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GROWTH OF ANIMAL CELLS IN A CHEMICALLY DEFINED SUSPENSION SYSTEM

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ABSTRACT

A medium consisting of 13 amino acids, 10 vitamins, 6 inorganic salts, glucose, pyruvate, and methylcellulose was developed for growth of animal tissue cells in suspension. Cultures were grown in 25 milliliters of medium in 100-ml serum bottles in a New Brunswick Gyrotory incubator shaker at 35°C. Populations (4.3×10^6 cells per ml) of cat kidney cells obtained in this medium were comparable with those reported for the lactalbumin hydrolyzate medium and the insulin-containing chemically defined medium reported earlier from our laboratories. Growth curves were obtained for cat kidney, HeLa, and L cells. Growth of these cells has been maintained continuously for several months by changing the medium on alternate days, and by reducing cell populations to between 5×10^5 and 10×10^5 at the time of medium change. The defined medium is relatively simple in chemical composition, allows high cell yields, and, combined with the suspension culture method, should be of value in studying various aspects of cell physiology and infectivity.

A chemically defined medium that was developed for the growth of animal cells in suspension was reported earlier from our laboratory.[1] The medium contains 13 amino acids, 10 vitamins, 6 inorganic salts, glucose, pyruvate, methylcellulose, insulin, phenol red, streptomycin, and penicillin (Table I). The requirements for the growth of a line of cat kidney cells had been established for each of the medium constituents at approximately the concentrations shown. This report deals with studies directed toward further simplification of the medium by replacement or elimination of insulin.

Incubation and cell enumeration were carried out as follows: Suspension cultures in rubber-stoppered 100-ml serum bottles containing 25 milliliters of medium were incubated at 34°C to 35°C in a New Brunswick Gyrotory incubator shaker operating at 124 to 130 rpm. At appropriate intervals, the numbers of viable cells were determined in the hemocytometer by the trypan blue procedure. Media were changed by centrifuging the serum bottle cultures at 1000 rpm for 10 minutes, decanting the supernatant, and replacing it with fresh medium. Three cell lines were employed: cat kidney cells, originally isolated in our laboratory, L cells, and HeLa cells. All cells had been growing continuously in suspension in defined medium for nearly a year prior to the present studies. Cell populations were maintained at 5×10^5 to 10×10^5 cells per milliliter by changing media on alternate days and reducing cell numbers as required. Populations as high as 2×10^6 to 4×10^6 cells per milliliter can be obtained. Growth curves of six cell lines previously obtained in the insulin medium are shown in Figure 1.

Preliminary studies indicated that cat kidney cells were capable of growth in the defined medium in which NPH insulin was replaced with the equivalent of 0.2 unit per milliliter of zinc insulin that had been hydrolyzed in 0.3 N HCl at 121°C for one hour. After growth for one month in the defined medium containing hydrolyzed insulin, these cat kidney cells had become adapted to growth in defined medium alone. Concurrently with these experiments, it was observed that a mixture of seven nonessential amino acids (i.e., nonessential for growth in the presence of insulin) would also substitute for NPH insulin for the growth of cat kidney cells. The requirement for each of these "nonessential" amino acids as a replacement for insulin was tested by omitting them individually from the complete chemically defined medium. Only glycine and aspartic acid were apparently not required and were omitted from subsequent experiments. The results are shown in Table II.

The ability of cat kidney, L, and HeLa cells to grow in the absence of insulin was tested in (a) the original defined medium, (b) the defined medium less insulin, and (c) the insulin-free medium fortified with five nonessential amino acids. Three types of cat kidney cells were available: (a) the original cat kidney cells requiring insulin, (b) cat kidney cells that had been adapted to growth in the defined unfortified medium, and (c) cat kidney cells that had been grown in the defined medium containing no insulin but fortified with five nonessential amino acids. From the results shown in Table III, the following observations were made: (a) the insulin-requiring cat kidney cells grew in the absence of insulin only where the mixture of five nonessential amino acids was used; (b) cat kidney cells that

had adapted to the unfortified medium grew equally well in all media; (c) cat kidney cells grown in medium containing nonessential amino acids were also capable of growth in all media; (d) L cells were capable of limited growth in unfortified media even though they had not previously been adapted to growth under these conditions; and (e) HeLa cells were also capable of some growth in all media. Cat kidney, L, and HeLa cells subsequently have been grown in the insulin-free chemically defined medium for several months. Growth curves for these cell lines are presented in Figure 2. Media were changed daily after the first two days of incubation. Maximum cell numbers obtained in the insulin-free medium compared favorably with those previously reported in the insulin medium.

The chemically defined medium described in this report may be compared with the two chemically defined media (medium NCTC 109 of McQuilken and coworkers,[2] and the minimum essential medium of Eagle[3]) that have been used for growth in suspension cultures of variants of two cell lines. Concentrations of most amino acids of medium NCTC 109 range from one-fifteenth to about one-third the levels of those in the medium described in this report. In addition, NCTC 109 contains 11 amino acids that are not present in our medium. Cysteine is the only amino acid used at a higher concentration in NCTC 109. Medium NCTC 109 also contains additional vitamins and cofactors (a total of 69 components with methylcellulose) not contained in the reported medium.

Our medium differs in the following respects from Eagle's minimum essential medium: Individual amino acid concentrations of Eagle are from one-sixth to approximately equal the levels of our medium; biotin and vitamin B_{12} are added to our medium in addition to the eight vitamins present in Eagle's medium.

The present findings indicate that the requirement for insulin in serum-free tissue culture media can be met in some cases by a mixture of non-essential amino acids. However, it is realized that the requirement for insulin by various tissue cell strains has been observed in serum-free media containing complex protein hydrolyzates,[4] which presumably are adequately supplied with these amino acids. Therefore, it is premature for us to draw any conclusions about the role of simple amino acids as substitutes for insulin.

In summary, the medium as described for suspension culture appears to be adequate for growth of the cell lines tested. The defined medium is relatively simple in chemical composition, allows high cell yields, and, combined with the suspension culture method, should be of value in studying various aspects of cell physiology and infectivity.

TABLE I. CHEMICALLY DEFINED MEDIUM FOR GROWTH OF ANIMAL CELLS IN SUSPENSION

COMPONENT	CONCENTRATION, Mg Per Liter	COMPONENT	CONCENTRATION, Mg Per Liter
AMINO ACIDS:		SALTS:	
L-Arginine·HCl	100	NaCl	7400
L-Cysteine·HCl	75	KCl	400
L-Histidine·HCl	60	$NaH_2PO_4 \cdot H_2O$	100
L-Isoleucine	150	$NaHCO_3$	500
L-Leucine	300	$CaCl_2 \cdot 2H_2O$	265
L-Lysine·HCl	300	$MgCl_2 \cdot 6H_2O$	275
L-Methionine	60	CARBON SOURCES:	
L-Phenylalanine	120	Glucose	1000
L-Threonine	135	Sodium pyruvate	110
L-Tryptophan	60	VITAMINS:	
L-Tyrosine	120	D-biotin	1.0
L-Valine	150	Choline Cl	1.0
L-Glutamine	450	Folic acid	1.0
ANTIBIOTICS, ETC.:		Niacinamide	1.0
Methocel, 15 Centipoise	1000	Ca pantothenate	2.0
NPH insulin	200 units/Liter	Pyridoxal·HCl	1.0
Streptomycin	100 mg/Liter	Thiamine·HCl	1.0
Penicillin	100,000 units/Liter	i-Inositol	1.0
Phenol red	10 mg/Liter	Riboflavin	0.1
		B_{12}	0.002

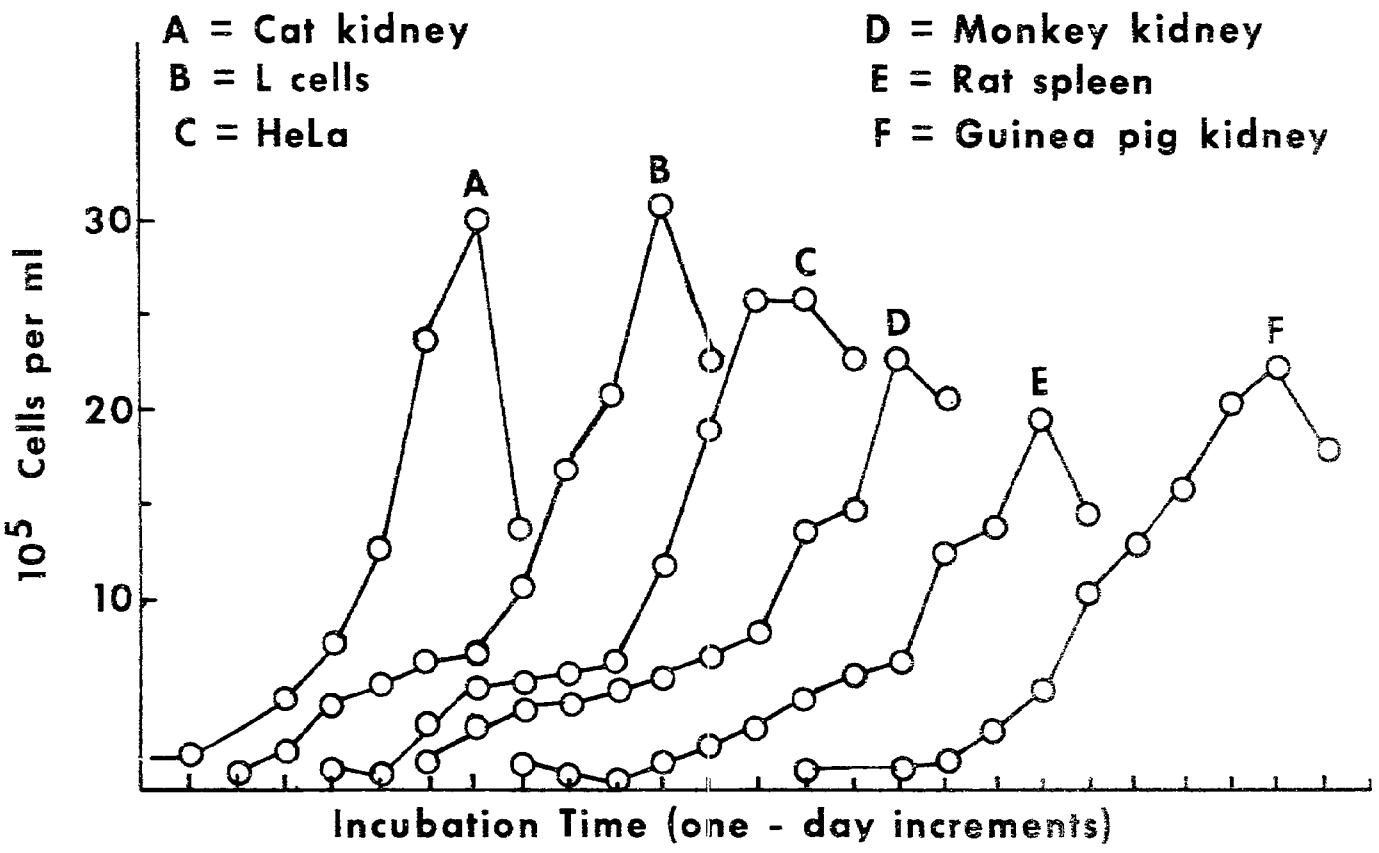

Figure 1. Growth of Animal Cells in Chemically Defined Medium.

TABLE II. GROWTH OF CAT KIDNEY CELLS IN INSULIN-FREE CHEMICALLY DEFINED MEDIUM

	MEDIUM VARIATION	HIGHEST CELL POPULATION [a] IN 8 DAYS, 10^5 PER ML
1.	Defined medium, complete	6.8
2.	Same plus 7 nonessential amino acids	8.4
3.	As #2 less insulin	7.2
4.	As #3 less glycine	7.7
5.	As #3 less serine	3.6
6.	As #3 less alanine	2.8
7.	As #3 less cystine	2.9
8.	As #3 less aspartic acid	7.3
9.	As #3 less glutamic acid	2.8
10.	As #3 less proline	4.8
11.	Defined medium, less insulin	2.5

a. Zero-hour cell count = 2.8×10^5 per ml.

TABLE III. GROWTH OF ANIMAL CELLS IN CHEMICALLY DEFINED MEDIA

CELL LINE	MEDIUM REQUIREMENT OF CELL LINE	HIGHEST CELL POPULATION IN 6 DAYS,[a] 10^5 Cells Per Ml		
		Defined Medium	Defined Less Insulin	Defined Less Insulin Plus Nonessential Amino Acids
Cat kidney	Chemically defined	18.1	4.9	11.1
Cat kidney	Chemically defined less insulin	15.6	14.7	15.6
Cat kidney	Chemically defined less insulin plus nonessential amino acids	13.8	14.3	18.0
L	Chemically defined	17.0	12.0	13.5
HeLa	Chemically defined	7.9	6.4	7.7

a. Zero-hour cell counts were approximately 2.0×10^5 per ml.

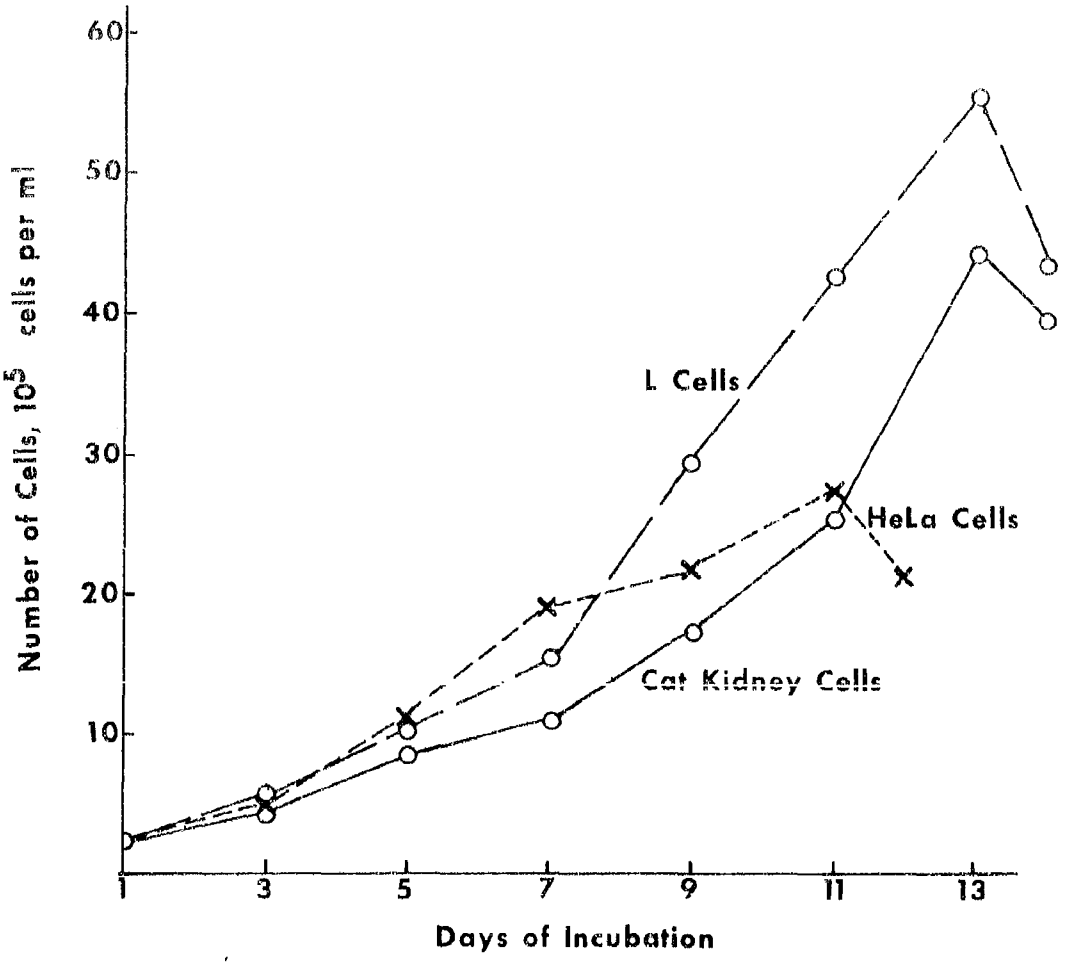

Figure 2. Growth of L Cells, Cat Kidney Cells, and HeLa Cells in the Unfortified Chemically Defined Medium.

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