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REQUIREMENTS OF CULTURED MAMMALIAN CELLS FOR VITAMIN B12 AND BIOTIN

Kiyoshi Higuchi

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REQUIREMENTS OF CULTURED MAMMALIAN CELLS FOR VITAMIN B_{12} AND BIOTIN

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BIOLOGICAL SCIENCES LABORATORIES

Project 1B562602A082

April 1969

ABSTRACT

Eagle reported in 1955 and 1957 that eight vitamins were essential for the growth of most mammalian cells in culture. No evidence for requirement of vitamin B_{12} or of biotin was presented. Sanford and co-workers in 1963 and 1964 reported evidence for requirement of both of these vitamins in certain cell strains, but no quantitative data were presented. In 1967, Ham reported that 3×10^{-10} M biotin permitted successful cloning of a Chinese hamster cell line. We have found that both HeLa and L cells required approximately 10^{-11} to 10^{-10} M vitamin B_{12} for optimal growth in a chemically defined medium. Requirement of HeLa cells for biotin was demonstrated initially with avidin, a biotin inactivator. The inhibitory activity of avidin on growth of HeLa cells was reversible by addition of biotin. Serial passage of both HeLa and L cells in presumably biotin-free medium resulted in achieving biotin-deficient cells. Both cell lines required approximately 10^{-8} M biotin for optimal growth in a chemically defined growth medium.

I. INTRODUCTION*

The minimum vitamin requirements of HeLa and L cells in tissue culture were reported by Eagle[1] in 1955 to be choline, folic acid, nicotinamide, pantothenate, pyridoxal, riboflavin, and thiamine. He pointed out that additional essential factors might be identified when better media and techniques became available. Eagle used a medium containing dialyzed serum in his studies. In 1957, he and others reported that myo-inositol was also essential for growth of most cell lines.[2] In 1963, Sanford and co-workers, using chemically defined culture media, described experiments in which an apparent variant of the mouse fibroblast, strain L, was isolated that required vitamin B_{12} for optimal rate of growth.[3] The parental L cells showed no clear-cut response to vitamin B_{12}. In a later publication, Sanford and Dupree[4] reported that nine other cell strains of various species of origin (including strain HeLa) grew in the absence of vitamin B_{12} for prolonged periods with no evidence of deficiency.

Biotin is another substance that has been studied extensively in efforts to demonstrate requirement for it by animal cells in vitro. Sanford, Dupree, and Covalesky[5] in 1963 described work in which evidence for biotin deficiency in a clonal line of strain L was observed in long term experiments. More recently, Ham[6] reported that successful cloning of a Chinese hamster cell line occurred in a chemically defined medium only if supplemented with 3×10^{-10} M biotin.

It seemed desirable, in view of the limited knowledge of the role of these two vitamins in animal cells in vitro, that further work be undertaken in this area. The work presented here shows that vitamin B_{12} and biotin are required by both HeLa and L cells.

II. MATERIALS AND METHODS

The chemically defined medium used for cultivation of HeLa and L cells is described in Table 1. The composition of the medium is, with certain exceptions, essentially similar to that of many described by others. Details on the development of the medium are not covered here. This medium permitted unlimited serial propagation of HeLa and L cells in a chemically defined nutritional environment.

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TABLE 1. COMPOSITION OF THE CHEMICALLY DEFINED GROWTH MEDIUM

L Amino Acids:	mg/Liter	Vitamins:	mg/Liter
arginine·HCl	32	D-biotin	1.0
asparagine·H_2O	150	choline·Cl	1.0
cysteine HCl·H_2O	22	D-Ca-pantothenate	2.0
glutamine	196	folic acid	1.0
histidine HCl·H_2O	63	inositol	1.0
isoleucine	33	niacinamide	1.0
leucine	26	pyridoxal·HCl	1.0
lysine·HCl	28	riboflavine	0.1
methionine	15	thiamine·HCl	1.0
phenylalanine	33	vitamin B_{12}	0.002
proline	115	Inorganic Salts:	
serine	105	NaCl	7,400
threonine	12	KCl	400
tryptophan	6.3	$NaH_2PO_4·H_2O$	200
tyrosine	46	$NaHCO_3$	720
valine	35	$CaCl_2·2H_2O$	265
Miscellaneous:		$MgCl_2·6H_2O$	275
		$Fe(NH_4)(SO_4)2$	4.8
glucose	1,800		
Na gluconate	218		
Na pyruvate	110		
methylcellulose (15 cps)	500		
phenol red	10		
insulin (0.05 U/ml)			

Tests for *Mycoplasma* contamination made at intervals showed no extraneous organism in either culture.

Plastic Falcon T-30 culture flasks containing 5 ml of culture medium were employed exclusively.

Cell growth response was assayed by measuring increased cellular protein in the cultures by the procedure of Oyama and Eagle.[6]

III. RESULTS AND DISCUSSION

A. VITAMIN B_{12}

The data in Figure 1 show the growth response of HeLa cells to vitamin B_{12} in the chemically defined medium. Data from duplicate cultures are presented. Cells used as inoculum were grown through six cell-doublings in medium free of vitamin B_{12} prior to use in the experiment. Approximately 22,000 cells/ml were inoculated. The cultures were fed three times during the 8-day incubation period before cells were harvested for cell protein assay. In this test less than 10 picograms/ml of vitamin B_{12} (about 10^{-11} M) were adequate to yield peak growth. The results indicate that vitamin B_{12} is essential for growth of HeLa cells.

It must be remembered that because of possible trace contamination of reagents with vitamin B_{12} and, probably more significantly, because of the substantial number of cells required as inoculum for initiation of cell multiplication regardless of vitamin B_{12} concentration, the so-called zero level of the vitamin in these experiments is only an approximation.

In similar experiments with L cells, growth responses were less clear-cut. Data from one experiment are shown in Figure 2. The cells used as inoculum were grown through at least 20 cell-doublings in medium free of vitamin B_{12} prior to use in the experiment. The cultures were fed twice during the 8-day incubation period. Approximately 30,000 cells/ml were used as inoculum. The concentration range for producing growth response in L cells was approximately the same as for HeLa cells; however, the data indicate that the L cell may need higher concentrations of vitamin B_{12} than the HeLa cell.

The ability of deoxynucleosides to substitute for vitamin B_{12} in the nutrition of certain microorganisms is well known. In the case of HeLa cells, thymidine in a wide range of concentrations failed to show any vitamin B_{12} - sparing effect. Data are shown in Table 12. It appears, therefore, that the response of HeLa cells to vitamin B_{12} is very much like that of the flagellate Ochromonas malhamensis, an organism commonly used in the bioassay of vitamin B_{12}. On the other hand, Sanford and Dupree also had reported that their cell strain 3654 required vitamin B_{12} even in the presence of certain nucleic acid derivatives.[4]

B. BIOTIN

The data in Table 3 show that a requirement for biotin was demonstrable when avidin was added to the growth medium. The activity of this specific inactivator of biotin was apparently reversible by biotin. The HeLa cells used in this experiment were grown twice in "biotin-free" medium but showed no evidence of biotin deficiency unless treated with avidin.

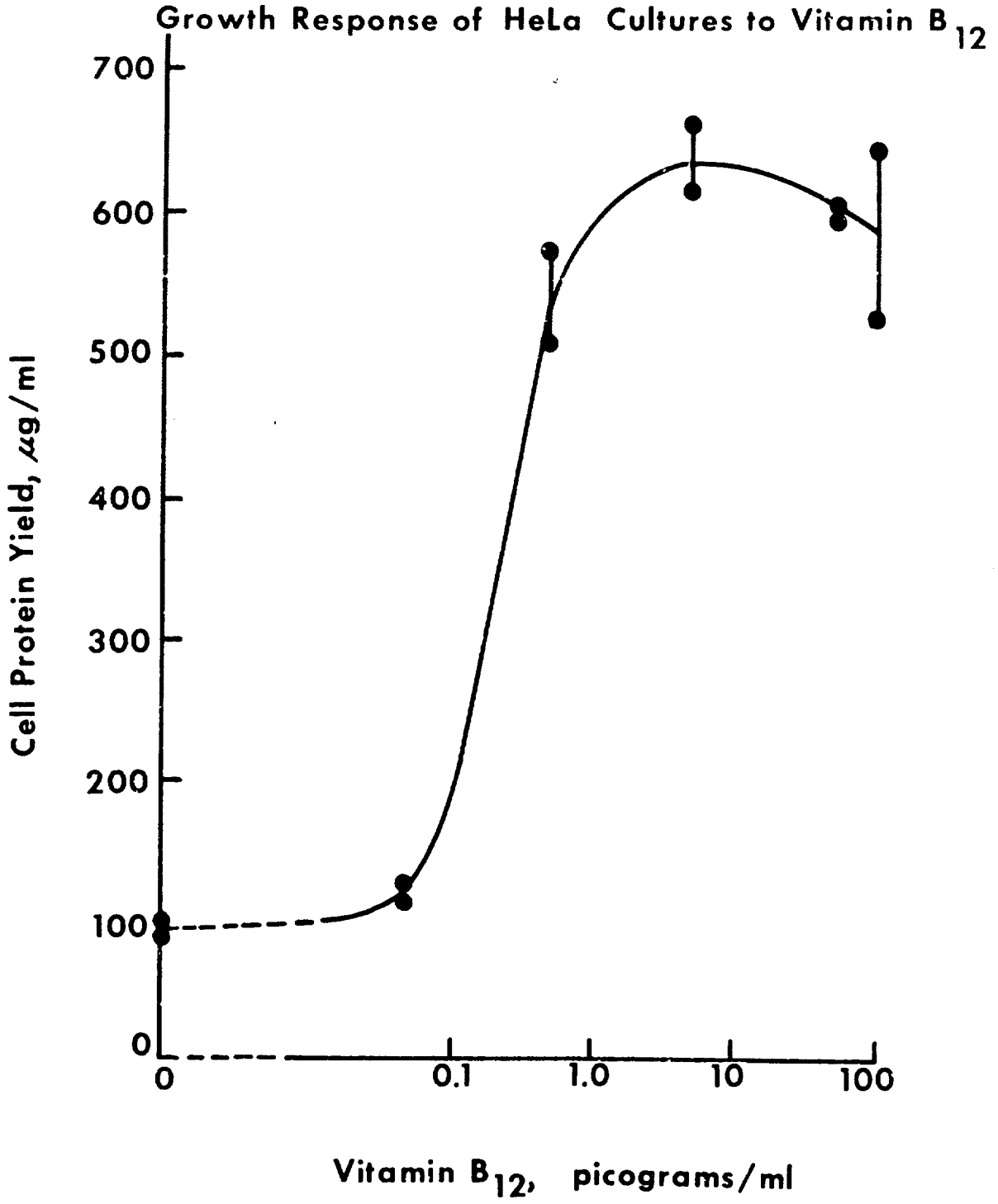

FIGURE 1. Growth Response of HeLa Cultures to Vitamin B_{12}. The graph was drawn as a smooth curve through points representing averages of duplicate values. The breaks in the curve in the region approaching zero concentration of the vitamin are required in order to avoid misrepresenting the scale and yet to present the zero point.

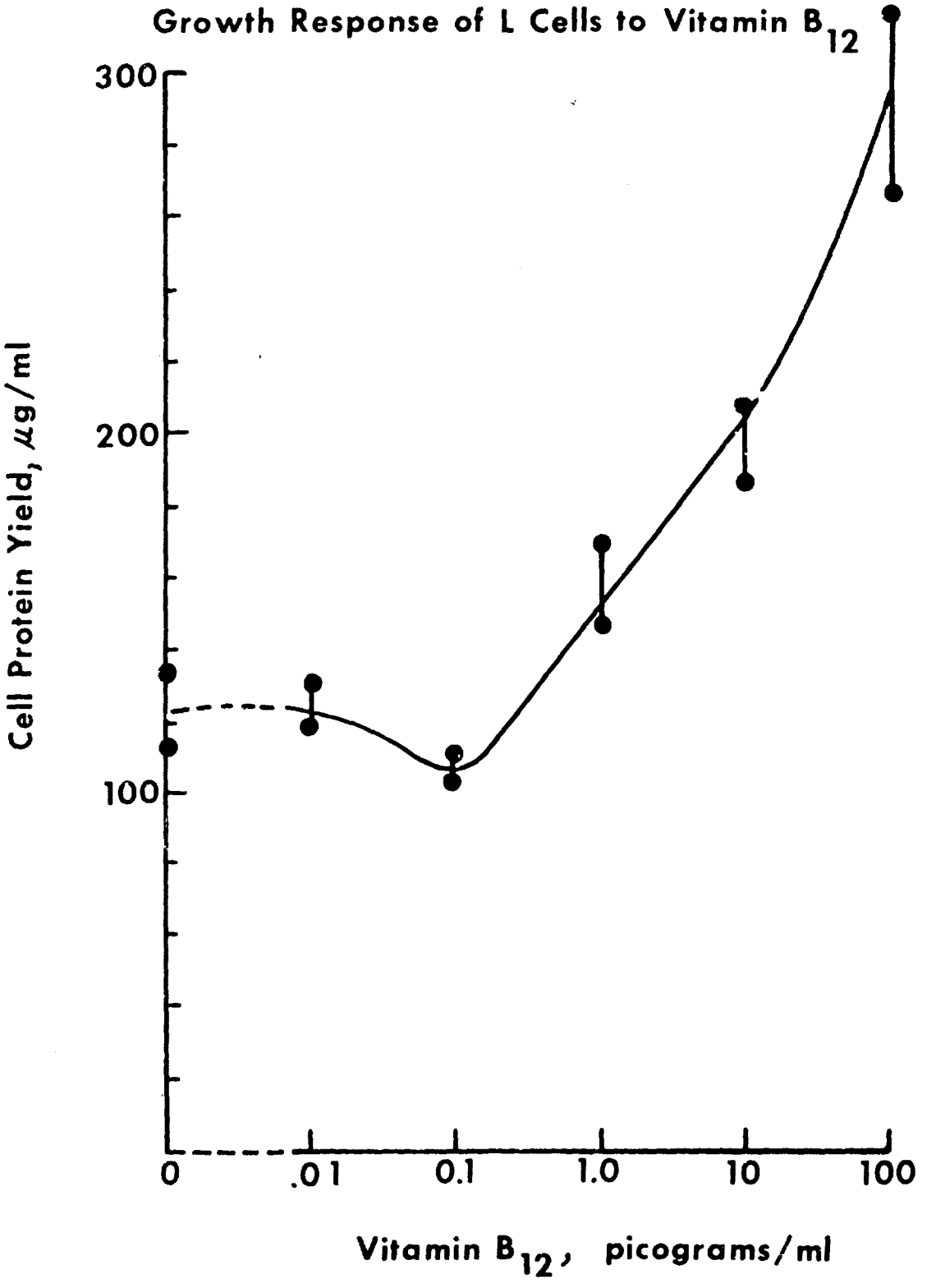

FIGURE 2. Growth Response of L Cells to Vitamin B_{12}. The graph was drawn as a smooth curve through points representing averages of duplicate values. The breaks in the curve in the region approaching zero concentration of the vitamin are required in order to avoid misrepresenting the scale and yet to present the zero point.

TABLE 2. FAILURE OF THYMIDINE TO SUBSTITUTE FOR VITAMIN B_{12} IN HeLa CELLS

Growth Conditions	Cell Protein Yield, μg/ml[a]
Control (no B_{12}, no thymidine)	86, 76
Control with 50 picograms/ml B_{12}	383, 375
Control with 10^{-6} M thymidine	88, 74
Control with 10^{-5} M thymidine	54, 50
Control with 10^{-4} M thymidine	32, 28

a. Values from duplicate cultures are presented.

TABLE 3. INHIBITORY EFFECT OF AVIDIN ON GROWTH OF HeLa CELLS AND ITS REVERSAL BY BIOTIN

Avidin, μg/ml	Biotin, μg/ml	Cell Protein Yield, μg/ml[a]
0	0	250, 245
4.4	0	17, 12
22	0	16, 15
0	0.02	205, 200
22	0.02	210, 225

a. Values from duplicate cultures are presented.

Because unequivocal evidence was obtained that biotin was needed for growth, it may be assumed that previous failures were due either to incomplete depletion of the vitamin in the inoculum or to trace contamination of the basal medium with biotin. Subsequently, after seven repeated passages of HeLa in presumably biotin-free medium, the data shown in Figure 3 were obtained. It appears that approximately 10^{-8} M biotin (2.44 nanograms/ml) is required for optimal growth of HeLa cells in the defined medium. Similarly, L cells were serially passed in the biotin-deficient medium four times and tested for response to biotin (Fig. 4). The results obtained with these two cell lines indicate that a requirement for biotin may be a characteristic of many or all cultured mammalian cells.

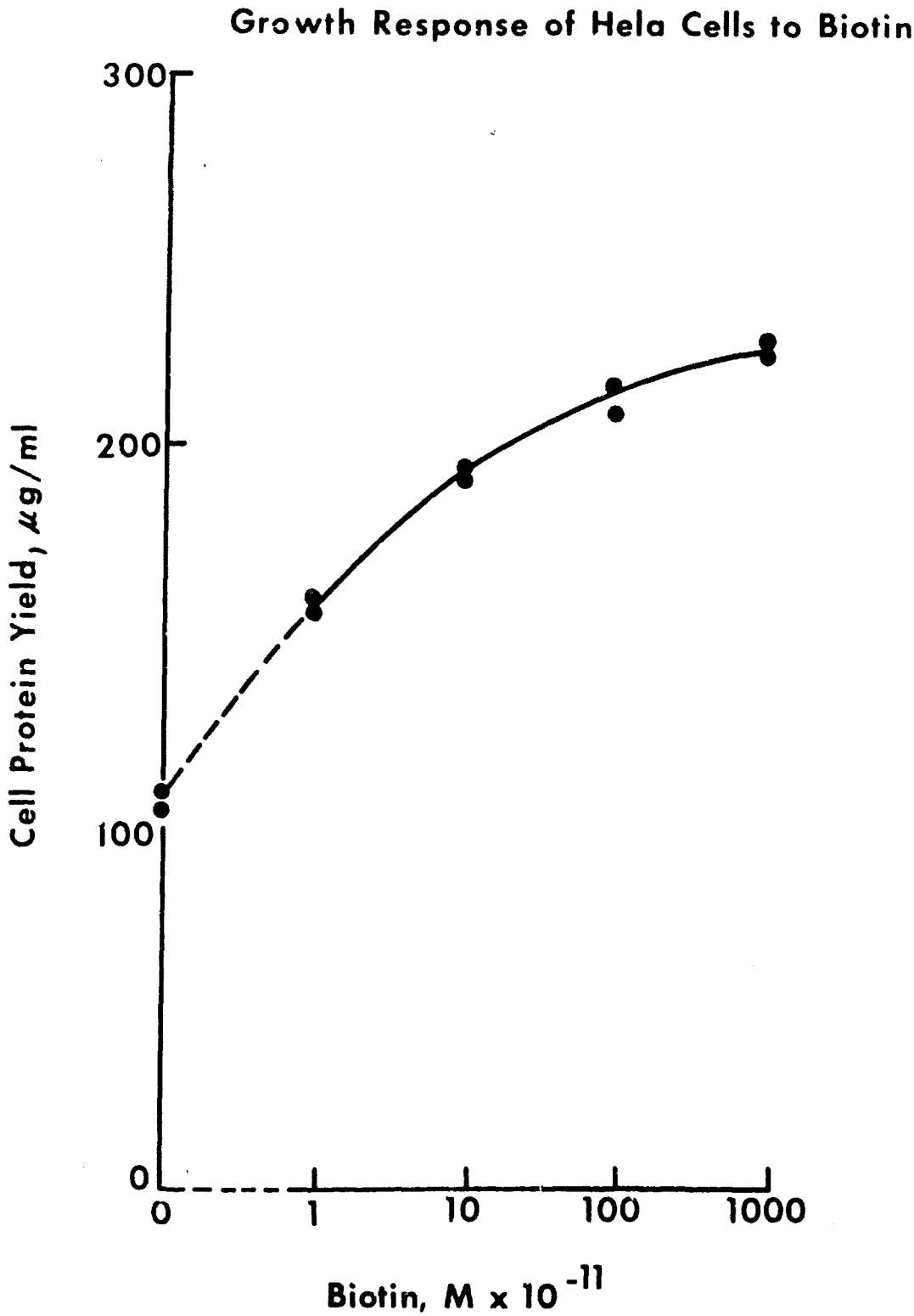

FIGURE 3. Growth Response of HeLa Cells to Biotin. The graph was drawn as a smooth curve through points representing averages of duplicate values. The breaks in the curve in the region approaching zero concentration of the vitamin are required in order to avoid misrepresenting the scale and yet to present the zero point.

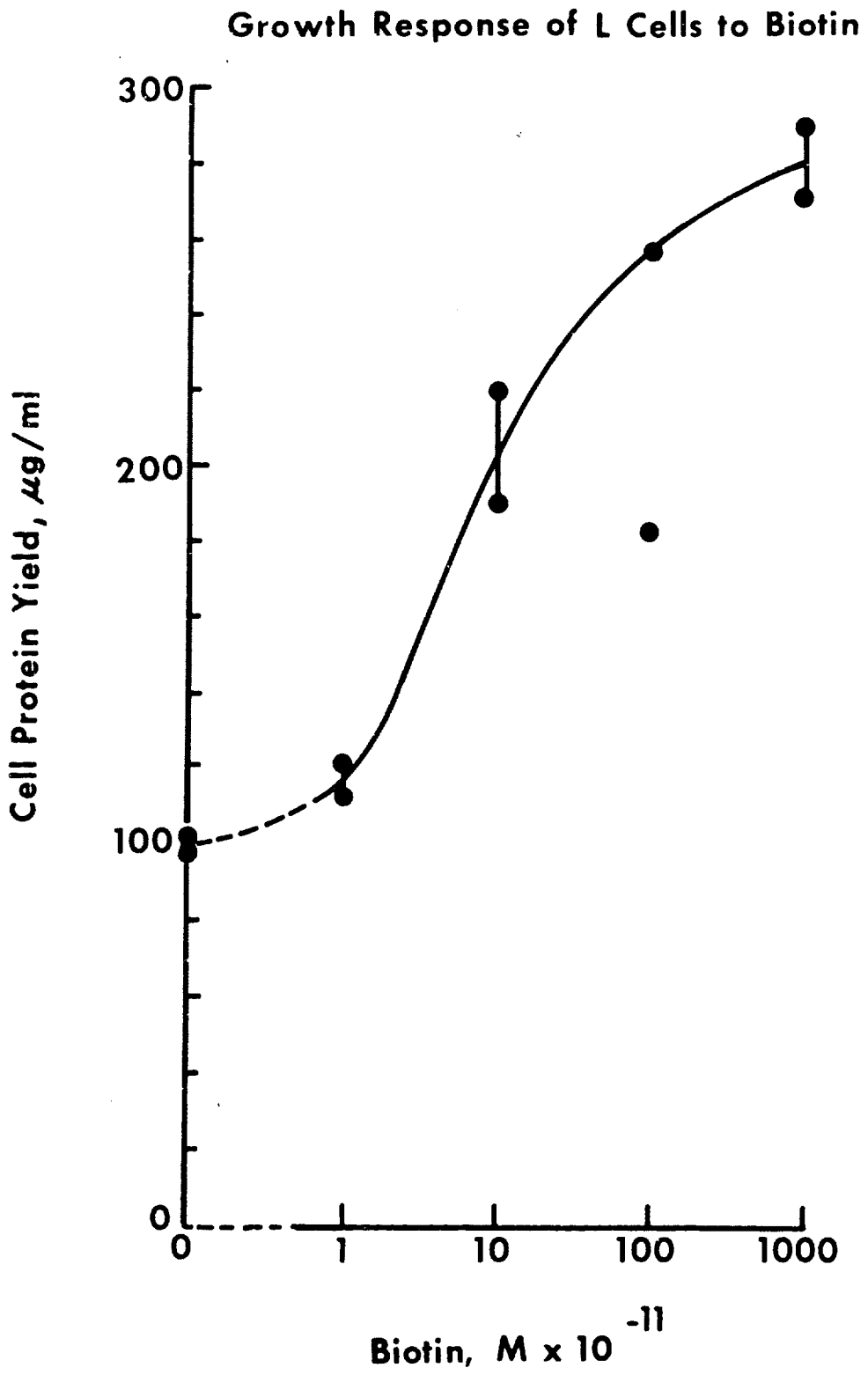

FIGURE 4. Growth Response of L Cells to Biotin. The graph was drawn as a smooth curve through points representing averages of duplicate values. The breaks in the curve in the region approaching zero concentration of the vitamin are required in order to avoid misrepresenting the scale and yet to present the zero point.

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Unclassified
Security Classification

DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author) Department of the Army Fort Detrick, Frederick, Maryland, 21701	2a. REPORT SECURITY CLASSIFICATION Unclassified 2b. GROUP

3. REPORT TITLE

REQUIREMENTS OF CULTURED MAMMALIAN CELLS FOR VITAMIN B_{12} AND BIOTIN

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)

5. AUTHOR(S) (First name, middle initial, last name)

Kiyoshi (NMI) Higuchi

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
April 1969	15	6

8a. CONTRACT OR GRANT NO.	9a. ORIGINATOR'S REPORT NUMBER(S)
b. PROJECT NO. 1B562602A082	Technical Manuscript 505
c.	9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)
d.	

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11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
	Department of the Army Fort Detrick, Frederick, Maryland, 21701

13. ABSTRACT

Eagle reported in 1955 and 1957 that eight vitamins were essential for the growth of most mammalian cells in culture. No evidence for requirement of vitamin B_{12} or of biotin was presented. Sanford and co-workers in 1963 and 1964 reported evidence for requirement of both of these vitamins in certain cell strains, but no quantitative data were presented. In 1967, Ham reported that 3×10^{-10} M biotin permitted successful cloning of a Chinese hamster cell line. We have found that both HeLa and L cells required approximately 10^{-11} to 10^{-10} M vitamin B_{12} for optimal growth in a chemically defined medium. Requirement of HeLa cells for biotin was demonstrated initially with avidin, a biotin inactivator. The inhibitory activity of avidin on growth of HeLa cells was reversible by addition of biotin. Serial passage of both HeLa and L cells in presumably biotin-free medium resulted in achieving biotin-deficient cells. Both cell lines required approximately 10^{-8} M biotin for optimal growth in a chemically defined growth medium.

14. Key Words

*Cells (biology)
*Biotin
*Vitamins
HeLa cells
L cells
Growth

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