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TECHNICAL MANUSCRIPT 563

AN IMPROVED CHEMICALLY DEFINED CULTURE MEDIUM FOR STRAIN L MOUSE CELLS BASED ON GROWTH RESPONSES TO GRADED LEVELS OF NUTRIENTS

Kiyoshi Higuchi

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TECHNICAL MANUSCRIPT 563

AN IMPROVED CHEMICALLY DEFINED CULTURE MEDIUM FOR STRAIN L MOUSE CELLS BASED ON GROWTH RESPONSES TO GRADED LEVELS OF NUTRIENTS

Kiyoshi Higuchi

Medical Bacteriology Division
BIOLOGICAL SCIENCES LABORATORIES

Project 1B562602AD01 October 1969

ACKNOWLEDGMENT

I thank Lawrence A. Swann for excellent technical assistance.

ABSTRACT

Nutritional factors were evaluated for effects on growth of mouse fibroblast cells in suspension in a chemically defined medium. Quantitative requirements for each essential amino acid, choline, inorganic phosphate, iron, and zinc were established. An improved chemically defined medium was formulated on the basis of the findings yielding populations of L cells in excess of 5×10^6/ml without nutrient replenishment. When spent medium was replaced periodically, yields approaching 30×10^6 cells/ml were attained. The efficiency of utilization of most amino acids in the new medium appears to be twofold to threefold better than results reported by others.

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I. INTRODUCTION*

The ability of serum-free, chemically defined media to support growth of a number of mammalian cells in culture is now well recognized. Various useful chemically defined culture media have been described by many workers.[1-8] The composition of most of these media was based primarily on qualitative rather than on quantitative requirements, and very little attention has been given to the efficiency of utilization of amino acids to produce cellular material. Mohberg and Johnson[9] and more recently Griffiths and Pirt[10] have examined this problem in some detail. Mohberg and Johnson showed that the ratios of the amount of amino acid consumed to the amount incorporated into cell protein ranged from 1.6 to 5.0 for the essential amino acids. Griffiths and Pirt, who employed a medium containing 10% bovine serum, described wide differences in ratios of amount of amino acid consumed to cell yield attained under various conditions of culture.

Because of a need for more complete information on the quantitative requirements for nutrients in the growth of animal cells in vitro, studies were undertaken in our laboratory that resulted in the development of an improved chemically defined cell culture medium.

II. MATERIALS AND METHODS

A. MEDIUM

The composition of the cell culture medium as finally formulated is shown in Table 1. Preliminary experiments were undertaken in media containing approximately one-fifth to one-half the levels of the individual amino acids shown in the table. In the original medium, proline and asparagine were also included at 1.0 mM concentration. Amino acid levels in the medium were subsequently changed on the basis of requirements for improved cell yields as indicated by a series of experiments of the type described in detail in this report.

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TABLE 1. IMPROVED CHEMICALLY DEFINED CELL CULTURE MEDIUM[a]

L-Amino Acids	Concn, mM
Arginine	0.50
Cysteine	0.60
Glutamine	6.00
Histidine	0.30
Isoleucine	0.75
Leucine	0.80
Lysine	0.75
Methionine	0.30
Phenylalanine	0.30
Serine	1.00
Threonine	0.40
Tryptophan	0.06
Tyrosine	0.30
Valine	0.80

Miscellaneous	Concn, mg/liter
Methylcellulose	500
Phenol red	10
Na penicillin	100
Streptomycin SO_4	100

Vitamins	Concn, µg/ml
Biotin	1.0
Choline·HCl	40.0
Folic acid	1.0
Nicotinamide	1.0
Pyridoxal·HCl	1.0
Thiamine·HCl	1.0
Inositol	1.0
Ca pantothenate	1.0
Riboflavin	0.1
Vitamin B_{12}	0.002

Inorganic Salts	Concn, mg/ml
NaCl	6.50
KCl	0.41
$NaH_2PO_4 \cdot H_2O$	0.28
$NaHCO_3$	0.84[b]
$CaCl_2 \cdot 2H_2O$	0.15
$MgCl_2 \cdot 6H_2O$	0.30
$FeNH_4(SO_4)_2 \cdot 12H_2O$	(10^{-5} M)
$ZnSO_4 \cdot 7H_2O$	(10^{-6} M)

Carbohydrate and Derivatives	Concn, mM
Glucose	25.0
Na pyruvate	1.0
Na gluconate	1.0

a. The levels of nutrients in this medium were adjusted to be enough to produce yields of at least 5×10^6 cells/ml of mouse fibroblast (strain 929-L, substrain LDR).

b. This level of $NaHCO_3$ in the final medium is composed of 0.25 mg/ml added as part of salts I mixture plus 0.42 to 0.60 mg/ml added as 1 M $NaHCO_3$ as described in Section II.

The following procedure is employed in preparation of the culture medium. The amount of each of 14 amino acids required for 4 liters of final volume, together with appropriate amounts of glucose and sodium pyruvate, is weighed into a 1-liter beaker and dissolved in a combination of 400 ml of salt mixture I (containing 6.5% NaCl, 0.41% KCl, 0.276% $NaH_2PO_4 \cdot H_2O$, and 0.25% $NaHCO_3$), 200 ml of salt mixture II (containing 0.294% $CaCl_2 \cdot 2H_2O$ and 0.61% $MgCl_2 \cdot 6H_2O$), and 40 ml of salt mixture III (containing 10^{-3} M $FeNH_4(SO_4)_2$ and 10^{-4} M $ZnSO_4$ dissolved in 0.1 M gluconic acid solution). Solution of the dry ingredients is promoted by heating the mixture to approximately 40 C while stirring. The medium is supplemented further by adding 40 ml of a 100-fold concentrated solution of vitamins (according to concentrations shown in Table 1) and 40 ml of a 100-fold concentrated solution of penicillin, streptomycin, and phenol red indicator. Finally, sufficient H_2O is added to make 800 ml of a fivefold concentrated solution of the basal medium. This material is filtered aseptically through a membrane filter (0.22 μ) and bottled as four 200-ml aliquots in tightly capped sterile 8-oz prescription bottles. The concentrated medium is diluted for use by aseptic addition of 40 ml to 155 ml of sterile (autoclaved) water in 8-oz bottles, and to each bottle, 5 ml of sterile (autoclaved) 2% methylcellulose (15 cp grade) solution and 1.0 to 1.5 ml of 1.0 M $NaHCO_3$ are added. The sterile bicarbonate solution is prepared by autoclaving a 1.0 M solution, cooling, and aseptically saturating the material with 100% CO_2 to restore the bicarbonate state. The medium as finally prepared is kept tightly capped to prevent loss of CO_2 during storage at 4 C. The use of methylcellulose was based on the report of Bryant et al.,[11] who showed that this nonionic polymer was useful in protecting animal cells in suspension cultures.

B. CULTURE CONDITIONS AND ASSAY OF CELL GROWTH

Experimental cell cultures were grown in suspension in 25 ml of test medium in 100-ml serum bottles. Early experiments were conducted in stoppered bottles; however, as improvements in cell yields were obtained, better aeration of cultures became necessary. Adequate ventilation of shaken cultures was achieved by use of cotton-plugged vents in the stoppers. The vents were kept closed until cell populations reached 2×10^6 cells/ml. Premature opening of vents caused undesired rises in medium pH owing to excessive loss of CO_2. Attention to proper ventilation resulted in maintenance of pH in cultures in the desired range (pH 6.7 to 7.4) during the entire growth cycle without further treatment. All cultures were grown at 35±0.2 C on the New Brunswick Gyrotory shaker operated at 130 rpm with an excursion radius of 1 inch.

Growth response to individual nutrients was tested by inoculating duplicate flasks containing graded amounts of the test substance with approximately 5×10^5 cells/ml. Growth was assessed by daily microscopic enumeration of cell numbers in hemocytometers. Viability of cells was determined by the dye-exclusion method employing trypan blue. Cell cultures were diluted when necessary in phosphate-buffered saline containing 0.05% methylcellulose prior to mixing 1:1 with 0.05% trypan blue solution.

C. CELL CULTURE STRAINS

The cell strains employed in these studies were derived from the mouse fibroblast cell, clone 929-L, originally isolated by Sanford, Earle, and Likely.[12] The major part of this work was done with a substrain designated LDR kindly provided by Dr. W.F. Daniels of Fort Detrick. The LDR cell was chosen for study because of its excellent adaptation to growth in suspension. Another subline of strain 929-L adapted for suspension culture whose growth characteristics were quite similar to those of strain LDR was also employed. Examinations for contaminants were made periodically to avoid cultures containing PPLO and other extraneous microorganisms. At intervals, return to frozen stock cultures was made in order to assure purity of inoculum.

III. RESULTS

A. GROWTH CURVES

A typical growth curve of mouse fibroblast cells in suspension culture in the improved chemically defined medium is presented in Figure 1. Approximately 2.5 x 10^5 cells/ml were used as inoculum in this experiment. Initiation of growth can be obtained in the medium from populations below 10^5 cells/ml; however, delays and sometimes reduced peak yields occurred unless larger inocula (about 2 x 10^5 to 5 x 10^5 cells/ml) were employed. When spent media in cultures were replaced periodically with fresh medium, continued increases in cell population occurred. An example of this type of growth is shown in Figure 2. Daily medium changes permitted attainment of cell populations approaching 30 x 10^6 cells/ml.

B. AMINO ACID REQUIREMENTS

Assessment of growth responses of L cells to graded levels of each of the essential amino acids has permitted determination of the quantity of each nutrient required for achieving peak cell population (5 x 10^6 cells/ml). The data presented in Figure 3 show the growth response of L cells to varied levels of arginine, histidine, and lysine. The level of histidine (0.2 mM) required to attain peak yields was distinctly less than for the other two amino acids shown. The growth responses of L cells to isoleucine, leucine, and valine are also presented in Figure 3. Each of these compounds was required in relatively high amounts (0.7 mM) to obtain peak populations.

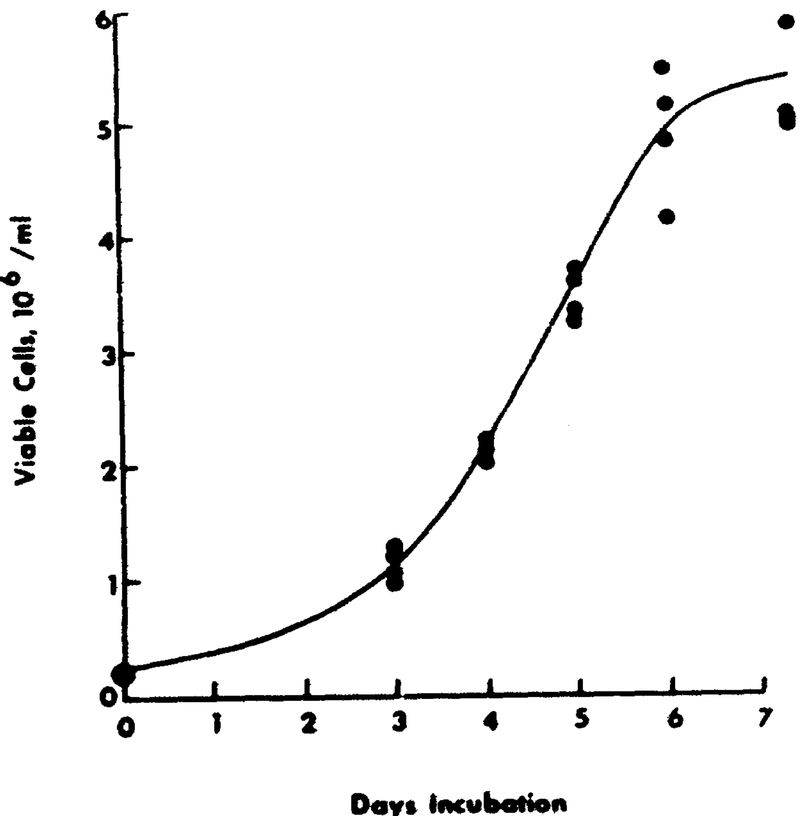

FIGURE 1. Growth Curve of Mouse Fibroblast Cells (Strain 929-L, Substrain LDR) in Chemically Defined Medium. Each point represents cell counts obtained in duplicate cultures. No medium changes were made during the period shown.

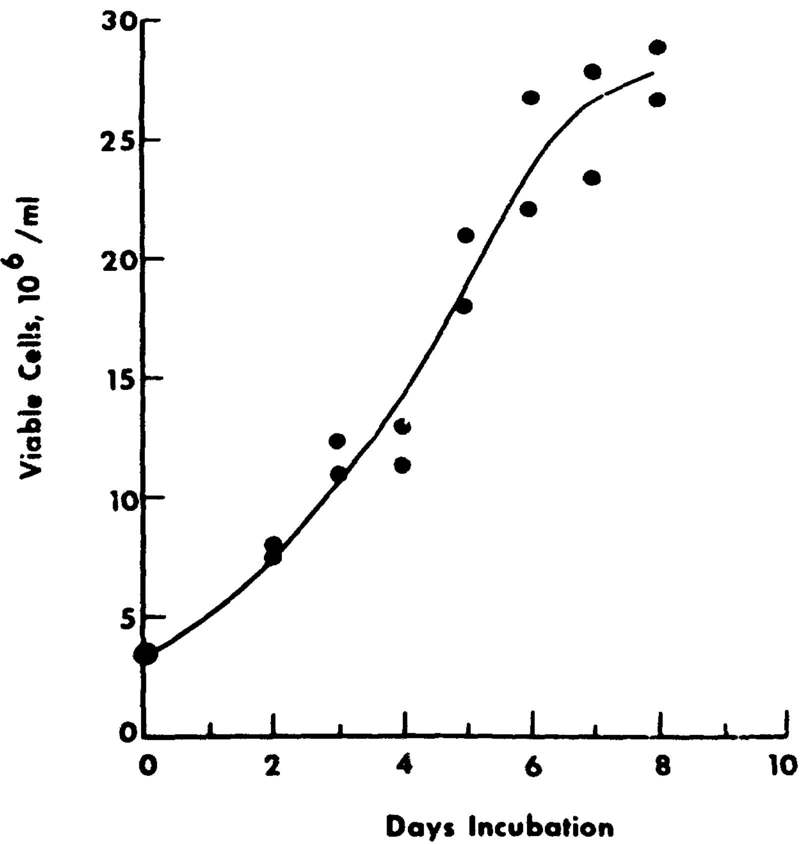

FIGURE 2. Heavy Cell Yields Obtained in Defined Medium by Daily Changes of Medium During 8 Days of Incubation. Initial inoculum was 3.6×10^6 cells/ml.

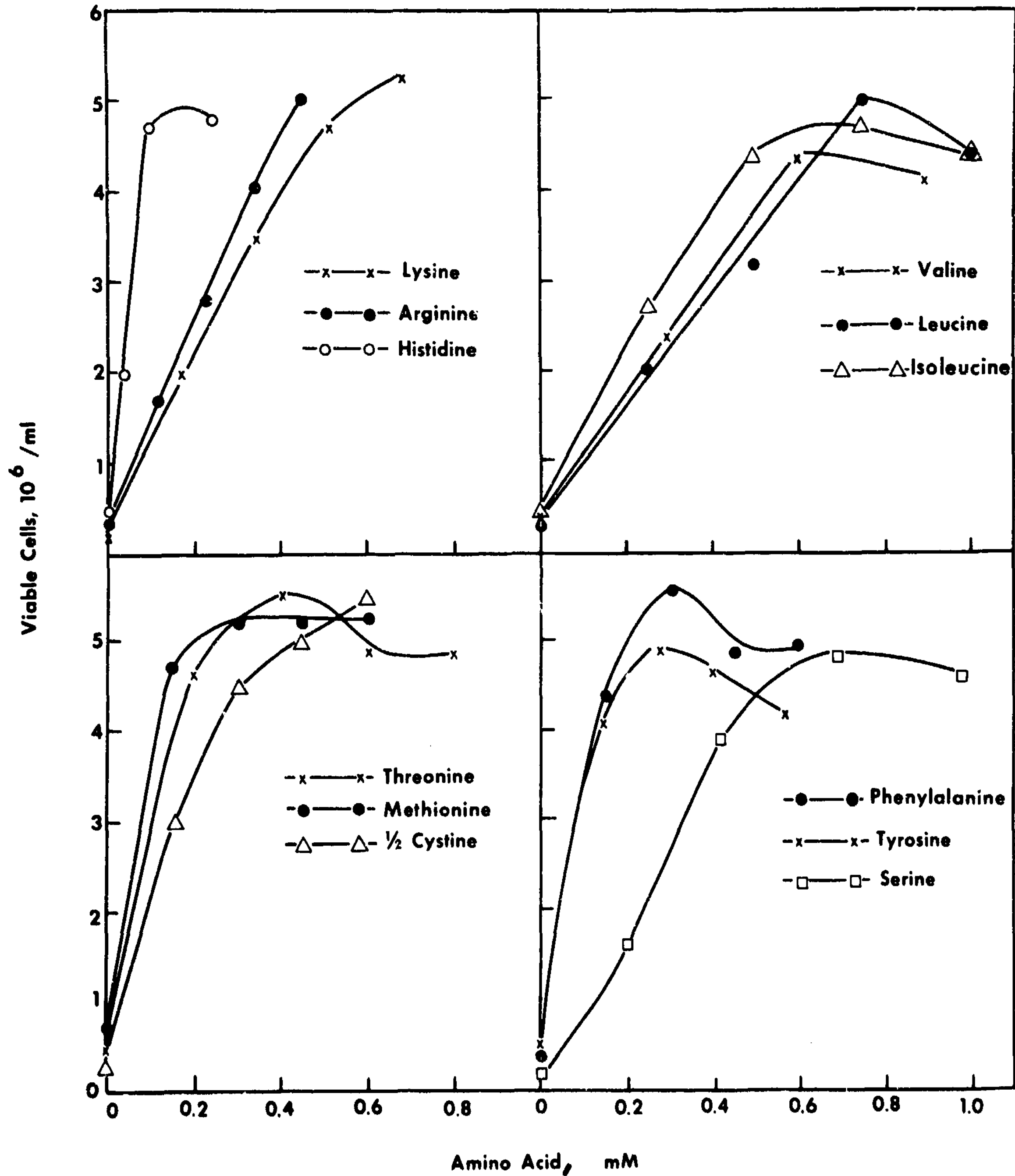

FIGURE 3. Growth Response of L Cells to Various Amino Acids. Each point is an average of values from duplicate flasks and represents the peak cell yield obtained during 6 days of incubation. The cystine data are plotted as half-cystine residues in order to permit comparison with cysteine.

The growth responses of L cells to the sulfur-containing amino acids, cystine and methionine, and to threonine are also presented in Figure 3. The reduced form of cystine (cysteine·HCl·H_2O) is more convenient to use in preparation of the medium because of its better solubility. It is rapidly converted to cystine by oxidation, but either form can be used equally well in the growth medium. Approximately 0.45 mM half-cystine residue, 0.20 mM methionine, and 0.25 mM threonine were required to yield peak cell population.

Both tyrosine and phenylalanine were required at approximately 0.25 mM to produce peak yields (Fig. 3). Excessive amounts of either amino acid appeared to be slightly inhibitory for cell growth. The growth response to serine in the defined medium (Fig. 3) was somewhat unexpected, but as observed previously,* certain substrains of L cells in suspension culture failed to grow unless serine was present. Glycine was able to replace the serine requirement only partially, and cell growth was distinctly reduced in a medium lacking serine.

Data presented in Figure 4 show the quantitative requirement for tryptophan. This requirement was significantly less than for any other amino acid employed. Only an estimated 0.045 mM tryptophan was required to yield 5 x 10^6 cells/ml. The growth response of L cells to glutamine is also presented in Figure 4. The amount of glutamine (5 mM) required to produce peak cell population was 100-fold the amount of tryptophan required. This unusually high requirement was due to an apparent inefficient utilization of glutamine. Large quantities of ammonium ions, approximately equimolar to the amount of glutamine that was consumed, were produced in cell cultures. Most of the ammonia liberation was caused by cell activity, since in cell-free incubated medium, spontaneous liberation of amide ammonia occurred at only one-fifth the overall rate observed in L cell cultures.

C. CHOLINE REQUIREMENT

Choline is an essential nutrient that is present in most defined culture media at inadequate levels for supporting good cell yields.[13] The growth response of L cells to graded levels of choline chloride (Fig. 5) demonstrates that a relatively large amount of this substance is needed. When growth was initiated from an inoculum of 2.4 x 10^5 cells/ml, approximately 30 µg/ml of choline chloride was required to produce peak cell yields.

D. REQUIREMENTS FOR INORGANIC IONS

In addition to the major cations and anions provided by NaCl, KCl, $NaHCO_3$, $MgCl_2$, and $CaCl_2$, certain other inorganic ions are needed for cell growth. The growth response of L cells to inorganic phosphate was approximately linear for cell populations up to 5 x 10^6 cells/ml with approximately 1 mM orthophosphate required for this population level (Fig. 6).

* Nagle, S.C. Jr., personal communication.

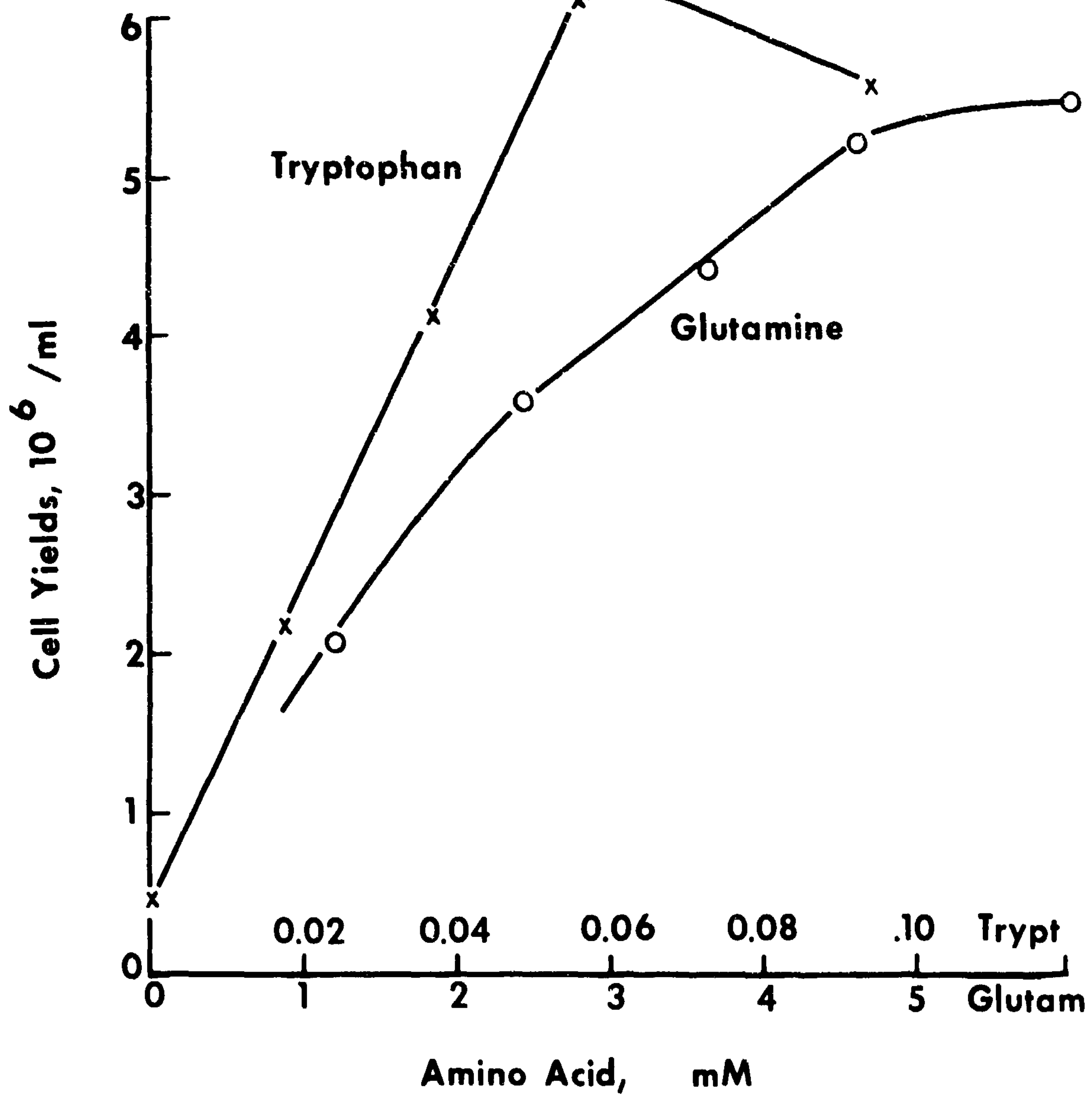

FIGURE 4. Growth Responses of L Cell Cultures to Graded Levels of Glutamine and Tryptophan. Each point is an average of values from duplicate flasks.

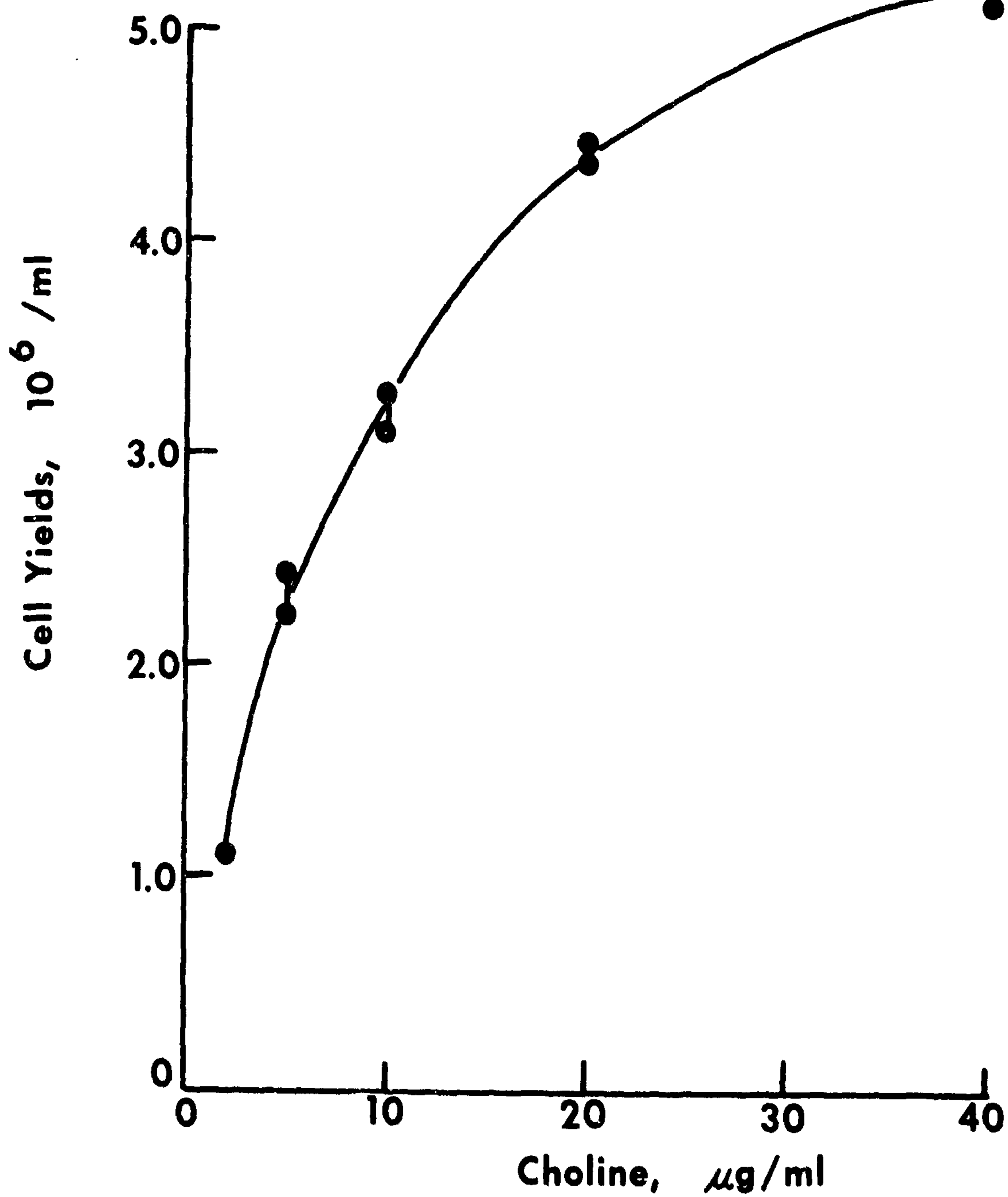

FIGURE 5. Growth Response of L Cell Cultures to Choline Chloride. Values from duplicate cultures are shown for each level of choline tested.

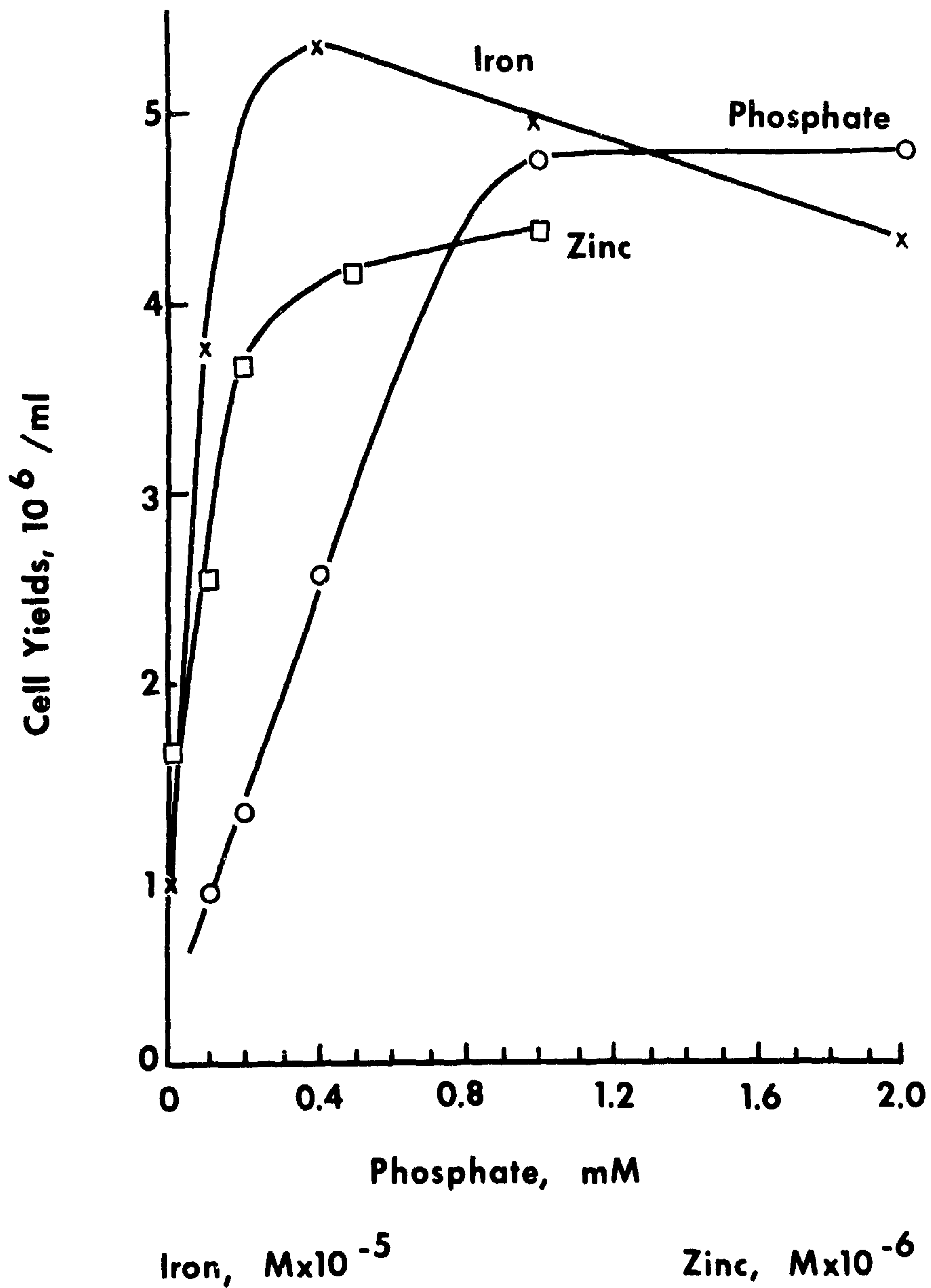

FIGURE 6. Growth Responses of L Cell Cultures to Graded Levels of Inorganic Phosphate, Inorganic Iron [as $FeNH_4(SO_4)_2$], and Inorganic Zinc (as $ZnSO_4$). Averaged values from duplicate cultures are presented.

Requirements for both iron and zinc ions have been demonstrable for L cells in the defined medium. The growth response to graded levels of ferric iron is shown in Figure 6. Approximately 4 x 10^{-6} M iron was required to attain peak cell yields. Evidence for a small but definite degree of inhibition of growth with higher levels (to 20 x 10^{-6} M) of iron was noted in several experiments.

A requirement for zinc ions for growth of L cells was also demonstrable (Fig. 6). The data show that approximately 5 x 10^{-7} M zinc was needed to produce peak cell yields.

IV. DISCUSSION

The data presented above show that the improved chemically defined culture medium provides an unusually good growth environment for propagation of L cells (substrain LDR). Growth in other defined media previously described required repeated replenishment of nutrients to attain populations of considerably less than 5 x 10^6 cells/ml during incubation periods of 6 to 9 days. In the present medium, no nutrient replenishment was needed to attain 5 million cells/ml, and when the spent medium was replaced daily, unprecedented yields approaching 30 x 10^6 cells/ml were obtained in batch culture. A recent report describes a device (spin filter) for continuous replacement of medium.[14] Extremely high cell densities approaching 100 x 10^6 cells/ml have been obtained in this system. It should be of interest to study cell population obtainable in a continuous spin filter culture system employing the present medium.

Although there was no serious inhibition of cell growth due to an excess of any amino acid in our experiments, there were a number of instances where trends toward decreased cell yields were evident with an excessive amount of certain amino acids. It was therefore necessary to adjust these concentrations to more favorable levels for optimal growth. Moreover, the use of great excess of nutrients in the medium is uneconomical.

The possibility of further improvement of the culture medium in order to obtain better cell yields has been considered. However, merely increasing the concentration of all nutrients has not been successful. The wasteful rapid utilization of glutamine during growth with resultant ammonia formation causes inhibition of growth either by depletion of the essential nutrient or by toxicity of accumulated ammonium ions. A simple solution to this problem may be obtained by replacing most of the glutamine with glutamic acid as shown feasible by the work of Griffiths and Pirt.[10] Unfortunately, the growth rate in glutamine-free medium is markedly reduced under present conditions.

Griffiths and Pirt[10] evaluated the efficiency of amino acid utilization in cell cultures. They showed that the efficiency of utilization was variable, depending on growth rate and cell cultivation method (batch or continuous). In general, they noted that the continuous method was more efficient. A comparison of their results with efficiency of utilization in our medium showed that distinctly better utilization was obtained in our system, even when compared with their data obtained with continuous cultures (Table 2). Although no significant difference occurred between the best values of Griffiths and Pirt and those obtained in our medium for cysteine, tyrosine, and valine, most other amino acids were utilized with twofold to threefold better efficiency in our medium.

TABLE 2. COMPARISON OF AMOUNTS OF AMINO ACID REQUIRED TO PRODUCE A MILLION MOUSE CELLS

L-Amino Acid	μg of Amino Acid/10^6 Cells	
	Griffiths and Pirt[a]	Present Study[b]
Arginine	43 to 90	16.0
Cysteine (or cystine)	12.5 to 39	10.5
Glutamine	340 to 1,080	132.0
Histidine	13.5 to 18	6.0
Isoleucine	38 to 76	18.5
Leucine	36.5 to 77	18.5
Lysine	26 to 60	16.0
Methionine	9 to 15	6.0
Phenylalanine	11 to 25	6.5
Serine	c/	15.0
Threonine	15 to 29	5.5
Tryptophan	5.5 to 9	1.8
Tyrosine	11 to 14.5	10.0
Valine	15 to 49	16.5

a. Data abstracted from Table 7 of Griffiths and Pirt.[10]
b. Calculated from data shown in Figures 3 and 4 and expressed as the free amino acid.
c. No data.

The demonstration in the present work of requirements for inorganic cations such as iron and zinc was not unexpected in view of the known role of these elements in animal nutrition. Thomas and Johnson[15] showed that in a defined medium treated with a chelating resin (Dowex A-1), additions of 0.6 to 1.4 μM iron and 0.6 μM zinc ions were required to restore maximum growth of L cells. They calculated that 0.16 nmole iron was required to produce 10^6 cells. In our medium an estimated 0.36 nmole iron was needed per 10^6 cells; however, as shown by the above authors, the presence of other trace ions, notably Cu^{++} and Mn^{++}, markedly influenced iron requirement; therefore the lack of agreement is not surprising. The apparent requirement of 0.6 nmole of zinc in their medium as contrasted to only 0.1 nmole in our medium to produce 10^6 cells is not quite clear; however, differences in the composition of test media may have contributed to this difference. It is hoped that further work will reveal nutritional requirements for other trace elements in animal cell cultures.

Further work in defined medium with other cell lines is certainly desirable. Preliminary studies along this line have indicated that differences in cellular nutrition and metabolism may necessitate modification of the present medium to obtain yields with other cell lines comparable to those obtained with cell strain LDR.

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13. ABSTRACT

Nutritional factors were evaluated for effects on growth of mouse fibroblast cells in suspension in a chemically defined medium. Quantitative requirements for each essential amino acid, choline, inorganic phosphate, iron, and zinc were established. An improved chemically defined medium was formulated on the basis of the findings yielding populations of L cells in excess of ~~5 x 10^6/ml~~ without nutrient replenishment. When spent medium was replaced periodically, yields approaching 30 x 10^6 cells/ml were attained. The efficiency of utilization of most amino acids in the new medium appears to be twofold to threefold better than results reported by others. ( )

5,000,000/ml

14. Key Words

L cells
Cell culture medium
Iron
Zinc
Nutrients
Amino acids
Choline
Phosphate

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