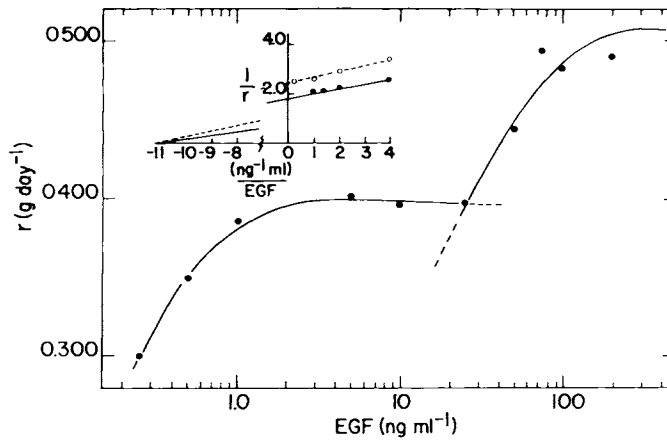
Fig. 3. Multiplication response of human foreskin fibroblasts to epidermal growth factor (EGF). Clonal multiplication of 100 ERAL-7 cells was analyzed after 14 days of incubation in medium MCDB 107 at pH 7.4 [23] containing 250 μg FBSP per ml and the indicated amounts of EGF (Collaborative Research, Waltham, MA). The indicated stimulation of multiplication rate by EGF was above that supported by medium MCDB 107 plus the FBSP. Stimulation by EGF absolutely required the presence of FBSP. The inset is the double-reciprocal plot of the same data. Data for the first phase of the response (broken line, open circles) is plotted on an abscissa of the units indicated. The phase of the response indicates an $S_{0.50}$ of 88 ± 13 pg EGF per ml and a R_{max} of 0.409 ± 0.008 g per day. Data from the second phase (solid line, solid circles) have an abscissa of 10^{-2} × the units indicated. These data yield an $S_{0.50}$ of 9.0 ± 1.4 ng EGF per ml and a R_{max} of 0.535 ± 0.013.

from the initial growth factor-receptor interactions. Regardless of the underlying mechanism, the biphasic multiplication response suggests that as multiplication rate increases as a consequence of increasing levels of growth factors in the medium, there is an increase in the quantitative requirement for the factors to increase growth rate further.

Kinetic Analysis of the Requirement for Nutrients for Multiplication

In contrast to FBSP and EGF, the multiplication rate of normal fibroblasts plotted against the concentration of most nutrients was fitted to a single hyperbola (niacinamide [29] was an exception) and a single $S_{0.50}$ value was assigned. Table I lists the $S_{0.50}$ values for individual nutrients for multiplication of N-HLF when all other nutrients and FBSP were held constant. $S_{0.50}$ values for amino acids ranged from 5.6 nM for tryptophane to 16 μM for glycine. The average $S_{0.50}$ value for the 15 amino acids indicated in Table I was about 4 μM. The multiplication response to alanine, aspartic acid, asparagine, glutamic acid, and proline was zero order under a variety of conditions tested (not shown). This indicates that cellular synthesis of these 5 amino acids was adequate to sustain a maximal rate of proliferation. $S_{0.50}$ values for the major ions ranged from about 1 μM for Pi to 510 μM for K^+. The assay system was sensitive enough to assign kinetic constants to two trace nutrients, Fe and Se. During the course of the study, the multiplication response of N-HLF to water- and fat-soluble vitamins, H^+, Na^+, Cl^-, SO_4^{2-}, HCO_3^-, CO_2, O_2, other trace elements, and over 100 other single metabolites, hormones, and growth factors was examined with different levels of FBSP in the medium. Although a variety of these agents detectably affected multiplication rate, the cellular response was either of insufficient magnitude for kinetic analysis or the response did not fit the Michaelis-Menten equation.

TABLE I. Kinetic Constants for Human Lung Fibroblasts[‡]

	$S_{0.50}$ values for multiplication of N-HLF	df	$S_{0.50}$ values for maintenance of N-HLF	df	$S_{0.50}$ values for multiplication of SV-HLF	df
FBSP[a]	4.3 ± 3.5	30	*Zero order	—	13.7 ± 12.7	9
	43.4 ± 26.6	30	*Zero order	—	43.7 ± 34.0	9
Arg	1.5 (±0.20) E-6	4	1.1 (± 0.10) E-6	4	1.3 (±0.10) E-6	4
Cys	2.4 (±0.83) E-6	9	3.3 (± 1.1) E-6	6	*9.3 (±0.73) E-7	6
Gln	1.1 (±0.18) E-5	5	*Zero order	—	*1.1 (±0.10) E-6	4
Gly[b]	1.6 (±1.2) E-5	9	*Zero order	—	***6.8 (±1.6) E-6	4
His	1.5 (±0.30) E-6	9	*1.1 (±0.10) E-8	4	*5.0 (±0.90) E-7	4
Ile	4.0 (±1.6) E-7	6	***2.1 (±0.20) E-7	4	***1.8 (±0.50) E-7	4
Leu	5.2 (±3.1) E-7	12	***1.1 (±0.10) E-7	4	3.8 (±0.20) E-7	4
Lys	1.5 (±0.30) E-5	4	*1.7 (±0.10) E-6	4	1.6 (±0.70) E-5	4
Met	9.6 (±2.2) E-8	4	***6.4 (±0.50) E-8	4	****4.9 (±2.0) E-8	4
Phe	3.0 (±0.95) E-6	4	*Zero order	—	*9.5 (±0.24) E-8	9
Ser	8.0 (±0.10) E-7	4	*Zero order	—	NA	—
Thr	1.8 (±0.30) E-6	12	2.0 (±0.10) E-6	4	*1.1 (±0.58) E-6	8
Trp	5.6 (±1.5) E-9	6	*Zero order	—	*Zero order	—
Tyr	1.4 (±0.35) E-7	9	*3.5 (±11.60) E-9	4	***2.0 (±0.47) E-7	6
Val	1.5 (±0.60) E-6	4	9.5 (±5.2) E-7	4	4.1 (±0.30) E-6	4
Ade[b]	1.4 (±0.08) E-8	6	*Zero order	—	*Zero order	—
Choline	9.5 (±1.5) E-7	4	1.2 (±0.53) E-6	6	*2.0 (±0.70) E-8	4
dThd[b]	1.3 (±0.60) E-8	4	*Zero order	—	*Zero order	—
Glc	2.7 (±0.92) E-5	6	***1.3 (±0.79) E-5	4	3.5 (±1.2) E-5	6
Inositol	3.3 (±0.60) E-7	6	*Zero order	—	*1.1 (±0.14) E-7	6
Niacinamide[c]	7.0 (±0.60) E-10	4	*6.0 (±4.0) E-10	4	NA	—
	9.2 (±2.1) E-8	4			NA	—
Pyruvic acid	3.4 (±0.71) E-6	15	*1.2 (±0.10) E-4	4	2.9 (±0.33) E-6	6
Ca²⁺	1.3 (±0.49) E-5	9	*1.8 (±0.20) E-6	4	2.4 (±0.61) E-5	12
Mg²⁺	3.5 (±1.5) E-5	9	*2.3 (±0.90) E-6	6	*2.5 (±0.92) E-6	12
K⁺	5.1 (±2.2) E-4	21	*1.3 (±0.50) E-3	6	3.2 (±2.0) E-4	13
Pi	1.0 (±0.34) E-6	15	*2.3 (±0.70) E-7	4	***6.7 (±0.95) E-7	6
Fe	3.3 (±0.90) E-8	4	*Zero order	—	*Zero order	—
Se	2.7 (±0.50) E-10	4	*Zero order	—	*Zero order	—
Lipid[d]	4.5 (±1.4) E-9	4	*Zero order	—	NA	—

†Kinetic constants for each indicated variable were determined as described in Materials and Methods. Determinations for FBSP were carried out in complete medium MCDB 104 to 107. Kinetic analysis for each nutrient was carried out in medium MCDB 104 to 107 minus the nutrient consideration. Multiplication assays for nutrients contained 250 μg FBSP per ml. Maintenance assays were carried out in FBSP-free medium MCDB 104 to 107 as described in Materials and Methods. The values indicated were assigned from pooled results obtained by the authors over a period of 4 years at the University of Colorado, Boulder, and the W. Alton Jones Cell Science Center, Lake Placid, NY. The $S_{0.50}$ value is the concentration of the component that promotes a half-maximal rate of cell multiplication or maintenance. Except for FBSP, $S_{0.50}$ values are in units of moles per liter. An abbreviated exponential notation is used here and throughout the text. For example, 1.5 ($\pm$0.20) E-6 (df = 4) denotes an $S_{0.50}$ value of 1.5×10^{-6} M. The error of the value is $\pm$0.20 $\times 10^{-6}$ M. For statistical analysis, the value has 4 degrees of freedom (df) associated with it. Generally, a single kinetic experiment involved determination of rates in triplicate dishes at 6 different concentrations of substrate. However, because the number of replicates for each concentration of substrate and the number of substrate concentrations tested varied among experiments, the mean rate at each substrate concentration was assigned a single df. For example, an $S_{0.50}$ derived from 6 data points, which are usually the mean of triplicate plates, has 4 df. Two df were utilized in the mathematical linear regression analysis that was used to calculate the kinetic parameters and their error. More than 4 df generally indicated values that were derived from pooled data from multiple experiments. "Zero order" indicates that no stimulatory effect on rate due to increasing the concentration of the component in the medium could be detected. NA = data not available. Asterisks indicate the results of a Student's t-test of the probability (P) that the difference between the indicated $S_{0.50}$ value and the $S_{0.50}$ value for multiplication for N-HLF was due to chance.
*P $\leqslant$ 0.001; **P $\leqslant$ 0.01; ***P $<$ 0.10; no asterisk, P $\geqslant$ 0.10.

aFBSP denotes "fetal bovine serum protein" prepared as described in Materials and Methods. $S_{0.50}$ values are in units of micrograms dry weight of non-dialyzable material per ml. The multiplication response to FBSP for both N-HLF and SV-HLF was biphasic (see Results and Discussion). The lower $S_{0.50}$ values for FBSP were calculated from the first phase of the response, and the higher values were calculated from the second phase (Figs. 1 and 7).

bThe response to extracellular glycine, serine, adenine (and other purines), and thymidine (and other pyrimidines) can be determined by manipulation of the relative level of these nutrients and the folic or folinic acid concentration in the medium. The MCDB 104 to 107 series of media contains a low level of folinic acid (1.0 nM). Multiplication of N-HLF is absolutely dependent on extracellular glycine, and the rate is stimulated by purines and pyrimidines. Under this condition, multiplication rate is independent of serine. However, a response to serine can be demonstrated in glycine- and serine-free medium MCDB 107 that contains 1 μM folinic acid.

cAt 250 μg FBSP per ml, the multiplication response of N-HLF to niacinamide is biphasic [29]. The first phase was that expected of a water-soluble vitamin ($S_{0.50}$ = 7.0 E-10). The second phase was indicative of a substrate effect ($S_{0.50}$ = 9.2 E-8). The maintenance response, however, exhibited a single hyperbola with $S_{0.50}$ = 6.0 E-10.

d"Lipid" refers to the response of cells to purified phosphatidylcholine (PC) introduced as purified vesicles or liposomes [30]. The response required polyunsaturated fatty acid moieties on the PC and was enhanced when PC was co-sonicated with cholesterol. The multiplication response of N-HLF to these lipids was most apparent in the presence of delipidated FBSP.

Nutrient and Serum Factor Requirements for Cell Maintenance Versus Multiplication

Multiplication rate of a population of cells is a composite of the average interdivision time per cell that divides during the assay and the rate of cell death or detachment during the incubation period. Therefore, the quantitative requirement for some nutrients and serum factors for multiplication of the population may simply reflect their requirement to maintain cells in a state where they are capable of division under the appropriate stimulus. However, certain nutrients, as well as serum factors, may play important roles in cell division processes in addition to their roles in cell maintenance. We explored this possibility by a comparison of the $S_{0.50}$ values for overall multiplication to the $S_{0.50}$ values for maintenance of nonproliferating cells. As pointed out earlier, complete medium MCDB 104 to 107 supports attachment and maintenance of cells in the absence of FBSP or other growth factors [15, 17]. Since FBSP-free medium is a nonpermissive medium for multiplication, the $S_{0.50}$ values for nutrients for maintenance were determined in FBSP-free medium as described in Materials and Methods. The quantitative requirement for the majority of nutrients (18 of 29 tested) was significantly higher for multiplication in the presence of serum growth factors than for maintenance in the absence of serum growth factors (Table I). Endogenous cellular metabolism or background levels of glutamine, glycine, phenylalanine, serine, tryptophane, adenine, thymidine, inositol, Fe and Se ions, and lipids were adequate for maintenance without addition of the nutrients to the medium. However, cell multiplication rate was stimulated by the exogenous addition of the same nutrients to the medium containing serum growth factors. The quantitative requirement for histidine, lysine, niacinamide, tyrosine, Ca^{2+}, Mg^{2+} and Pi for cell multiplication was also significantly less ($P < 0.001$) than the requirement for multiplication. The results suggest that specific nutrients are quantitatively important in cellular processes that are related to cell division in addition to the role of the nutrients in cell maintenance processes. These specific nutrients provide clues about cellular processes whose rate is limiting to cell multiplication and whose rate is controlled by these nutrients or their products. In striking contrast to the majority of nutrients, the requirement for pyruvic acid and K^+ was higher for maintenance than for multiplication (Table I). Conclusions about pyruvic acid and K^+ as well as Ca^{2+}, Mg^{2+}, and Pi are complicated by the fact that their requirement in multiplication (and maintenance) is modified by the presence of serum factors in the medium.

Modification of Nutrient Requirements by Serum Growth Factors

Steroid and polypeptide regulatory factors may affect cell multiplication rate in a manner analogous to "allosteric effectors" of enzyme-catalyzed reactions [1, 4, 5, 31, 32]. Growth factors may change multiplication rate by causing a change in the cellular requirement ($S_{0.50}$ value) for a nutrient that is analogous to a change in the K_m of an enzyme for its substrate. To examine for this, we applied the kinetic approach to relate simultaneously the level of FBSP or other growth factors and the level of individual nutrients in the medium to cell multiplication rate [19, 20, 23, 33–36]. Three different types of relationships were observed between FBSP and individual nutrients (Figs. 4–6). Some nutrients (class I) exhibited a sharp inverse relationship with FBSP. These were Ca^{2+} (Fig. 4a), K^+ (Fig. 4b), 2-oxocarboxylic acids (Fig. 4c), and lipids (not shown). The relationship of the requirement for class II nutrients, Mg^{2+} (Fig. 5), Pi (Fig. 5), Fe ions, Se ions, and niacinamide (not shown), exhibited a region where the requirement for multiplication was initially independent of FBSP and then had an inverse relationship with it. The requirement for the third type of nutrient (class III) had no relationship with FBSP. The majority of nutrients fell
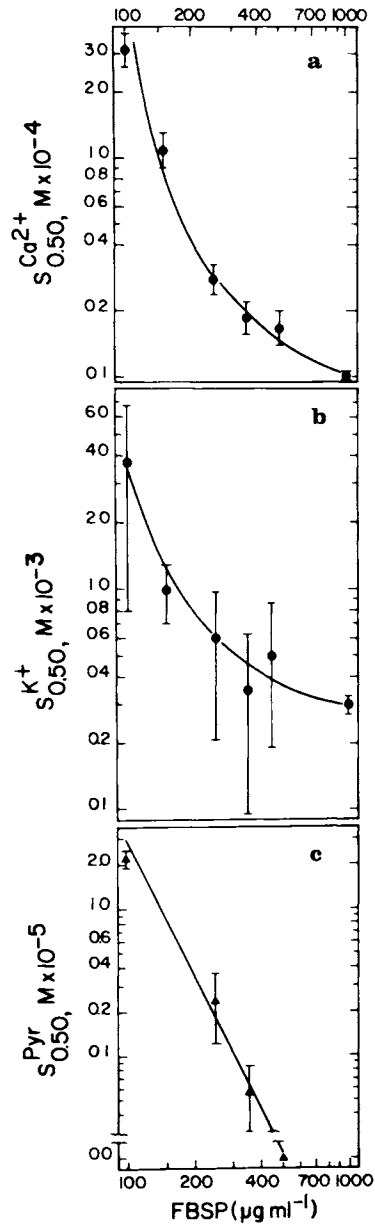
Fig. 4. Effect of FBSP concentration on the extracellular requirement for Ca^{2+}, K^+, and pyruvic acid. Ca^{2+}- or pyruvic acid-deficient medium was prepared by omission of $CaCl_2$ or sodium pyruvate, respectively, from medium MCDB 105. K^+-deficient medium MCDB 105 was prepared by omission of KH_2PO_4 and replaced with 3.0×10^{-3} M $Na_2HPO_4 \cdot 7H_2O$. The K^+ response was determined by addition of KCl. Vertical bars indicated SE estimated by a Gaussian analysis of variance due to linear regression and deviation from linearity of the $1/r$ versus $1/[S]$ data. Data in a and c are the mean $S_{0.50}$ values from several different experiments. Data in b are from a single representative experiment. From McKeehan WL, McKeehan KA [35].
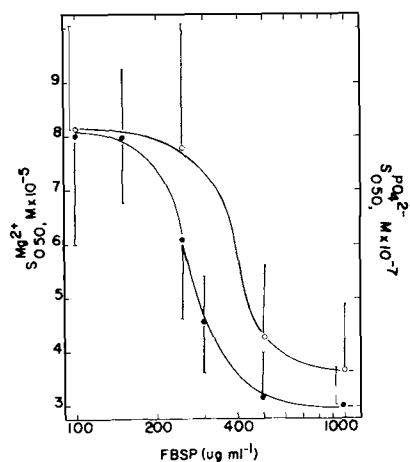
Fig. 5. Effect of FBSP concentration on extracellular requirement for Mg^{2+} and Pi. The $S_{0.50}$ for each ion was determined in Mg^{2+}- or Pi-deficient medium MCDB 107 containing the indicated amounts of FBSP. Multiplication responses to Mg^{2+} were determined by addition of $MgCl_2 \cdot 6H_2O$. Pi-deficient medium was prepared by omission of KH_2PO_4 from medium MCDB 107 and replacement with an equimolar amount of KCl. Responses to Pi were determined by addition of $Na_2HPO_4 \cdot 7H_2O$. Vertical bars indicate SE. Data are from single representative experiments. ●, $S_{0.50}$ for Mg^{2+}; ○, for Pi. From McKeehan WL, McKeehan KA [35].

into class III, which included the 20 amino acids, glucose, adenine (and other purines), thymidine (or other pyrimidines), choline, inositol, and putrescine (or other polyamines). Although some lipids [30], niacinamide [29] and the ions of Fe and Se showed class I and II relationships with FBSP, a functional modification of their requirement in multiplication by FBSP could not be distinguished from their addition to deficient culture medium as a contaminant in the FBSP [35]. Table II summarizes the classification of all the nutrients tested. We concluded from these results that Ca^{2+}, Mg^{2+}, K^+, Pi, and 2-oxocarboxylic acids (and possibly lipids, niacinamide, Fe, and Se) are likely to be involved in the mechanism by which serum factors affect multiplication rate. By the same reasoning, amino acids, glucose, purines, pyrimidines, polyamines, choline, and inositol are unlikely to be involved. Because of technical limitations in the approach, no conclusion could be made about Na^+, H^+, HCO_3^-, SO_4^{2-}, Cl^-, and several other trace nutrients that affected multiplication rate under certain conditions.

Chemically purified growth factors modify the cellular requirements for nutrients in a manner similar to crude FBSP. We reported elsewhere that 10 ng of pure epidermal growth factor (EGF) per ml of culture medium reduced the Ca^{2+} requirement for multiplication of human skin fibroblasts to 2% of the normal Ca^{2+} requirement in the absence of the factor [23]. Lechner and Kaighn also showed that EGF reduced the Ca^{2+} requirement for multiplication of a normal prostatic epithelial cell line [20]. Fibroblast growth factor (FGF) also reduced the Ca^{2+} requirement for proliferation of bovine endothelial cells [personal communication, Dr. J. R. Smith, W. Alton Jones Cell Science Center]. At present, it is not clear if single growth factors reduce the cellular requirement for Ca^{2+}, K^+, Mg^{2+}, Pi, and 2-oxocarboxylic acids in a manner similar to crude serum proteins or if different factors modify the requirement for one or a few nutrients of the set. So far we have demonstrated that EGF reduces the requirement for Ca^{2+}, Mg^{2+}, and pyruvic acid in a manner similar to crude serum factors [23; unpublished results]. Data for K^+ and Pi are not yet available. These results support the suggestion that Ca^{2+}, Mg^{2+}, K^+, Pi, and 2-oxo-
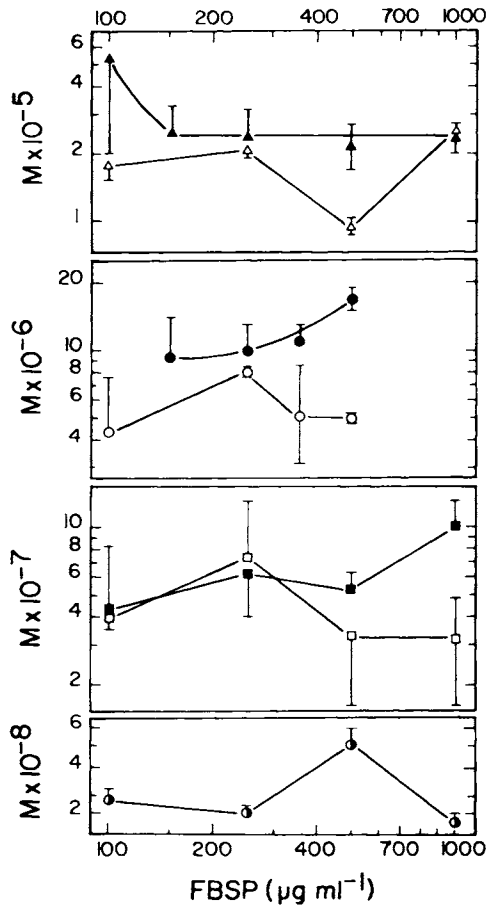
Fig. 6. Effect of FBSP on extracellular requirement for several class III nutrients. $S_{0.50}$ (ordinates) was determined for each nutrient in medium MCDB 105 lacking the individual nutrient and containing the indicated level of FBSP. ▲, glucose; △, glutamine; ●, glycine; ○, serine; ■, leucine; □, isoleucine; ⊙, adenine. From McKeehan WL, McKeehan KA [35].

TABLE II. Kinetic Relationships Between Extracellular Nutrients and FBSP*

Class I	Class II	Class III
Ca^{2+}	Mg^{2+}	Amino acids
K^+	P_i	Choline
2-Oxocarboxylic acids[a]	Fe^{2+}	Glucose
Lipids	SeO_3^{2-}	Inositol
	Niacinamide	Polyamines
		Purines
		Pyrimidines

*Individual nutrients were ranked according to the type of kinetic relationship that they exhibited with FBSP (see Results and Discussion). Amino acids tested include all 20 common amino acids. Polyamines include putrescine, spermidine, and spermine. Purines include adenine, guanine, hypoxanthine, their corresponding nucleosides, and the deoxyribonucleoside equivalents. Pyrimidines include uridine, cytidine, their deoxyribonucleoside equivalents, and thymidine.
[a]Although pyruvic acid is the most common 2-oxocarboxylic acid in culture medium, the requirement is for any one of a class of 2-oxocarboxylic acids [34].

carboxylic acids may be closely involved in the mechanism by which growth regulatory factors exert their controls on cell multiplication [23, 35]. On the one hand, if the reduced dependence on extracellular Ca^{2+}, Mg^{2+}, K^+, Pi, and 2-oxocarboxylic acids that is caused by growth factors reflects an increase in availability of the nutrients to key intracellular processes that control multiplication, then these 5 nutrients can be considered mediators of the primary action of serum growth factors. On the other hand, the reduction in the requirement for extracellular Ca^{2+}, Mg^{2+}, K^+, Pi, and 2-oxocarboxylic acids that is caused by serum factors may reflect a reduced requirement for the nutrients in intracellular processes that limit multiplication. Concentration of the nutrients in the microenvironment of key processes may normally be constant. If this is the case, then serum growth factors may control multiplication rate by modification of the affinity for Ca^{2+}, Mg^{2+}, K^+, Pi, and 2-oxocarboxylic acids in processes that otherwise limit multiplication. Alternatively, serum factors and their messengers may activate alternate pathways that bypass processes that are otherwise dependent on the 5 nutrients in a chain of events that control multiplication.

Serum Factors and Nutrients in Cell Maintenance

Since the requirement for Ca^{2+}, Mg^{2+}, K^+, Pi, and pyruvic acid in the multiplication assay was modified by factors in FBSP, the question arises whether it is the requirement for these nutrients for cell division or cell maintenance that is modified by factors in FBSP. The higher requirement for pyruvic acid and K^+ (class I nutrients) for maintenance in the absence of FBSP than the requirement in the complete multiplication assay in the presence of FBSP suggested that serum factors may affect the pyruvic acid and K^+ requirement for maintenance (Table I). Indeed, serum factors decreased the maintenance requirement for Ca^{2+}, K^+, and pyruvic acid (Table III). The addition of 100 μg of serum factors per ml completely eliminated a requirement for Ca^{2+} for maintenance. Data for Pi were not available. Separate experiments further revealed that when the Ca^{2+}, K^+, or pyruvic acid content of medium MCDB 107 was reduced below a critical level, cell maintenance then required the presence of macromolecular serum factors (unpublished results). In this case, serum-derived factors play a role as cell "survival factors" that probably act by modifying

TABLE III. Effect of Serum Factors on Requirements for Class I and II Nutrients for Cell Maintenance*

	Maintenance		Multiplication
Nutrient	FBSP-free	100 μg/ml dFBSP	100 μg/ml FBSP
Ca^{2+}	1.8 ($\pm$0.20) E-6	Zero order	3.0 ($\pm$0.30) E-4
Mg^{2+}	2.3 ($\pm$0.90) E-6	1.9 ($\pm$0.70) E-6	8.0 ($\pm$2.0) E-5
K^+	1.3 ($\pm$0.50) E-3	4.8 ($\pm$0.70) E-4	3.8 ($\pm$3.2) E-3
Pi	2.3 ($\pm$0.70) E-7	NA	8.1 ($\pm$1.9) E-7
Pyruvic acid	1.2 ($\pm$0.10) E-4	6.8 ($\pm$1.3) E-6	2.2 ($\pm$0.30) E-5

*The effect of factors in FBSP on the requirements for maintenance was determined with delipidated FBSP (dFBSP) [37]. Delipidation of FBSP removes or inactivates one or more factors that are essential for multiplication of N-HLF [30]. Therefore, medium MCDB 107 containing dFBSP is a nonpermissive condition for multiplication. The indicated values are the $S_{0.50}$ $\pm$ SE in units of moles per liter for maintenance or multiplication and were determined in medium containing the indicated amounts of dFBSP or FBSP. NA = data not available.

TABLE IV. Interactions Between Ca^{2+} and Mg^{2+} in Multiplication of Normal Human Lung Fibroblasts*

Ca^{2+} (M)	$S_{0.50}^{Mg^{2+}}$	$R_{max_-}^{Mg^{2+}}$
1.0 E-3	3.9 (±0.32) E-5	0.463 ± 0.01
	(P < 0.001)	(NS)
1.0 E-4	1.1 (±0.80) E-4	0.481 ± 0.08
Mg^{2+} (M)	$S_{0.50}^{Ca^{2+}}$	$R_{max}^{Ca^{2+}}$
1.0 E-3	1.2 (±0.70) E-5	0.433 ±0.025
	(NS)	(P < 0.001)
1.0 E-4	1.5 (±1.25) E-5	0.314 ± 0.039

*Kinetic parameters for Ca^{2+} and Mg^{2+} for multiplication of N-HLF were determined as a function of each other in medium MCDB 105 containing 250 μg FBSP per ml. Values ±95% confidence limits are indicated. The probability (P) that the difference between values is due to chance is indicated between rows. NS = not significant, or P > 0.10. $S_{0.50}$ is in units of moles per liter. R_{max} is the maximal rate of multiplication in cell generations per day.

the cellular requirement for Ca^{2+}, pyruvic acid, and K^+ in processes that are essential to basic maintenance of nonproliferating cells. Under these conditions, the role of factors in FBSP in the multiplication assay can no longer be considered as purely "regulatory." The requirement for FBSP in multiplication reflects a requirement for factors that support basic cell maintenance as well as cell division. Even though the requirement for maintenance of Ca^{2+}, K^+, and pyruvic acid was modified by serum factors, at an equivalent level of serum factors in the medium, the multiplication requirement for Ca^{2+}, K^+, and pyruvic acid was still much greater than the requirement for maintenance (Table III). This suggests that Ca^{2+}, K^+, and pyruvic acid are quantitatively important in cell division processes beyond their role in maintenance. Whether the same or different serum factors are involved in modification of the requirement for Ca^{2+}, K^+, and pyruvic acid in both maintenance and cell division awaits a similar study with the purified factors responsible for the effects.

Effect of One Nutrient on the Quantitative Requirement for Others in Multiplication

The kinetic approach has been employed to determine the interplay between individual nutrients in multiplication of N-HLF [33]. Table IV shows that, when FBSP was constant, Ca^{2+} decreased the requirement for Mg^{2+} without an effect on the maximal multiplication rate that could be achieved by addition of saturating levels of Mg^{2+}. In contrast, Mg^{2+} determine the maximal growth rate and had no effect on the requirement for Ca^{2+}. We interpreted this pattern to indicate specific effector relationships between Ca^{2+} and Mg^{2+} in cell multiplication [33, 36]. The results suggested that part of the effect of Ca^{2+} on multiplication rate is probably mediated by Ca^{2+}-dependent changes in the cellular requirement for Mg^{2+}. Mg^{2+} exerts its effects on multiplication rate more directly via R_{max}. In sequential processes that lead to multiplication, Mg^{2+} probably exerts its effect on multiplication rate in a process that is more proximal to actual cell multiplication than those events where Ca^{2+} exerts its influence.

Experiments are in progress to determine the interplay among all 5 class I and II nutrients (Table II) to derive clues about how they fit into a regulatory network of processes that control cell multiplication rate. Preliminary results indicate specific relationships among the effects of single nutrients of the group, in addition to Ca^{2+} and Mg^{2+}, on the
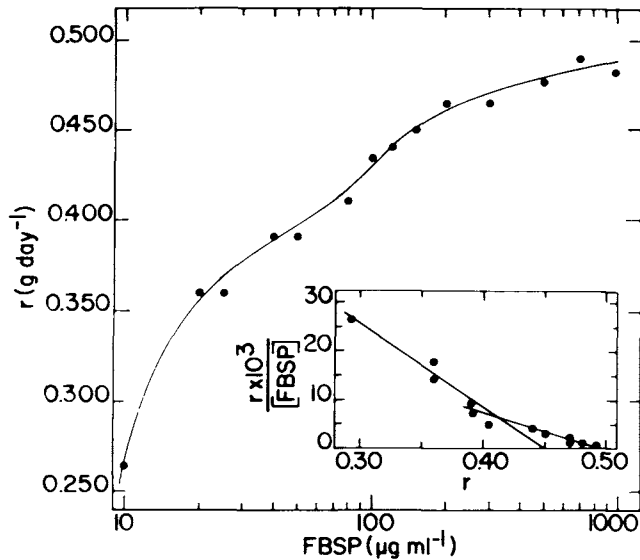
Fig. 7. Multiplication response of SV-HLF to FBSP. Multiplication of 100 SV-HLF was assessed in medium MCDB 105 at the indicated concentrations of FBSP as described for N-HLF in Figure 1. The data are a composite of three separate experiments. Data points are the mean of triplicate dishes. The inset is an Eadie-Scatchard plot of the data.

quantitative requirement for others in multiplication. These results suggest that specific quantitative relationships among external nutrient concentrations may be important in control of cell multiplication rate.

Differences in Requirements for Nutrients and Serum Growth Factors Between Normal and Transformed Cells

The kinetic approach was applied to probe for differences in the requirements for nutrients and serum growth factors for multiplication of a continuous, transformed cell line (SV-HLF) and the normal lung fibroblasts (N-HLF). When all nutrient concentrations were optimal, the multiplication response of SV-HLF to serum factors was similar to N-HLF (Fig. 7). SV-HLF also exhibited a biphasic response to FBSP (Fig. 7), although the biphasic response was less marked than that of N-HLF (Fig. 1). From the pooled results of multiple experiments, no significant difference in the quantitative requirement for FBSP for multiplication of N-HLF and SV-HLF could be demonstrated (Table I). $S_{0.50}$ values that were calculated from similar regions of the biphasic response of both N-HLF and SV-HLF were similar (Figs. 1, 2, and 7, Table I).

At a constant level of FBSP in the medium, the quantitative requirement for a number of individual nutrients for multiplication of SV-HLF was significantly less than the requirements of N-HLF (Table I). Individual nutrients are ranked in Table I according to statistical significance of the difference. The multiplication response of SV-HLF was zero order with respect to tryptophane, adenine, thymidine, and Fe and Se ions. Each of these nutrients significantly stimulated the multiplication rate of N-HLF. The $S_{0.50}$ values for multiplication of SV-HLF for cysteine, glutamine, histidine, phenylalanine, choline, inositol, and Mg^{2+} were also significantly less (P < 0.001) than those for N-HLF. Although the pooled results in Table I indicated a higher Ca^{2+} requirement for multiplication of SV-HLF

than for N-HLF, the multiplication of SV-HLF to Ca^{2+} is complex and different from that of N-HLF [36]. SV-HLF actually multiply at a suboptimal rate in the absence of Ca^{2+}, and the response to Ca^{2+} was zero order at medium Ca^{2+} concentration below 50 μM [36]. In contrast, a detectable rate of multiplication of N-HLF absolutely required the addition of Ca^{2+}. Thus, transformation actually caused a marked reduction in the requirement for Ca^{2+} for detectable multiplication of SV-HLF. However, SV-HLF responded to added Ca^{2+} above 50 μM in a hyperbolic fashion [36]. At 250 μg FBSP per ml, the $S_{0.50}^{Ca^{2+}}$ for SV-HLF was higher (P < 0.001) than the $S_{0.50}^{Ca^{2+}}$ that was calculated in multiple experiments from the single hyperbolic respons of N-HLF to Ca^{2+} [36] (Table I). The $S_{0.50}$ value calculated from the hyperbolic region of the response of SV-HLF to added Ca^{2+} was modified by the level of serum factors in the medium, but much less stringently than the $S_{0.50}$ value for N-HLF [36] (Fig. 4a). Although the $S_{0.50}^{Ca^{2+}}$ for SV-HLF was equal to or higher than that for N-HLF at FBSP concentrations above 250 μg per ml, at 100 μg FBSP per ml the $S_{0.50}^{Ca^{2+}}$ for SV-HLF was less than 10% of the $S_{0.50}^{Ca^{2+}}$ for N-HLF. Therefore, when the concentration of serum factors was limiting for multiplication of N-HLF, the requirement for Ca^{2+} for optimum multiplication of SV-HLF was much less than that of N-HLF [36].

The foregoing results suggest that alterations in cell nutrient requirements may confer a growth advantage on SV-HLF relative to N-HLF. When all nutrient concentrations are optimal, SV-HLF have no growth advantage over N-HLF, regardless of the level of serum factors in the medium. However, SV-HLF possess a growth advantage over N-HLF under a number of conditions where nutrient concentrations in the medium limit the growth of N-HLF. Since the normal cell multiplication requirement for certain nutrients is under the transient, but stringent control of serum factors, constitutive alterations in the requirement for specific nutrients may override the control of cell multiplication rate by some individual serum-derived growth factors. Indeed, we have shown this to be the case for specifically Ca^{2+}, K^+, and Mg^{2+} in human lung fibroblasts transformed with SV40 virus [W. L. McKeehan, K. A. McKeehan, D. Calkins, in preparation].

The specific alterations in nutrient requirements of SV-HLF as reported here should provide valuable clues to cellular processes that were altered during viral transformation and evolution of the transformed cell line.

Does Transformation Reduce the Quantitative Requirement for Nutrients for Multiplication Whose Quantitative Requirement is Normally Higher for Multiplication Than for Maintenance?

The results indicate that the $S_{0.50}$ values for several class III nutrients (gln, his, trp, ade, dThd, and inositol) were greater (P < 0.001) for multiplication than for maintenance of N-HLF (Table I). SV-HLF required significantly less (P < 0.001) of these nutrients for multiplication than did N-HLF. Conclusions about class I and II nutrients (Table II) were more difficult because of their complicated kinetic relationships with serum factors in both maintenance (Table III) and multiplication [35, 36] (Tables II and III). However, when compared at equivalent and limiting levels of serum factors in the medium, a reduction in the requirement for Ca^{2+}, Mg^{2+}, K^+, Fe, and Se ions for multiplication of SV-HLF was apparent (Tables II and III) [36; W. L. McKeehan, K. A. McKeehan, D. Calkins, in preparation]. Under the same conditions, the requirement for each of these nutrients for multiplication of N-HLF was higher than for maintenance (Tables II and III) [W.L. McKeehan, K. A. McKeehan, D. Calkins, in preparation]. The requirement for Mg^{2+}, K^+, Fe, and Se ions for multiplication of SV-HLF was not significantly different from simply the requirement for maintenance of nonproliferating normal cells. In summary, multiple, but

specific nutrients appear quantitatively important in cell division beyond their roles in cell maintenance, and the quantitative requirement for multiplication of several (but not all) of these nutrients was reduced as a consequence of viral transformation and evolution of the transformed cell line.

Does Transformation Constitutively Reduce the Requirement for Nutrients Whose Quantitative Requirement for Multiplication of Normal Cells is Transiently Controlled by Serum Factors?

The normal cell multiplication requirement for Ca^{2+}, Mg^{2+}, K^+, Pi, and 2-oxocarboxylic acids is under the control of serum factors [35] (Tables II and III). In a previous report [36], we showed by kinetic analysis that the Ca^{2+} requirement for multiplication of SV-HLF was still influenced by FBSP, but to a much lesser extent than N-HLF. A reduction in the Ca^{2+} requirement of SV-HLF relative to N-HLF was apparent at FBSP concentrations below 250 μg per ml. More striking was the reduction in the Mg^{2+} requirement for multiplication of SV-HLF. The reduced Mg^{2+} requirement of SV-HLF was apparent at all levels of FBSP in the medium. The Mg^{2+} requirement for multiplication of SV-HLF was completely independent of the level of FBSP in the medium [36]. Although the results in Table I suggested that there was no difference in the K^+ requirement for multiplication of N-HLF and SV-HLF, a difference is masked by the level of FBSP in the medium under the experimental conditions [W. L. McKeehan, K. A. McKeehan, D. Calkins, in preparation]. At 100 μg FBSP per ml, the K^+ requirement for multiplication of SV-HLF is less than 5% of that of N-HLF [W. L. McKeehan, K. A. McKeehan, D. Calkins, in preparation]. The K^+ requirement for multiplication of SV-HLF was independent of the level of serum factors in the medium [W. L. McKeehan, K. A. McKeehan, D. Calkins, in preparation]. In contrast to the Ca^{2+}, K^+, and Mg^{2+} requirement, the requirement for pyruvic acid and Pi for multiplication of SV-HLF appear to be similar to that of N-HLF at all levels of FBSP between 100 μg and 1,000 μg FBSP per ml [W. L. McKeehan, K. A. McKeehan, D. Calkins, in preparation]. It is noteworthy that the requirement for Fe and Se ions for multiplication of N-HLF was reduced by increasing levels of FBSP in the medium [35] and that the requirement for both ions for multiplication of SV-HLF was also reduced (Table I). However, because of the presence of both ions as contaminants in the FBSP, a functional modification of the normal cell requirement could not be distinguished from a simple addition of the ions to deficient medium by FBSP [35]. Nevertheless, both ions warrant further study in a more refined assay system.

In summary, the multiplication requirement for Ca^{2+}, Mg^{2+}, and K^+ is under the stringent control of serum factors in normal cells. The requirement for the 3 ions for multiplication of SV-HLF was less than that of N-HLF and was partially removed from the influence of serum factors. If specific serum factors control the rate of normal cell proliferation by a transient reduction of the cellular requirement for Ca^{2+}, Mg^{2+}, and K^+, then the constitutive reduction in the requirement for the ions will override the control of cell proliferation by the relevant serum growth factors. Others have reported similar results using different cell types that were transformed by different methods. Balk [38] demonstrated that the loss of requirement of a factor uniquely in serum (as opposed to cell-free plasma) for multiplication of Rous sarcoma virus-infected chicken fibroblasts was related to a reduced cellular Ca^{2+} requirement. Subsequently, the unique factor in serum has been identified as a polypeptide growth factor from platelets [39]. Lechner and Kaighn [20] recently demonstrated that the multiplication of normal prostate epithelial cells required much higher levels of Ca^{2+} requirement for the normal cells to that required by the tumor cells. Multiplication rate of the tumor cells was independent of EGF. These studies support the

hypothesis that a transformation-dependent reduction in nutrient requirements may cause a reduction in requirement for specific purified polypeptide growth factors.

ACKNOWLEDGMENTS

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APPENDIX: APPLICATION OF THE PRINCIPLES OF HENRI-MICHAELIS-MENTEN KINETICS TO CELL GROWTH IN CULTURE

To study the kinetics of cell growth in culture, we considered the following criteria that are essential to kinetic analysis of unireactant enzymes [1]:

1) The enzyme is a catalyst.

2) The enzyme (E) and substrate (S) react rapidly to form an enzyme-substrate complex (ES).

3) The enzyme-substrate complex breaks down to form free enzyme and product (P).

4) Enzyme, substrate, and the enzyme-substrate complex are at equilibrium; that is, the rate at which ES dissociates to E + S is much faster than the rate at which ES breaks down to form E + P.

5) The substrate concentration is much larger than the enzyme concentration so that the formation of an ES complex does not significantly alter the substrate concentration.

6) The overall rate of the reaction is limited by the breakdown of the ES complex to form free enzyme and product.

7) The velocity is measured during early stages of the reaction so that the reverse reaction is insignificant.

To employ these principles, consider the stoichiometry of the reaction under consideration. For cell proliferation, we assumed a reaction C + S ⇌ CS → C + P = 2C, where C = cell, S = substrate, CS = cell-substrate complex, and P = product. In the reaction, the cell is the catalyst that interacts with external substrates to convert them into a product (P), which is another cell (P = C). For the model cell types employed in this study, the catalyst emerges from the reaction unchanged and ready for another round of reaction. However, the number of catalysts doubles during each cycle of the reaction. It was assumed that a substrate (S) interacts with sites on or in each cell to form a cell-substrate complex (CS) that is equivalent to the ES complex of first-order enzyme reactions. Cell multiplication is an extremely complex higher order reaction in regard to extracellular variables. However, in practice, conditions can be developed where the interaction between cellular sites and S is such that the formation of CS can be considered a first-order function of S. For simplicity, an experimental system was designed to allow the study of the effect of single extracellular factors on cell multiplication while holding all other variables constant and at optimum levels. The rate of cellular multiplication can then be considered a first-order function of the concentration of a single CS complex, where S is the single substrate or ligand under consideration. It should be pointed out here that we have treated inorganic nutrients and non-nutritive, hormone-like growth factors the same as conventional substrates in the kinetic analysis. However, neither type of extracellular factor is actually converted into new cell product like a conventional substrate (eg, amino acids). Growth factors are envisioned to act as activators of other more conventional C + S ⇌ CS reactions. Inorganic ions probably act in complex with conventional substrates or as activators of other C + S ⇌ CS reactions.

We assumed that the rate of equilibration among cells, S, and CS complex is rapid relative to the rate of cellular multiplication or other processes that deplete the concentration of CS (postulate 4 above). The interaction between nutrients and polypeptide or

steroid regulatory factors and cellular binding sites fits these assumptions. Nutrients [2, 3] and polypeptide hormones [4, 5], as well as other macromolecular factors [6, 7], initially interact rapidly and reversibly with sites in or on cells. The slowest of these interactions are faster than the 24 h or more required for division of the model cell types considered in this study.

To apply the fifth principle, we designed a system where all extracellular variables were at optimum for and at a concentration sufficient to promote a near-maximal rate of cell multiplication. Multiplication rates were determined as the number of cells increased from an initial inoculum of 20 to a maximum of about 10^4 cells per ml (Fig. 1A). If it is assumed that an average cell is 300 pg protein and that the average, active protein is 100,000 daltons distributed among 1,000 cellular proteins with an average of 3.3 active binding sites for low-molecular weight ligands [1], then the concentration of a cellular site for the average ligand increases from 2×10^{-13} to 1×10^{-10} during the assay. This is $2 \times 10^{-4}\%$ to 0.10% of the lowest concentration, 10^{-7} M, that was employed in this study to assess the effect of low molecular weight ligands other than trace elements and niacinamide. Sample calculations for regulatory growth factors are less definite, since we usually employed a crude mixture of multiple, uncharacterized regulatory factors (FBSP). Assume that single factors in the fraction act as hormone-like mitogens and that each cell has about 50,000 receptors for a factor [8, 9]. Then, 200 μg FBSP per ml would support an increase in receptor site concentration for a single factor of 1.7×10^{-15} to 5.1×10^{-13} M
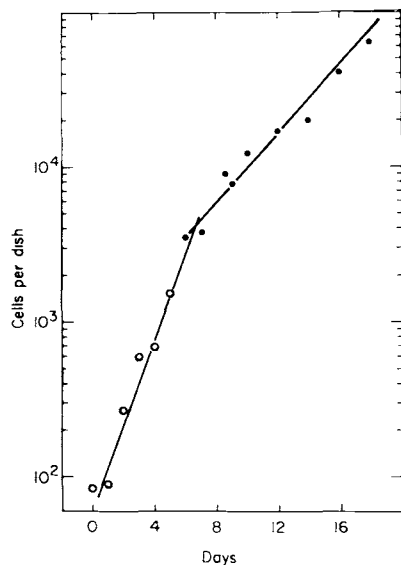
Fig. 1A. Complete time course of multiplication of N-HLF. One hundred N-HLF (Flow 2000; PDL 16) were inoculated into complete medium MCDB 105 containing 200 μg FBSP per ml. For the data points for days 1 through 6 (o—o), cells on the dish were fixed, stained, and counted directly with a grid system under a dissecting microscope. For data points at 7 to 17 days (●—●), cells were removed by trypsinization and counted with a Coulter Counter. The data from 0 to 6 days (o—o) represent a mean of cell counts from 3 separate dishes. The data for 7 to 17 days were the total count of cells pooled from 3 dishes, and then divided by 3.

TABLE IA. Steady-State Properties of Assay System*

Component	$[S]_{0.90}$	$[S]_{t=0\ days}/[S]_{0.90}$	$[S]_{t=12\ days}/[S]_{0.90}$
Arg	1.4 E-5	71.4	73.5
Cys	2.2 E-5	2.3	ND
Gln	9.9 E-5	25.3	25.8
Gly	1.4 E-4	2.1	2.7
His	1.4 E-5	7.1	8.0
Ile	3.6 E-6	8.3	9.4
Leu	4.7 E-6	21.3	22.4
Lys	1.5 E-4	1.3	1.2
Met	8.7 E-7	34.5	44.9
Phe	2.7 E-5	1.1	1.1
Thr	1.7 E-5	5.9	7.5
Trp	5.0 E-8	200.0	ND
Tyr	1.3 E-6	23.1	35.1
Val	1.4 E-5	7.1	8.5
Ade	1.2 E-7	83.3	53.3
Choline	8.6 E-6	11.6	9.7
dThd	1.2 E-7	2.5	2.5
Glc	2.4 E-4	16.7	16.1
Inositol	2.9 E-6	34.5	22.7
Niacinamide	8.3 E-7	60.2	ND
Pyruvic acid	3.1 E-5	32.3	35.9
Ca	1.2 E-4	8.3	8.3
Mg	3.2 E-4	3.1	3.6
K	4.3 E-3	1.2	1.6
Pi	9.0 E-6	333.3	346.5
Fe	3.0 E-7	16.7	14.7
Se	2.4 E-9	12.5	13.5

Other conditions		
	t = 0	t = 12 days
pH	7.25	7.25
Osmolality (mOsm/kg)	288	317
Lactic acid (M)	<1.0 E-5	<1.0 E-5
NH_4^+	7.0 E-5	2.0 E-4
Protein (μg/ml)	500	500

From McKeehan WL, McKeehan KA [11], reproduced by permission of the Tissue Culture Association.
*Medium MCDB 105 containing 500 μg FBSP per ml was incubated with 100 N-HLF cells under standard multiplication assay conditions. After 12 days, the medium was collected and analyzed for the indicated components by methods described elsewhere [11]. $[S]_{0.90}$ is the concentration of the component calculated from kinetic data that is required to promote a 90% of maximum multiplication rate. In the abbreviated exponential notation, E means "10 to the power." $[S]_{t=0\ days}$ is the concentration of the component in medium MCDB 107. $[S]_{t=12\ days}$ is the concentration of the component in the medium that was indicated by analysis after 12 days of incubation. ND = not determined because of the lack of an adequate assay method.

over a period of 14 days (data taken from Fig. 1A from 200 μg FBSP per ml, which is equivalent to 0.40% whole serum). A 200-μg FBSP per ml solution contains 1.7×10^{-11} M insulin. This represents an insulin concentration of about 10^4 to near 320-fold higher than the concentration of cellular binding sites for insulin during the assay. These theoretical calculations suggest that the concentration of low molecular weight nutrients and

regulatory factors are in excess concentration of their cellular sites of action at the levels used in the study. However, this assumption is contingent on the chemical stability of growth factors throughout the incubation period. There is disagreement on the stability of growth factors in culture (insulin in particular) at low medium protein concentrations [10].

Table IA demonstrates more directly the near steady-state properties of our model experimental system. The medium content of each component was assayed after 12 days of incubation under standard conditions and compared to the concentration required to promote a 90% of maximum rate of multiplication. The concentration of most components increased with incubation. This was partially due to the loss in volume of the medium due to evaporation (see data for osmolality). Except for tyr, adenine, K^+, osmolality, and NH_4^+, the differences between [S] at t = 0 and [S] at t = 12 days were within experimental error of the analysis. The reason for the increase in tyr, K^+, and NH_4^+ is unknown. The increase in osmolality correlated with the loss of medium volume due to evaporation in the incubator. The decrease in adenine may reflect substantial uptake, since the cells are dependent on it for a purine source at the low folinic acid level (1 nM) in medium MCDB 105. Despite the decrease, the concentration of adenine at 12 days was still over 50-fold in excess of that required to support a 90% of maximum multiplication rate. We showed separately that the changes in all 5 of the above parameters have little effect on multiplication rate [11]. Frequent changes of complete medium had little effect on multiplication rate of N-HLF or SV-HLF [11]. Furthermore, FBSP recovered from the standard incubation mixture after 12 days still exhibited multiplication-stimulating activity.* These results demonstrate directly that nutrients and macromolecular growth factors from serum do not become limiting for multiplication at the low cell densities and during the incubation period. We further assumed from these data that the reverse of the reaction, CS → 2C (ie, 2C → CS = C + S), was insignificant and did not have impact on the concentration of substrates that were examined in kinetic studies. The analysis suggested that cell-derived products did not contribute significantly to the concentration of most substrates and other factors that affect cell multiplication.

Once an experimental system was designed that satisfied the principles discussed above, we applied the equations that were derived by Henri-Michaelis-Menten to relate the concentration of substrates to the velocity of enzyme-catalyzed reactions. Rate of cell proliferation, r, and the concentration of any extracellular substrate or ligand can be substituted for v (velocity) and [S], respectively, in

$$v/V_{max} = [S]/K_m + [S].$$

The equation yields the useful numerical parameters, V_{max}, the maximal rate (velocity) of cell proliferation, and K_m (the Michaelis-Menten constant), which is substrate concentration where rate of cell proliferation is half-maximal. To avoid confusion with enzyme kinetics and because the terms K_m and V_{max} are not constants but are dependent on the

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*The activity of FBSP was assessed in two ways. One method involved incubation of a saturating level of FBSP (1–2 mg per ml) in the cell multiplication assay and then assessing the activity of the incubated medium by dilution into a fresh assay at a concentration of FBSP from 10 to 200 μg per ml. In the other method, the FBSP was recovered from a pooled batch of incubated medium (about 1 liter) from multiplication assays. The pooled medium was dialyzed, lyophilized, reconstituted in buffer, and assayed for growth-stimulatory activity as described for native FBSP.
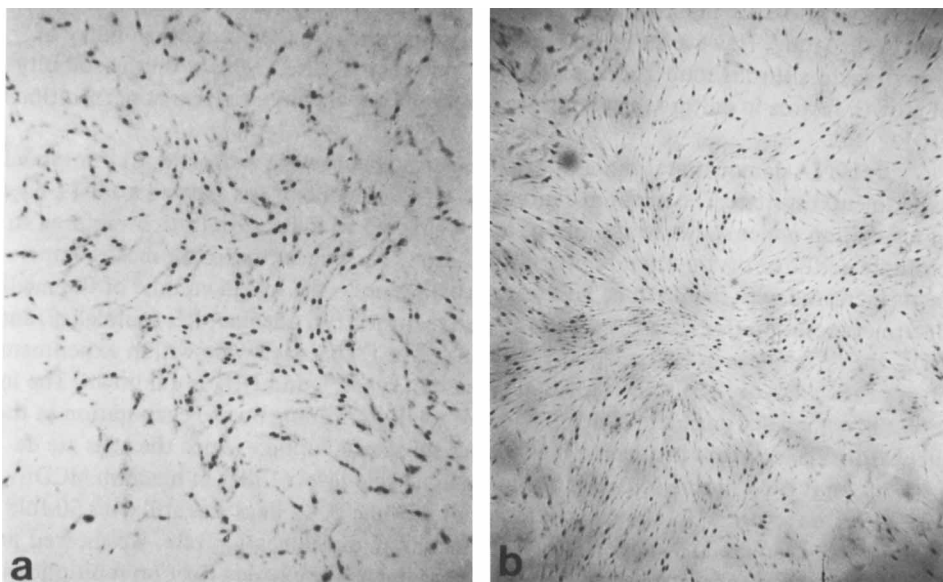
Fig. 2A. Autoradiography of nuclear [³H]-thymidine incorporation in a colony of N-HLF. Clonal mul-
tiplication of 50 N-HLF (Flow 2000) was carried out in 35-mm polylysine-coated tissue culture dishes
containing 2.0 ml of medium MCDB 107 and 1,000 μg FBSP per ml. After the time of incubation indi-
cated below, 0.10 μCi per ml of [³H]-thymidine (20 Ci/mol) was added, and the incubation was con-
tinued for 24 h. Cells were then fixed with methanol/acetic acid (3:1) and overlaid with Kodak NTB-2
photographic emulsion. The plates were developed after 3 days and counter-stained with hematoxylin.
a, a representative colony labeled after 7 days of incubation; b, a colony labeled after 13 days of incu-
bation.

status of other culture variables, we substituted the symbols r for v, R_{max} for V_{max}, and
$S_{0.50}$ for K_m in application of the formula to analysis of cell multiplication.

For kinetic analysis, a simple analytical procedure to reproducibly determine the
rate of reaction for large numbers of replicate samples is needed. For analysis of cell pro-
liferation, the measurement of increase in cell number (product) per unit time [d(P or C)/
dt] is a logical parameter. However, measurement of other parameters that are proportional
to cell number is feasible. Figure 1A shows the increase in cell number of 100 normal di-
ploid human lung fibroblasts (N-HLF) with time. Increase in cell number is sufficient for
measurement during a period when the concentration of cells has little impact on the con-
centration of extracellular nutrients or growth factors (Table IA). However, the non-linear-
ity of the growth curve complicated the calculation of the instantaneous multiplication
rate of the cell population from cell number at all different times during the incubation.
The reason for this pattern is under study and is beyond the scope of this treatise. When
the complete time curve is examined more closely, the cell population actually exhibits a
continuously decreasing rate of growth from day 0 until depletion of nutrients and growth
factors in the medium begins to severely limit multiplication rate after 18 to 20 days of in-
cubation (unpublished results). This pattern has been confirmed by careful quantitation of
the incorporation of [³H]-thymidine into cells both by counting percent-labeled nuclei
within colonies (Fig. 2A) or by determination of cpm [³H]-thymidine per μg DNA harv-
ested from the dishes (unpublished results). Autoradiographic analysis of the incorporation

of [^{3}H]-thymidine revealed that incorporation of the isotope was random among cell nuclei within colonies of variable density (Fig. 2A). The above results, and the fact that nutrients and serum factors are in steady state (Table IA), suggest that the non-linearity of the growth curve is unlikely due to cell–cell contacts or depletion of medium components.

In practice, we assumed that the growth curve (Fig. 1A) was biphasic and exhibited regions where growth rates are sufficiently uniform for kinetic studies. Since it was difficult to rapidly and accurately count small numbers of cells directly for large numbers of replicate assays, the second linear phase of growth was used for measurement of cell number and calculation of multiplication rate (Fig. 1A). Visible macrocolonies from single cells in the original inoculum began to appear after 6 to 7 days of incubation. Therefore, an in situ densitometric measurement of the average size of the macrocolonies formed during the 7- to 14-day period was used to measure cell number. The increase in colony size, when measured photometrically, accurately reflected the population multiplication rate as measured by the direct count of cells after harvesting them from the dish (Fig. 3A). Since the observed multiplication rate was similar when the size of all detectable colonies or only the larger class of colonies was measured (Fig. 3A), the average size of only the five largest colonies per experimental Petri dish was routinely used as a measure of total cell number.

Multiplication rate is the slope of the linear regions of the plots in Figs. 1A and 3A. Multiplication rate is described by the logarithmic transformation, $r = 3.32(\log_{10} C_t - \log_{10} C_0)/t$, of the exponential equation, $C_t = C_0 2^{rt}$, which describes the increase in cell number in a population when individuals are multiplying by fission. In the equation, $r =$
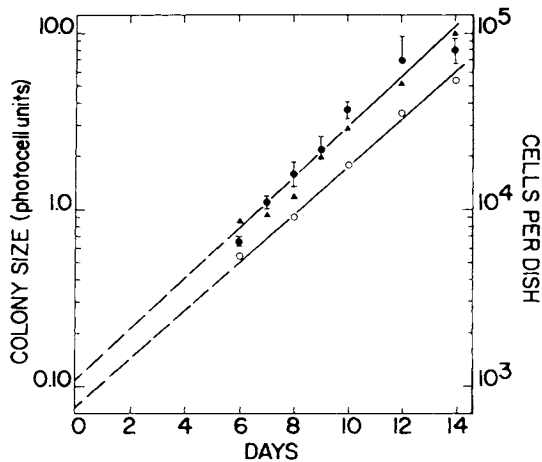

Fig. 3A. Time course of increase in cell number of N-HLF. One hundred N-HLF (Flow 2000; PDL 17) were inoculated into complete medium MCDB 105 containing 500 μg FBSP per ml. In one set of plates, cells were fixed and stained after the indicated time of incubation. In a replicate set of plates, cells were removed from the Petri dishes with trypsin and counted in a Coulter Counter. ●–●, photometric measurement of the five largest colonies per plate; the data points are means ± SE (triplicate dishes) of the mean size in arbitrary photocell units of the 5 largest colonies on a dish. ▲, photometric measurement of the total colonies per plate; the data points are the mean of the size in photocell units of all measurable colonies from a single dish. ○, total number of cells per dish; the count was derived from a suspension of pooled cells that were harvested from triplicate dishes. –––, linear regression of the courses that were fit to the data by "least squares" estimate. (From McKeehan WL, McKeehan KA [11], reproduced by permission of the Tissue Culture Association.)
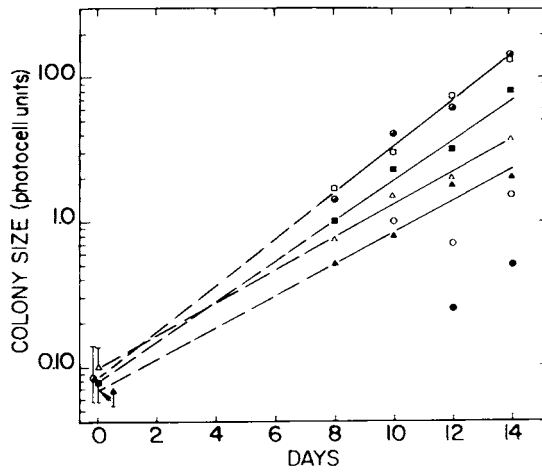
Fig. 4A. Time course of increase in cell number at different levels of FBSP in the medium. Clonal multiplication of 100 N-HLF (MRC-5; PDL 19) was analyzed in complete medium MCDB 105 containing the indicated levels of FBSP. Cells were fixed and stained at the indicated times. Each data point is the mean size in photocell units of the 5 largest colonies from a single dish. Linear curves were fit to the data by "least squares" and regression to the t = 0 axis (dotted lines). The vertical bars indicate the SE of each intercept due to deviations from linearity and the regression analysis. No curves were drawn through the data for 20 and 40 μg FBSP per ml because of the lack of measurable colonies at early times. FBSP: •, 20 μg per ml; ○, 40 μg per ml; ▲, 100 μg per ml; △, 200 μg per ml; ■, 400 μg per ml; □, 700 μg per ml; ⊙, 1,000 μg per ml. (From McKeehan WL, McKeehan KA [11]. Reproduced by permission of the Tissue Culture Association.)
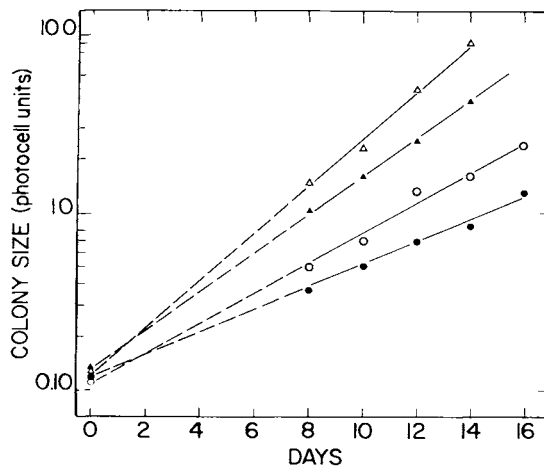

Fig. 5A. Time course of increase in cell number at different extracellular Ca^{2+} concentrations. Clonal multiplication of 100 N-HLF (Flow 2000; PDL 19) cells were analyzed. The amounts of $CaCl_2 \cdot 4H_2O$ indicated below were added to $CaCl_2 \cdot 4H_2O$-deficient medium MCDB 107 containing 500 μg FBSP per ml. At the indicated times, colonies were fixed and stained. Each data point is the mean size in photocell units of the 5 largest colonies from a single dish. Data points at t = 0 represent intercept points of the regression lines from data obtained, where t = 8 to 16 days. $CaCl_2 \cdot 4H_2O$: •, 30 μM; ○, 100 μM; ▲, 300 μM; △, 1.0 mM. (From McKeehan WL, McKeehan KA [11], reproduced by permission of the Tissue Culture Association.)

multiplication rate of the population in cell generations (g) per day; 3.32 = reciprocal $\log_{10}2$; C_t = cell number after t days of incubation; t = time of incubation; and C_0 = cell number at t = 0. The value of C_0, the t = 0 intercept (Fig. 3A) is a constant and independent of the multiplication rate or the extracellular condition that limits multiplication. Figure 4A illustrates the increase in cell number measured by size of the 5 largest colonies in photocell units during an 8- to 14-day incubation period at different concentrations of FBSP. Figure 5A shows similar data for Ca^{2+} when FBSP was constant. Multiplication rate was essentially uniform at all concentrations of FBSP or Ca^{2+}. A similar pattern resulted when other nutrients were varied (unpublished results). Regression lines established from the values where colony size could be measured clustered around the same point (C_0) on the t = 0 axis. This confirmed that the general nature of the growth curve shown in Figure 1A was independent of rate of multiplication as well as the extracellular condition that limited multiplication rate. This was also confirmed for several extracellular variables by examination of their effect on multiplication rate during the first linear phase of growth (Fig. 1A) between 0 and 6 days of incubation (unpublished results). Therefore, the instantaneous rate of cell multiplication can be calculated from colony size values determined at any point in the linear region between 6 and 14 days using the constant C_0 in the equation described above. The grand mean of the t = 0 intercept (C_0) was determined for individual cell types from regression lines of experimental time curves using a variety of extracellular conditions. Generally, multiplication rates were determined from data obtained after 12 to 14 days of incubation to yield colony sizes large enough to be measured accurately by densitometry at very low multiplication rates.

Cell multiplication as a function of the concentration of both FBSP and nutrients exhibited saturation kinetics with a typical transition from first order to mixed order, to
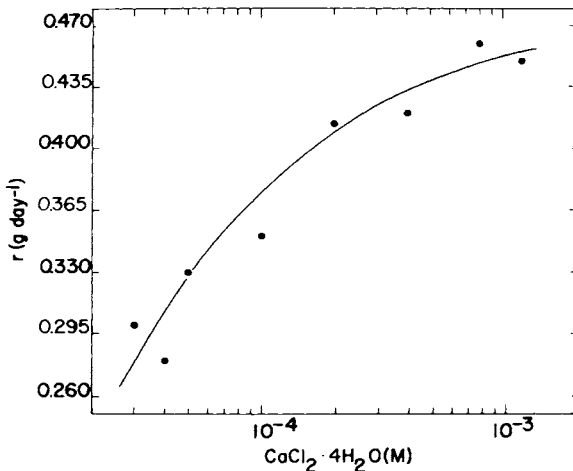

Fig. 6A. Multiplication rate of N-HLF as a function of increasing $CaCl_2 \cdot 4H_2O$ concentration. Clonal multiplication of 100 N-HLF was analyzed after 14 days of incubation in $CaCl_2 \cdot 4H_2O$-deficient medium MCDB 105 containing 500 μg FBSP per ml with the indicated levels of $CaCl_2 \cdot 4H_2O$ added to the medium. The data are combined from experiments using Flow 2000 N-HLF at PDL 19 and 20. Each data point is the mean of triplicates. The data were fitted to a single rectangular hyperbola. (From McKeehan WL, McKeehan KA [12].)
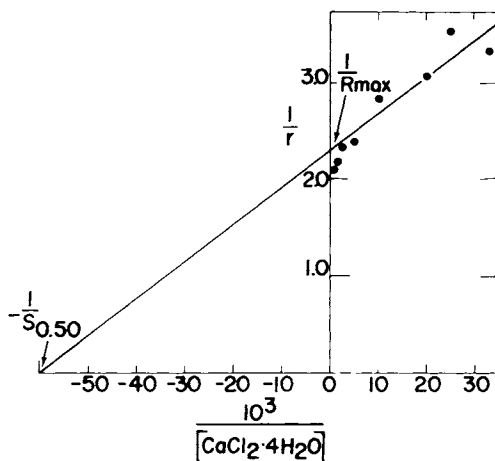
Fig. 7A.   Double-reciprocal plot of multiplication rate versus $CaCl_2 \cdot 4H_2O$ concentration. The best linear plot was fitted to the data from Figure 6A by "least squares" estimate. Regression to the intercepts yielded an $S_{0.50}$ value of 1.7 E-5 M and a R_{max} value of 0.444 g day^{-1}. (From McKeehan WL, McKeehan KA [12].)

essentially a zero order response. Multiplication rate, r, plotted against substrate or ligand concentration, [S], yielded right rectangular hyperbolas described by the Henri-Michaelis-Menten equation (Fig. 6A) and the linear relationship between $1/r$ and $1/[S]$ described by the Lineweaver-Burk transformation (Fig. 7A). Linear regression of the double-reciprocal plot yielded from the intercepts a maximal rate of multiplication, R_{max}, and the $S_{0.50}$, which is the substrate or ligand concentration that supports a half-maximal rate of cell multiplication. Other linearization methods for the r versus [S] data yielded similar results. However, the double-reciprocal transformation was used in most experiments. In contrast to enzyme kinetics, the ordinate, $1/r$, in the double-reciprocal treatment was a meaningful parameter that described the doubling time in days of the cell population.

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