

## THE AMINO ACID REQUIREMENTS OF MONKEY KIDNEY CELLS IN FIRST CULTURE PASSAGE

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PLATE 57

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Although 8 amino acids are known to suffice for nitrogen balance in feeding experiments in man (1), a number of cultured human and cell lines have been found to require 13 amino acids for survival and growth (2-5). These serially propagated culture strains all required arginine, cystine, glutamine, histidine, and tyrosine, over and above the 8 "essential" amino acids (isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine); and in this respect no differences were noted between cell lines deriving from normal and malignant human tissues (5). Individual cell lines have shown additional requirements for asparagine (6) and serine (7).

One possible explanation for the anomalous and uniform requirement for at least 13 amino acids was that the cell strains had altered in the course of their prolonged cultivation, to the degree that normal biosynthetic mechanisms leading to the formation of these 5 amino acids were no longer operative. The present experiments were undertaken to explore this possibility, by determining the amino acid requirements of monkey kidney cells in their first culture passage, prior to extensive proliferation.

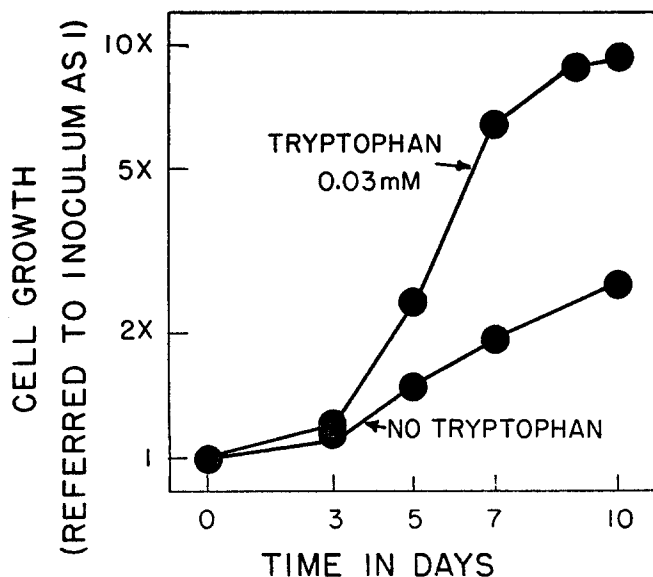
### *Methods and Materials*

Trypsinized suspensions of monkey kidney cells were obtained from three different laboratories. These were prepared as described by Youngner (8) or Melnick (9); and we are indebted to Dr. James Crawford and Dr. Karl Habel of the National Institutes of Health, and to Dr. A. J. Gerardi and Dr. Alan Gray of Microbiological Associates, Inc., for their cooperation in making these suspensions available for study. Modified Earle-Highhouse T-15 flasks (3) were inoculated with 300,000 to 1,500,000 cells in a volume of 2.5 to 3 cc. Twenty-four to 48 hours later, after some of the cells had adhered to the glass, the supernatant fluid and cell debris were withdrawn, the adherent cell layer washed, and the experimental fluids added. The basal medium, modified in detail as described in the text, was that previously described (10), and included the 13 amino acids shown to be essential for a wide variety of cells, the 8 essential vitamins, glucose, and salts. This minimal medium was supplemented in these experiments with 2 to 3 per cent dialyzed horse serum. At the time of the first feeding the protein content of the cells which had adhered to the glass was determined in 6 control flasks by a

modified Folin-Lowry method (11). The cultures were then refed every other day, and the experiments were terminated on the 7th to 12th day, at which time the amount of cell protein was again determined. Toward the end of the experiment, after the cell population had become fairly large, the cell cultures were sometimes fed daily rather than every other day.

#### RESULTS

The 13 amino acids previously found necessary for the growth of a variety of serially propagated human and animal cell lines proved equally essential for monkey kidney cells in their first culture passage. In particular, arginine,



TEXT-FIG. 1. The growth response of monkey kidney cultures to tryptophan. The initial growth sometimes seen in a medium lacking an essential amino acid is illustrated.

histidine, cystine, tyrosine, and glutamine were as necessary for survival and growth as were the 8 amino acids essential for nitrogen balance *in vivo*. On the omission of any one amino acid, there was an initial and variable period of slight multiplication, after which the cells slowly deteriorated, and eventually died. The degenerative changes produced by the omission of either arginine, cystine, glutamine, histidine, or tyrosine are illustrated in Figs. 1 to 6.

The slight initial multiplication sometimes seen in a medium supposedly lacking an essential amino acid is illustrated for tryptophan in Text-fig. 1. The cells which had survived the first culture passage regularly died on attempted subculture in the same deficient medium. It is not clear from the present experiments whether this initial growth in a deficient medium depends on (a) a large intracellular pool of amino acids (*cf.* reference 12), (b) the presence

in the medium of tissue debris necessarily included in a trypsinized tissue, or (c) a limited capacity of the cells to synthesize some of these amino acids from other components of the medium.

The quantitative aspects of the amino acid requirements are shown in Table I; and Text-fig. 2 shows the growth response to the 5 amino acids not essential for nitrogen balance in man, but essential for the survival and growth of cells in culture. Approximately the same concentration of the amino acids was neces-

TABLE I  
*The Amino Acids Essential for the Survival and Growth of Monkey Kidney Cells in First Tissue Culture Passage*

Amino acid tested	Concentration of specific amino acid, mM*							Approximate concentration permitting optimum growth, mM
	0	0.001	0.003	0.01	0.03	0.1	0.3	
	Growth response in 8-10 days, referred to inoculum as 1							
Arginine.....	1.2		2.0	2.9	5.4	9.8	10.00	0.1
Cystine.....	1.7		4.9	8.6	9.2	8.9	8.7	0.01
Glutamine.....	2.2		2.4	3.8	7.5	9.2	12.3	0.3
Histidine.....	1.3		2.1	3.9	7.4	8.8	9.4	0.03-0.1
Isoleucine.....	1.3		2.4	4.1	7.5	8.3	8.9	0.03-0.1
Leucine.....	1.1		1.1	4.6	6.1	8.5	7.6	0.1
Lysine.....	1.5		2.2	3.5	6.7	11.0	12.1	0.1
Methionine.....	0.95		1.9	3.7	8.2	8.9	7.4	0.03
Phenylalanine.....	1.8		2.4	4.7	6.6	7.0	6.8	0.03
Threonine.....	1.3		2.2	3.6	6.8	9.7	9.4	0.1
Tryptophan.....	3.9	4.7	6.6	10.7	10.2	10.7		0.01
Tyrosine.....	1.4		1.96	4.2	6.8	8.9		0.03-0.1
Valine.....	1.2		2.0	3.9	6.7	8.5	9.5	0.1±

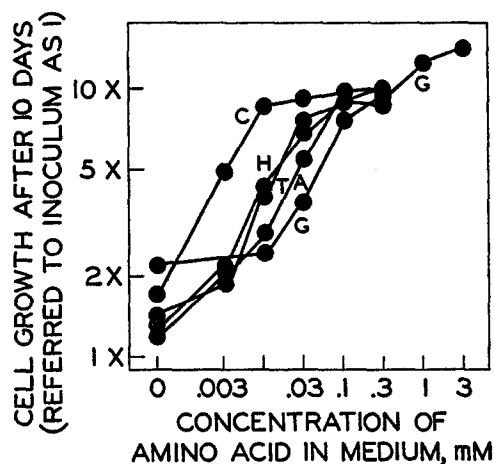
\* In a medium containing the other essential amino acids at maximally effective concentrations, and including glycine at 0.1 mM (see page 647).

sary as had been previously demonstrated for a variety of normal and malignant human cell lines (5).

In the experiments of Table I the medium contained, in addition to the other 12 essential amino acids, 0.1 mM glycine (*cf.* page 647). Qualitatively similar results to those of Table I were, however, obtained in the absence of glycine. The average generation time was then increased to approximately 4 days; but even under these conditions of slow growth, all 13 amino acids proved essential for survival. The cells again stopped growing after a few days, and died either in the first passage, or on attempted subculture.

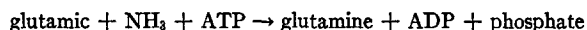
*The Interchangeability of L-Glutamine, L-Glutamic Acid, Asparagine, and Aspartic Acid in Primary Monkey Kidney Cultures.*—Glutamine had previously been found to be essential for the survival and growth of 7 serially prop-

agated cell lines (4). With  $C^{14}$ -labelled glutamine in the medium, both protein glutamine and glutamic acid residues were labelled, and to approximately the same degree (13). Although glutamic acid at extremely high concentrations (10 to 20 mM) could substitute for glutamine, at physiological levels it failed to support growth, and the cells rapidly died in the absence of added glutamine. Aspartic acid and asparagine were similarly inactive. When  $C^{14}$ -labelled glutamic acid was added to the medium, only the glutamic acid residues in the protein were heavily labelled, and the protein glutamine was labelled to only a minor degree.



TEXT-FIG. 2. The 8 to 10 day growth response of monkey kidney cells in primary culture to cystine (C), histidine (H), tyrosine (T), arginine (A), and glutamine (G) (*cf.* Table I).

It is evident that in the cell lines previously examined the reaction



was normally in the direction of glutamic acid.

A quite different situation obtained in the primary monkey kidney cultures under present consideration. Both glutamic acid and glutamine were found to be effective in promoting growth. Their maximally effective concentrations were of the same order of magnitude (approximately 0.3 to 1 mM, *cf.* Table II); and the rates of growth at that optimal level were also comparable (Text-fig. 3). Glutamine and glutamic acid together were no more effective than either alone. When labelled glutamic acid was used as a precursor, both the glutamic acid and glutamine residues of the protein were equally labelled, indicating their ready interconvertibility by this cell. Aspartic acid and asparagine also substituted for glutamine (Table II) in supporting the growth of monkey kidney epithelium, but growth on asparagine was significantly

slower (Text-fig. 3). Alanine, proline, and serine were inactive, as were a number of Krebs cycle intermediates and congeners (Table II).

*The Effect of Glycine on the Growth of Monkey Kidney Cells.*—In a limiting medium containing only the 28 components essential for growth (13 of which were the essential amino acids), supplemented with dialyzed serum, the generation time of most cell lines previously studied had averaged between 30 and 40 hours over a 7 day period of growth. In the same medium, the monkey kidney cells in primary culture grew both more slowly and more variably, with an average generation time of approximately 4 to 5 days; and even that slow growth was not sustained. On continued incubation, the rate of growth fell off;

TABLE II

*The Interchangeability of Glutamic Acid, Glutamine, Aspartic Acid, and Asparagine for Growth of Monkey Kidney Cells in Primary Culture and the Inactivity of Krebs Cycle Intermediates*

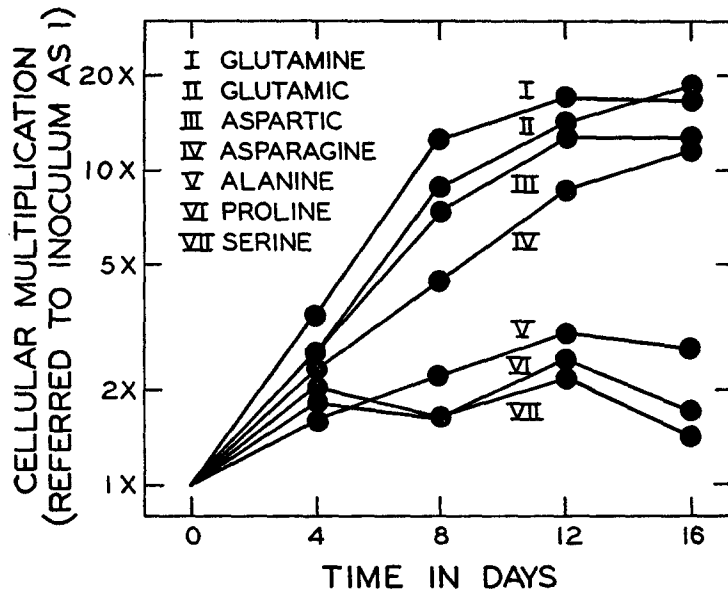
	Concentration of amino acid, <i>mM</i>						
	0	0.01	0.03	0.1	0.3	1	3
	Growth response in 12 days, referred to inoculum as 1						
Glutamine.....	2.7	3.2	7.3	10.7	12.5	16.7	18.4
Glutamic acid.....	2.7	3.1	4.6	9.9	11.3	13.8	14.4
Asparagine.....	2.7	8.1	7.3	7.1	9.0	8.6	7.6
Aspartic acid.....	2.7	2.8	5.4	9.4	11.1	12.5	11.1
Proline.....	2.7	2.4		2.5		2.4	2.1
Alanine.....	2.2	2.1		2.2		3.0	1.9
Serine.....	2.7	1.7		1.8		2.2	1.8

In the same concentration range, acetate, citrate, *cis*-aconitate, allosocitrate,  $\alpha$ -ketoglutarate, succinate, malate, and oxalacetate proved wholly inactive as substitutes for glutamine or glutamic acid.

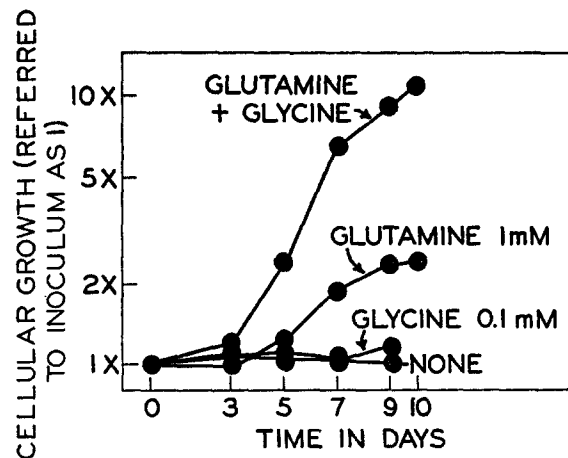
and on attempted subculture in the same limiting medium, the cells usually failed to survive. The cells obviously needed one or more factors for growth which were not being supplied in the limiting medium in which the other cell strains could be indefinitely propagated.

One of those missing components proved to be glycine. In confirmation of the findings of Rappaport and Melnick (14), on the addition of glycine to the limiting medium here used, there was a pronounced increase in the rate of growth. This growth-stimulating effect was superimposed upon that of glutamine, but the presence of the latter was essential (Text-fig. 4 and Table III).

Glycine was not only growth-stimulatory in the primary culture of the monkey kidney cells, but also permitted their subculture. Cells grown in a medium containing glutamine, but lacking glycine, usually failed to survive attempted subculture; while parallel cultures containing both glycine and glutamine con-



TEXT-FIG. 3. The interchangeability of glutamine, glutamic acid, aspartic acid, and asparagine in supporting the growth of monkey kidney cells in primary culture, and the failure of alanine, proline, or serine to substitute for glutamine. The basal medium, less glutamine, was supplemented with (a) 3 per cent dialyzed serum, (b) 0.1 mM glycine, and (c) the indicated amino acid at 1 mM concentration.



TEXT-FIG. 4. The growth response of monkey kidney cells in primary culture to glutamine and glycine. In order to deplete the cellular reserves of glutamine and glycine, cells were planted in the basal medium lacking both amino acids, but supplemented with 1 per cent whole horse serum. Twenty-four hours later, the adherent cell layer was washed and refed with basal medium supplemented with 3 per cent dialyzed horse serum, and either glutamine (1 mM), glycine (0.1 mM), or both. Results qualitatively similar to those of the figure were obtained when cells were planted in a complete growth medium containing both amino acids; but under these conditions there was some initial growth when glutamine-deficient media were added, not observed in the present experiment (*cf.* bottom two curves of the figure).

tinued to grow for several subcultures, representing a total of 10 to 20 generations. The ultimate death of the cells indicates the need for yet other metabolites essential for the serial propagation of monkey kidney cells, and not required by the other cell lines hitherto studied. The nature of this additional requirement is under present study.

TABLE III  
*The Independent Growth-Promoting Effects of Glycine and Glutamine in Primary Cultures of Monkey Kidney Cells*

Amino acid under test	Basal medium* supplemented with	Concentration of indicated amino acid, mM								Maximally effective concentration, mM
		0	0.003	0.01	0.03	0.1	0.3	1	3	
		Growth response after 10 days, referred to inoculum as 1								
Glycine	0	0.91	0.86	0.93	0.95	1.0	1.2	1.1	1.1	—
	Glutamine, 1 mM	1.2	1.7	2.3	5.5	7.0	8.2	8.7	6.6	0.1
Glutamine	0‡	0.98	1.0	1.1	1.1	1.2	1.2	1.0	1.0‡	—
	Glycine, 0.1 mM	0.83	0.83	1.7	1.7	2.4	5.4	7.6	7.5	1.0

\* Containing 12 of the essential amino acids, but lacking glycine and glutamine.

‡ In this experiment, unlike that of Text-fig. 4, the cells planted in a glycine- and glutamine-deficient medium failed to grow on the following addition of glutamine alone.

#### DISCUSSION

The present experiments show that the requirement for at least 13 amino acids by a wide variety of serially propagated human and animal cell lines does not necessarily result from either their prolonged cultivation *in vitro*, or their relatively rapid rate of growth. Monkey kidney cells in primary culture, tested within 24 hours after their removal from the animal host, and prior to extensive multiplication, required the same 13 amino acids for survival and growth; and these were just as necessary whether the cells were growing rapidly, in the presence of glycine, or slowly, in its absence. The term "essential amino acid" therefore requires qualification and definition. Eight dietary amino acids are essential and sufficient for nitrogen balance in man (1); but over and above those 8, arginine, cyst(e)ine, glutamine, histidine, and tyrosine are regularly essential for the growth of a variety of cell lines, including monkey kidney cells in first culture passage. It remains to be determined whether these 5 amino acids are similarly essential to most cells *in vivo*, and are supplied to the rest of the body by *e.g.*, the liver; or whether instead all cultured cells would have the capacity to make these specific amino acids if they were supplied the appropriate precursors or cofactors, available *in vivo*, but presumably not supplied

in the limiting medium here used. The latter is apparently the case for cystine<sup>1</sup>; and a similar situation may obtain for one or more of the other 4 amino acids.

In their ability to utilize low concentrations of glutamic acid, aspartic acid, or asparagine in lieu of glutamine, the monkey kidney cells differ from the cell lines previously studied. The latter can use glutamic acid for the biosynthesis of glutamine only at the extremely high and non-physiological levels of 10 to 20 mM (4), at which the cells form a glutamine synthase (15). That synthase is apparently present in the monkey kidney cells as isolated from the organ. In primary culture, monkey kidney cells grew on glutamic acid without a lag period (Text-fig. 3); the effective concentrations of glutamic acid and glutamine were of the same order of magnitude (Table II); and when the cells were grown on C<sup>14</sup>-labelled glutamic acid, both the glutamine and glutamic acid residues of the cell protein were found to have the same specific activity (13). Studies with other cells in primary culture will be necessary to determine whether the presence of glutamine synthase is generally characteristic of cells freshly isolated from the animal host, or uniquely characterizes kidney cells.

The growth-stimulating effect of glycine in primary monkey kidney cultures, and its requirement for sustained growth in subculture, are of particular interest. Three cell lines have now been described which in a limiting medium require 14 amino acids rather than 13 for survival and sustained growth: a rabbit fibroblast requiring serine (7), a rat carcinoma cell requiring asparagine (6), and monkey kidney cells requiring glycine. The need for glycine is particularly puzzling because these cells, like all others examined, can make significant amounts of glycine from either glucose or serine (16). Its metabolic function in the growth of the monkey kidney cell is under present study.

The trypsinized cell suspension used as inoculum in the present experiments is obviously a mixture of many cell types; and the experimental results must be considered in the light of that fact. (a) Although many cells grew out in a medium containing 14 amino acids, other cell types may have failed to grow because of an unrecognized requirement for yet other amino acids (*e.g.* alanine, serine, proline, aspartic acid, glutamic acid, asparagine). (b) Since all the cells eventually died in media lacking any one of the regularly essential 13 amino acids, these acids may be presumed to be essential for the cell types which grew out in the minimal medium here used. They might not, however, be similarly essential for the cell types which failed to grow. (c) The equally rapid growth response to glutamic acid and glutamine implies that most, if not all, of the cell types capable of growing in this limiting medium could synthesize glutamine from glutamic acid. (d) It is not clear from the present experiments whether the omission of glycine caused a uniform retardation of growth in all

<sup>1</sup> In the minimal medium here used, embodying only essential factors, cystine had proved to be essential for the survival and growth of a wide variety of animal cells. It has since been found that the requirement for cystine is eliminated by the addition of any one of a number of reduced inorganic sulfur compounds.



the cell types of the culture, or whether instead some cell types stopped growing entirely, while others continued to multiply at a "normal" rate. The problems of interpretation raised by the heterogeneity of the population here studied would presumably be resolved by similar experiments with clones of the several cell types, assuming that they can indeed be distinguished in culture.

#### SUMMARY

Monkey kidney cells tested in their first culture passage, 24 hours after their isolation from the animal host, required the same 13 amino acids for survival and growth as cell lines serially propagated in culture for years. Under the conditions of the present experiments, arginine, cystine, glutamine, histidine, and tyrosine proved necessary, over and above the 8 amino acids required for nitrogen balance in man.

With the serially propagated lines, glutamic acid substituted for glutamine only at extremely high and non-physiological levels. In the monkey kidney cell cultures, however, glutamic acid and glutamine were interchangeable, mole for mole; and aspartic acid and asparagine were also effective as glutamine substitutes.

Glycine was growth-stimulatory for monkey kidney cells in primary culture, and the cells grown in a glycine-deficient medium usually failed to survive subculture.

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## EXPLANATION OF PLATE 57

FIGS. 1 to 6. The cytopathogenic effect of specific amino acid deficiencies in primary cultures of monkey kidney cells. (Cells were planted in a complete growth medium, supplemented with 3 per cent dialyzed horse serum, but lacking a single amino acid as indicated in the figure. The cultures were refed every other day, and photographed at a magnification of 160 on the 13th day.)

FIG. 1. Control.

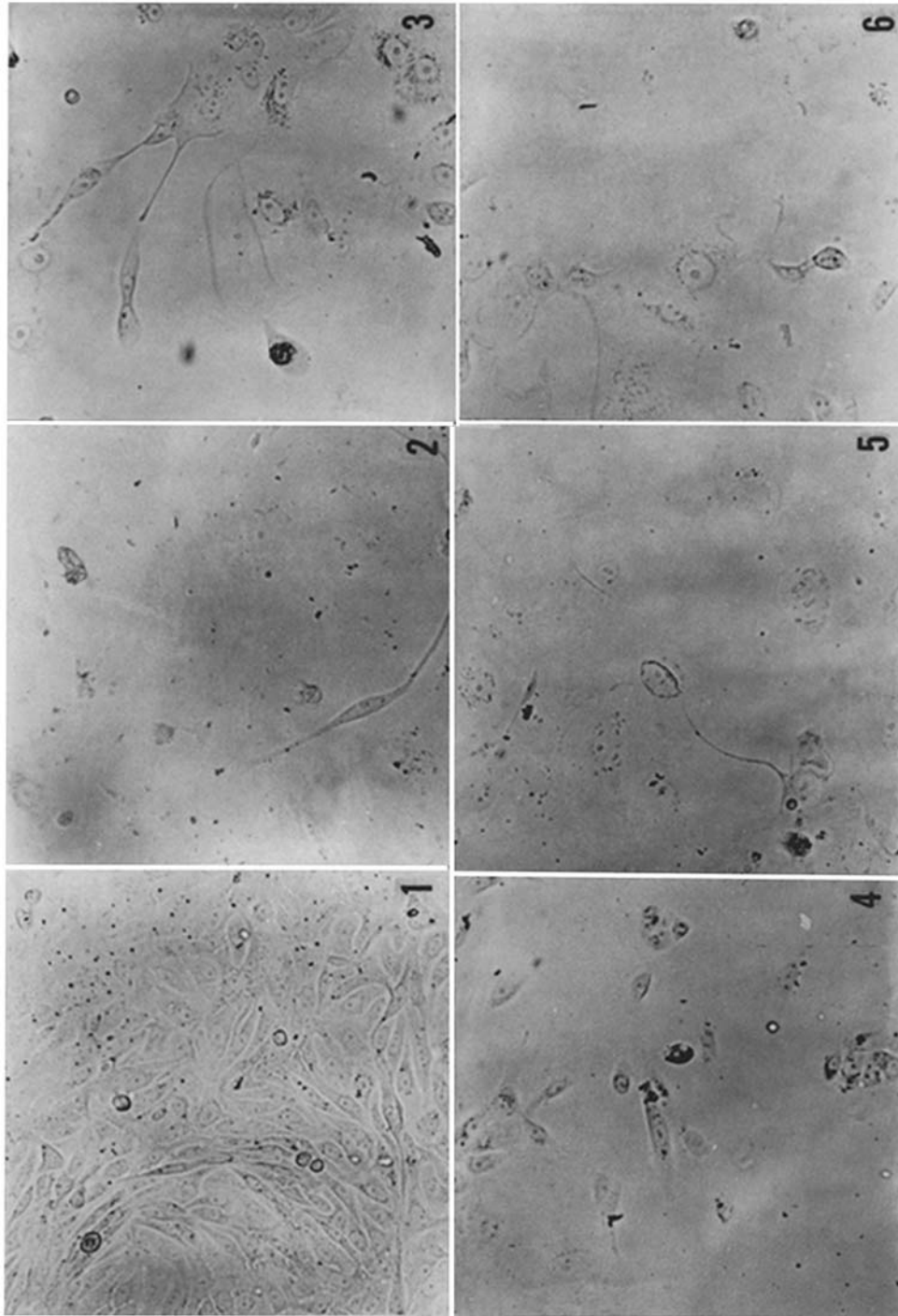
FIG. 2. Cystine-deficient cells.

FIG. 3. Histidine-deficient cells.

FIG. 4. Tyrosine-deficient cells.

FIG. 5. Arginine-deficient cells.

FIG. 6. Glutamine-deficient cells.



(Eagle *et al.*: Amino acid requirements, monkey kidney cells)