THE MINIMUM VITAMIN REQUIREMENTS OF THE L AND HeLa CELLS IN TISSUE CULTURE, THE PRODUCTION OF SPECIFIC VITAMIN DEFICIENCIES, AND THEIR CURE

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The specific amino acid requirements of a mouse fibroblast (strain L) and a human carcinoma cell (strain HeLa) have been described in previous communications (1, 2). Both cells lines were found to require the same 13 amino acids, and in approximately the same concentration. The present paper deals with their minimum vitamin requirements. Seven vitamins (choline, folic acid, nicotinamide, pantothenate, pyridoxal, riboflavin, and thiamin) have so far been identified as essential for both cell lines.

EXPERIMENTAL

Methods.—The methods used in maintaining stock cultures, in setting up the experiments in replicate small flasks containing varying concentrations of the metabolite under study, and assessing the results in terms of the cell population have been described in the preceding papers of this series (1, 2). In those studies, when a single amino acid was omitted from the medium, growth and multiplication usually stopped within 24 to 48 hours; and after 5 to 7 days' incubation, the difference between the complete medium, in which there had been 5- to 10-fold multiplication of the cells, and the deficient medium, in which the cell count had decreased rather than increased, was clearly evident. With most of the vitamins, however, presumably because of the cellular store of either the vitamin itself, precursors, or of the conjugate into which it is incorporated, multiplication sometimes continued for as long as 1 to 2 weeks after the vitamin had been removed from the medium. It was therefore necessary to grow the cells for varying periods in the appropriately deficient medium before the vitamin requirement could be demonstrated. At intervals, aliquot suspensions were inoculated into a series of small flasks containing graded concentrations of the vitamin under study. If the cells in all the flasks grew out equally, it was apparent that the deficiency had not yet been established, and propagation was continued in the vitamin-deficient medium. Eventually, after the cells had been depleted of their vitamin reserves, when they were inoculated into media containing graded concentrations of the specific vitamin, typical growth-response curves were obtained, ranging from degeneration and death in the absence of the vitamin, to normal growth and multiplication at an optimally effective level. A technical difficulty was posed by the fact that, if the cells were allowed to remain in the vitamin-deficient medium for too long a period, the cytopathogenic effects of the vitamin deficiency were no longer reversible, and no growth was obtained on the addition of the vitamin in any concentration. This was particularly evident with the HeLa cell.

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Materials.—The basal medium used for the identification of the specific vitamin requirements is shown in Table I. The concentrations of the individual amino acids were approximately twice the concentrations required to give maximal growth. The vitamins incorporated in the medium were an arbitrary mixture which did not, however, include the fat-soluble vitamins, the absence of which has been shown in a number of previous experiments to have no demonstrable effect over a 6 week period of cultivation under the conditions of the present experiments.

TABLE I

Basal Medium Used for the Determination of Vitamin Requirements

L amino acids	*mM*
Arginine,	0.1
Cystine,	0.05 (0.02)*
Glutamine,	2.0 (1.0)*
Histidine,	0.05 (0.02)*
Isoleucine,	0.2
Leucine,	0.2 (0.1)*
Lysine,	0.2 (0.1)*
Methionine,	0.05
Phenylalanine,	0.1 (0.05)*
Threonine,	0.2 (0.1)*
Tryptophan,	0.02 (0.01)*
Tyrosine,	0.1
Valine,	0.2 (0.1)*

Vitamins	*gm./ml.*
Biotin,	10^{-6}
Choline,	10^{-6}
Folic acid,	10^{-6}
Nicotinamide,	10^{-6}
Panthothenic acid,	10^{-6}
Pyridoxal,	10^{-6}
Thiamin,	10^{-6}
Riboflavin,	10^{-7}

Salts	*per cent*
NaCl	0.68
KCl	0.04
$NaH_2PO_4 \cdot H_2O$	0.014
$NaHCO_3$	0.22
$CaCl_2$	0.02
$MgCl_2$	0.008

Miscellaneous		
Glucose,	0.1	per cent
Penicillin,	0.005	per cent
Strepto-mycin,	0.005	per cent
Phenol red,	0.0005	per cent
For studies of cell nutrition		
Serum protein (Dialyzed horse serum—1 per cent*) (Dialyzed human serum—5 per cent)		
For stock cultures		
Serum protein (Whole horse serum—5 per cent*) (Whole human serum—10 per cent)		

* For mouse fibroblast.

RESULTS

The progressive growth of the L fibroblast and of the HeLa cell in the medium of Table I has been described in preceding communications (1, 2). If a single essential vitamin, and its congeners, were omitted from that medium, the cells continued to grow for a period which varied in individual experiments and with the particular vitamin, from approximately 5 to 15 days. During this initial period of exhaustion, the cells usually multiplied from 2.6- to 6-fold (*cf.* Tables II and III). When these vitamin-deficient cells were now resuspended, and transferred to a series of small flasks containing graded concentrations of the missing vitamin, typical growth-response curves were

obtained. In the absence of added vitamin, the cells progressively deteriorated and died, as illustrated for the fibroblast and a pantothenic acid deficiency in Figs. 1 to 3. With added vitamin, the cells grew out at a rate which varied with its concentration. This is illustrated for the mouse fibroblast and thiamin in Figs. 4 to 9, and for the HeLa cell and pyridoxal in Figs. 10 to 13. The

TABLE II

The Growth Response of a Mouse Fibroblast to Seven Essential Vitamins

Vitamin	Period of preliminary incubation in vitamin-free media*	Degree of multiplication‡ in vitamin-free media§	Inoculum of vitamin-depleted cells (× 10⁴)‖	Time of incubation	Concentration of specific vitamin§, *gm./ml.*							Concentration permitting maximal growth
					10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	0	
					Degree of multiplication‡							
	days			*days*								*gm./ml.*
Choline	7	3.7	28	14		2.7	1.9	0.75			0.75	10^{-8}
	8	0	19	8	4.4	7.1	2.0	0.75	0.9		0.9	10^{-6}
Folic acid	14	3.9	16	6		10.6	9.6	4.6	2.0	2.1	0.6	10^{-7}¶
	14	1.4	10	15	10.0	11.0	13.0	13.0	8.0		0.77	10^{-8}
Nicotinamide	8	4	22	6		6.8	6.9	6.5	1.5		1.0	10^{-8}
	10	2.1	24	6		7.1	5.5	4.0	2.0	1.3	0.5	10^{-6}
	13	7.6	29	12			7.3	5.5	0.93		0.24	10^{-7}
Pantothenic acid	5	6.0	30	7	3.5	3.7		4.4	2.1	1.1	<0.1	10^{-8}
	7	2.0	18	15	8.3	9.5	10.9	11.0	0.33		0	10^{-8}
	8	3.3	24	12			4.2	3.5	0.18	0.13	0.13	10^{-8}
Pyridoxal	8	2.5	13	6		6.7	10.4	8.2	4.3		0.8	10^{-8}
	8	3.1	12.5	9		12.0	10.4	10.2	8.25		0.97	10^{-8}
Riboflavin	4	3.1	29	6		7.5	6.2				0.6	10^{-7}
	7	0.8	14	7			1.2(?)	7.6	9.0	2.8	1.1	10^{-9}
	6	2.7	36	5	<0.1	2.8	3.0	2.3	1.2		0.8	10^{-8}
Thiamin	12	4.1	18	8	7.2	9.3	8.6	8.5	5.8	2.0	0.6	10^{-8}¶
	4	4.4	31	8		5.7	5.8				1.1	?

* To exhaust cellular reserves of vitamin, its precursors and conjugates.
‡ Referred to inoculum of column 3 as 1.
§ In a medium containing the other 6 vitamins at the concentrations shown in Table I.
‖ Viable cells, adhering to culture flask in 24 hours, and not removed when culture fluid was changed.
¶ Molar, rather than gravimetric.

slow rate of growth observed in some of the experiments of Tables II and III reflects the fact that it sometimes required several days for the vitamin-depleted cells to recover from the cytopathogenic effects of that depletion, and to resume multiplication. Conversely, the fact that in some of the experiments there was slight multiplication even in the absence of added vitamin (*cf.* last column of Tables II and III) reflects the fact that the cells in those experiments had not been wholly depleted of their vitamin reserves at the time of inoculation.

In this manner, 7 vitamins (choline, folic acid, nicotinamide, pantothenic acid, pyridoxal, riboflavin, and thiamin) were shown to be essential for the growth and multiplication of both the L fibroblast and the HeLa cell. The early cytotoxic effects produced with each of these 7 specific vitamin deficiencies are illustrated for the fibroblast in Figs. 14 to 20; and 4 specific deficiencies in the HeLa cell are similarly illustrated in Figs. 21 to 24. If such vitamin-deficient cells were tested at the appropriate time, just before or just after the cytotoxic effects of the deficiency became evident, it was possible to obtain typical growth-response curves, and to delimit the minimum concentration necessary for survival and the concentration at which maximal

TABLE III

Illustrating the Growth Response of the HeLa Cell to Some of the Essential Vitamins

Vitamin	Period of preliminary incubation in vitamin-free media*	Degree of multiplication‡ in vitamin-free media§	Inoculum of vitamin-depleted cells (× 10^4)‖	Time of incubation	Concentration of specific vitamin,§ *gm./ml.*					Concentration permitting maximal growth
					10^{-6}	10^{-7}	10^{-8}	10^{-9}	0	
					Degree of multiplication‡					
	days			*days*						*gm./ml.*
Choline	6	7.2	16	7	9.3	12.4	13.3	2.8	1.3	10^{-8}
Folic acid	6	7.5	14	7	3.9	9.0	14.1	0.8	0.4	10^{-8}
Panthothenic acid	6	?	25	7	5.3	6.6	4.6	2.7	1.1	10^{-7} ±
Pyridoxal	12	?	39	7	9.5	6.8	5.8	1.2	0.3	10^{-7} ±
Riboflavin	6	?	25	7	4.8	8.0	6.6	3.3	0.4	10^{-7} to 10^{-8}
Thiamin	5	6.5	19	7	9.1	6.6	6.3	4.3	2.4	10^{-8} ±

* To exhaust cellular reserves of vitamin, its precursors and conjugates.

‡ Referred to inoculum of column 3 as 1.

§ In a medium containing the other 6 vitamins at the concentrations shown in Table I.

‖ Viable cells, adhering to culture flask in 24 hrs., and not removed when culture fluid was changed.

growth was obtained (*cf.* Figs. 4 to 13). Those effective concentrations are shown for each of the 7 vitamins in Tables II and III.

Although not shown in the figures and tables, nicotinic acid had qualitatively the same effect as nicotinamide; and pyridoxine, pyridoxal, pyridoxamine, and pyridoxal phosphate were similarly interchangeable, although not quantitatively equivalent.

The Revival of Vitamin-Deficient Cells.—After the cells had been exhausted of their reserves of a single vitamin, its precursors and conjugates, and when the cytotoxic effects of the resulting deficiency were well established, the addition of the missing vitamin usually brought about a rapid recovery. The cells regained their normal microscopic appearance, and multiplication was re-

sumed at a normal rate (*cf.* Figs. 25 to 33 and 34 to 36). In some cases this recovery was strikingly rapid. Cells which had been deprived of a vitamin for 4 to 8 days and were in consequence shrunken, granular, and presumably "dead" sometimes resumed their normal appearance within 24 to 48 hours. However, if the cells were kept in the deficient medium for too long a period after the cells had stopped multiplying, the recovery process was much slower; and with yet longer periods of deprivation, the cytopathogenic effects of vitamin deficiency proved irreversible. The mouse fibroblast remained viable for much longer periods after the effects of the vitamin deficiency had become evident than did the HeLa cell. The experiments of Table III illustrate successful revivals of vitamin-deficient HeLa cells. In many other experiments, however, the cells were either not sufficiently depleted of their vitamin reserves (in which case there was active multiplication in the control tube, containing no vitamin), or the cytopathogenic effects were already irreversible at the time of the experiment, with no growth in any of the tubes.

DISCUSSION

It is apparent that the 7 vitamins here shown to be essential for the growth and multiplication of the L fibroblast and the HeLa cell (choline, folic acid, nicotinamide, pantothenic acid, pyridoxal, riboflavin, and thiamin) are the minimum and not necessarily the total requirement of these cells. There may well be, and probably are, a number of other substances the essentiality of which would become evident only after more prolonged cultivation in an appropriately deficient medium. It is possible also that there are additional essential vitamins which are present as trace contaminants either in the other vitamins, in the amino acids, or bound to the dialyzed serum protein, and which in consequence appear to be non-essential under the conditions of the present experiments.

With these necessary reservations, it is to be noted that to date it has not been possible to demonstrate a nutritional requirement by these cells for biotin, tocopherol, vitamins A, D, or K, inositol, or lipoic acid.

There were striking differences in the microscopic changes produced by the various vitamin deficiencies. The details of those specific cytopathogenic effects, and the nature of the recovery process, are under present study. Further, the technic here used lends itself to a rapid and simple quantitative evaluation of vitamin antagonists. It may prove possible also to explore directly the metabolic reactions affected by specific vitamin deficiencies.

The degree to which various vitamin precursors and conjugates (diphospho- and triphosphopyridine nucelotides, flavin mononucleotide and flavin adenine dinucleotide, coenzyme A, and cocarboxylase) can substitute for the corresponding vitamins is under present study.

SUMMARY

Seven vitamins have to date proved essential for the survival and multiplication of a mouse fibroblast (strain L) and a human carcinoma cell (strain HeLa) in tissue culture: choline, folic acid, nicotinamide, pantothenic acid, pyridoxal, riboflavin, and thiamin. It was necessary to cultivate the cells for 5 to 15 days in a medium lacking the specific vitamin before the deficiency became apparent in the cessation of multiplication and the development of specific cytopathogenic effects. In their early stages these changes could be reversed by the addition of the missing vitamin, an *in vitro* "cure" of a vitamin deficiency. The maximally effective concentrations were in the range 10^{-7} to 10^{-8} gm. per ml.

The probability that additional vitamins not demonstrably essential under the conditions of the present experiments are nevertheless required for survival and growth is discussed in the text.

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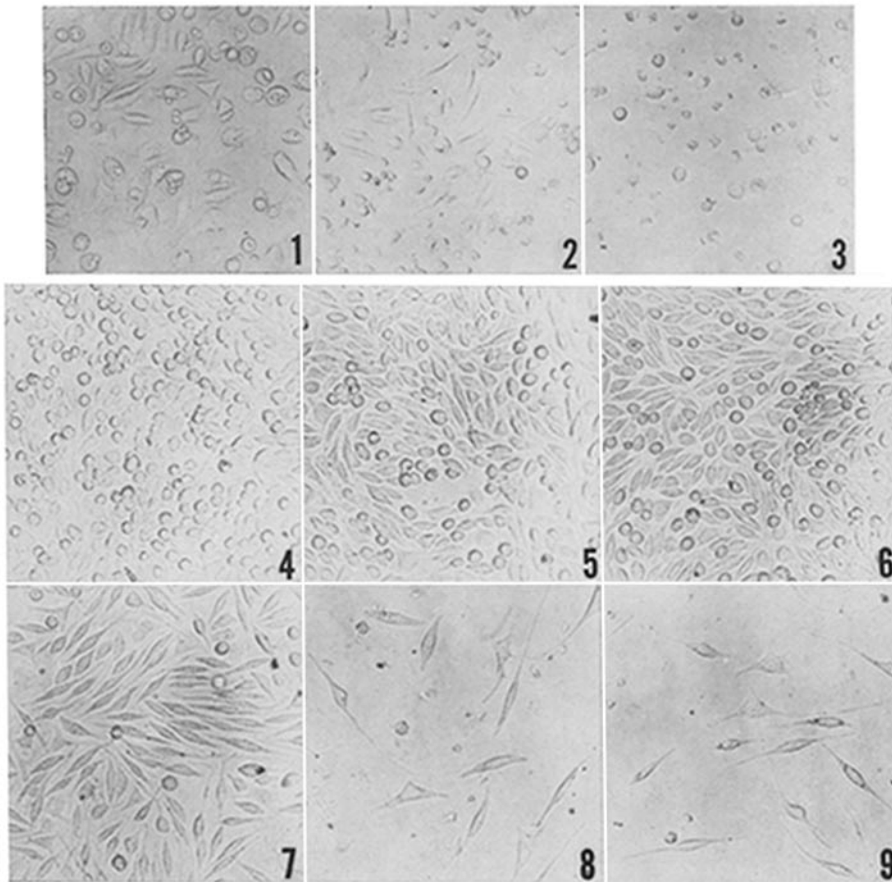
2. Eagle, H., *J. Exp. Med.*, 1955, **102**, 37.

EXPLANATION OF PLATES

Plate 68

Figs. 1 to 3. The progressive degeneration of a mouse fibroblast in the absence of pantothenate. Fig. 1, 11 days. Fig. 2, 15 days. Fig. 3, 18 days. × 112.

Figs. 4 to 9. The growth response of the L fibroblast to graded concentrations of thiamin. Fig. 4, 10^{-5} gm./ml. Fig. 5, 10^{-7}. Fig. 6, 10^{-8}. Fig. 7, 10^{-9}. Fig. 8, 10^{-10}. Fig. 9, 0. × 112.

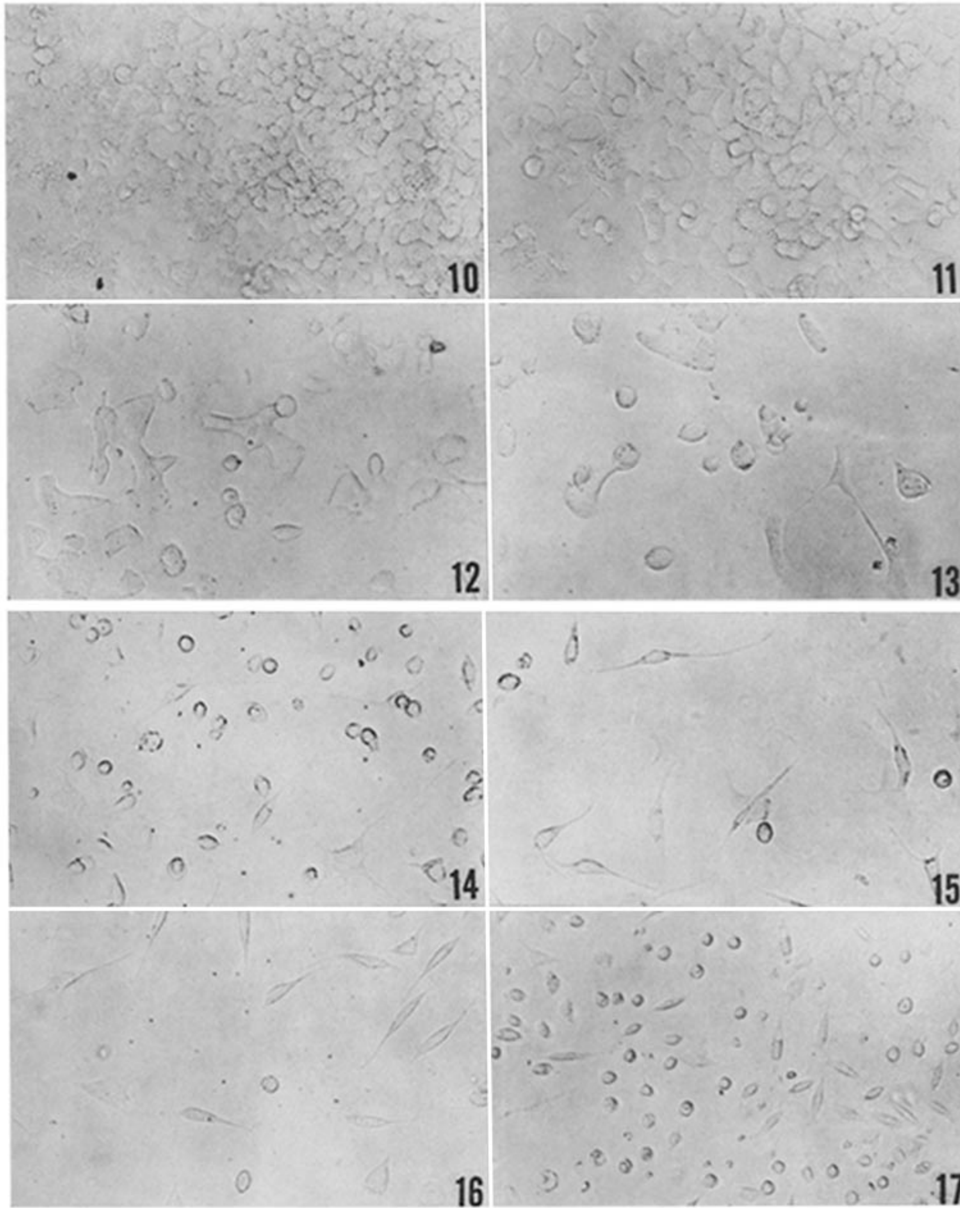


(Eagle: Vitamin requirements of mammalian cells)

Plate 69

Figs. 10 to 13. The growth response of the HeLa cell to pyridoxal. Fig. 10, 10^{-6} gm./ml. Fig. 11, 10^{-8}. Fig. 12, 10^{-9}. Fig. 13, 0. $\times$ 112.

Figs. 14 to 17. Specific vitamin deficiencies produced in a mouse fibroblast in tissue culture. Fig. 14, choline—8 days. Fig. 15, folic acid—8 days. Fig. 16, nicotinamide—12 days. Fig. 17, pantothenate—12 days. $\times$ 112.



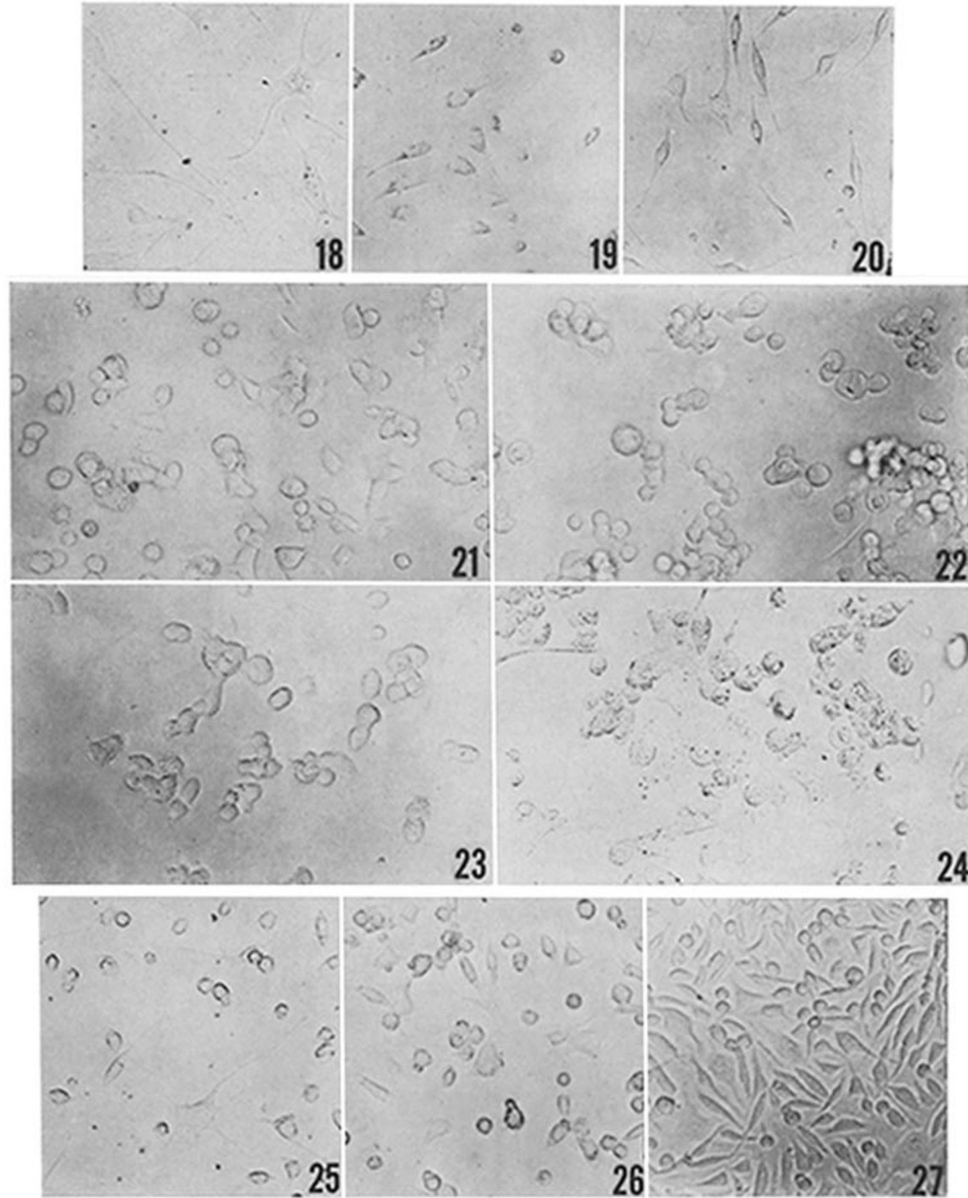
(Eagle: Vitamin requirements of mammalian cells)

PLATE 70

FIGS. 18 to 20. Specific vitamin deficiencies produced in a mouse fibroblast in tissue culture. Fig. 18, pyridoxal—14 days. Fig. 19, riboflavin—15 days. Fig. 20, thiamin—12 days. × 112.

FIGS. 21 to 24. Specific vitamin deficiencies produced in a human carcinoma cell in tissue culture. Fig. 21, nicotinamide—15 days. Fig. 22, pantothenate—11 days. Fig. 23, pyridoxal—15 days. Fig. 24, riboflavin—8 days. × 112.

FIGS. 25 to 27. The "cure" of specific vitamin deficiencies in mouse fibroblasts in tissue culture. Recovery from a 4 day choline deficiency. × 112.

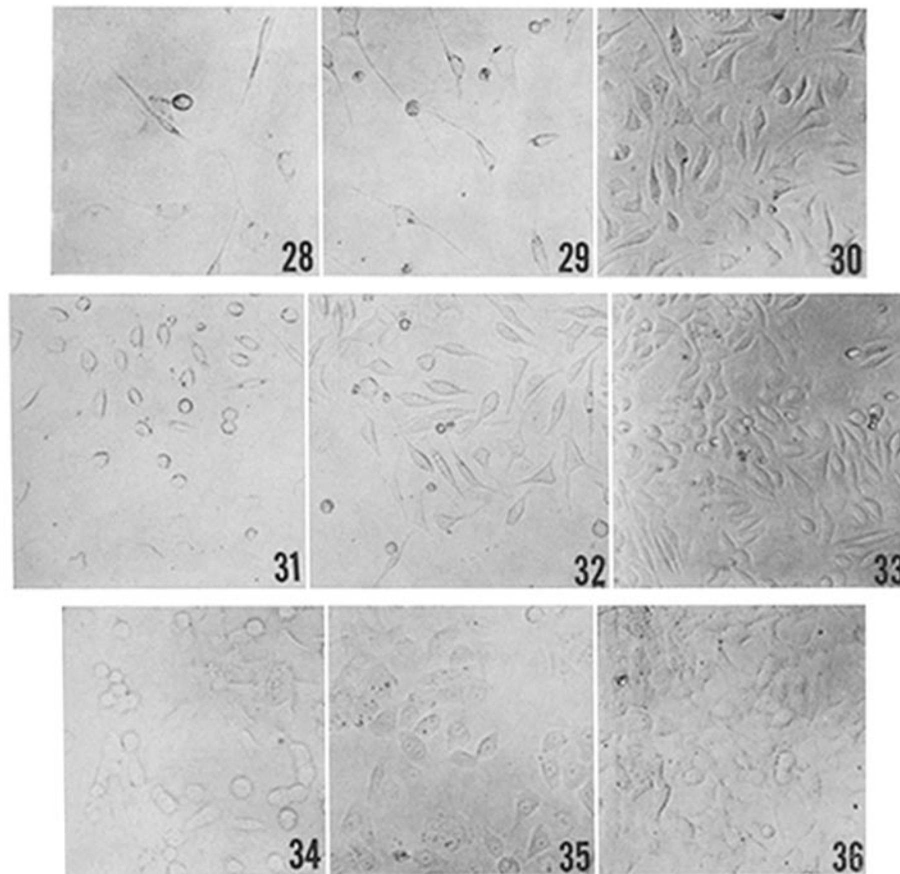


(Eagle: Vitamin requirements of mammalian cells)

Plate 71

Figs. 28 to 33. The "cure" of specific vitamin deficiencies in mouse fibroblasts in tissue culture. Figs. 28 to 30, recovery from a 7 day folic acid deficiency. Figs. 31 to 33, recovery from a 7 day riboflavin deficiency. × 112.

Figs. 34 to 36. The recovery of HeLa cells from an 11 day nicotinamide deficiency. Fig. 34, 11 day deficiency. Fig. 35, 1 day recovery. Fig. 36, 4 day recovery. × 112.



(Eagle: Vitamin requirements of mammalian cells)