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Nutrition Needs of Mammalian Cells in Tissue Culture

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Although cells grown in tissue culture are usually imbedded in a supporting structure such as a plasma or fibrinogen clot, a number of cell lines have been propagated that do not require this support but, instead, adhere to the surface of the glass container. As they multiply, they spread out horizontally on the surface of the glass to form a thin adherent sheet. Several cell lines, notably the single-cell line of mouse fibroblasts (strain L) isolated by Sanford, Earle, and Likely (*1*) and a human uterine carcinoma cell (strain HeLa) isolated by Gey (*2*), have been propagated in this manner for years. The media ordinarily employed consist of a "balanced" salt solution enriched with serum, embryonic tissue extracts, and ultrafiltrates of these materials, in varying combination. Such complex systems do not, however, lend themselves to the identification of the specific requirements for growth.

The problem was simplified with the finding (*3, 4*) that these two cell lines could be propagated in a medium consisting of an arbitrary mixture of amino acids, vitamins, cofactors, carbohydrates, and salts, supplemented with a small amount of serum protein, the latter supplied as *dialyzed* horse or human serum. In such a system the omission of a single essential component resulted in the early death of the culture. It thus proved possible to determine most of the specific nutrients that are essential for the growth and multiplication of mammalian cells in tissue culture, to produce specific nutritional deficiencies, to study the microscopic lesions thereby produced, and to "cure" these deficiencies by the restoration of the missing component.

With the optimum medium as defined for the HeLa cell, another human carcinoma has been cultured directly onto glass (*5*), without intervening culture in a plasma clot; and a number of human cell lines cultured by other workers (*6–8*) have been propagated in the same medium for many months, with an average generation time of 40 to 60 hours. It has become possible to compare the growth requirements of normal and malignant human cells, to approach the problem of the specific metabolic requirements for the propagation of virus in such cells, and to study the incorporation of various nutrilites into cellular protein and nucleic acid. The results obtained to date are summarized in this report (*9*).

Amino Acid Requirements of a Mouse Fibroblast and of a Human Carcinoma Cell

These two cell lines proved remarkably similar with respect to their amino acid requirement. For both, 13 amino acids proved to be essential (arginine, cyst(e)ine, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine). Only the L-amino acids were active. The D-enantiomorphs, although inactive, did not inhibit the growth-promoting action of the L-isomers. For the mouse fibroblast, the requirements for optimal growth varied from 0.005 millimolar (m*M*) in the case of L-tryptophan, to 0.1 to 0.2 millimolar for L-isoleucine (*3*), and 0.2 to 0.5 millimolar for L-glutamine (*10*). The optimal concentrations for the growth of the HeLa cell (*4, 10*) were from 1 to 3 times those required by the mouse fibroblast.

Both cell species have been found to have active transaminating systems (*11*); and the carbon sources used for the synthesis of the six nonessential amino acids are under study.

Particular interest attaches to the glutamine requirement. The optimal requirement for growth proved to be 0.2 to 0.5 millimolar for the L cell and 1 to 2 millimolar for the HeLa cell. An unexpected finding was the fact that glutamic acid at any concentration, even supplemented by NH_4^+ and adenosinetriphosphate (ATP), failed to permit the growth of the L-fibroblast. This was not due to the impermeability of the cell, for isotopically labeled glutamic acid could be shown to be actively incorporated into protein, even from concentrations as low as 0.01 millimolar. With the HeLa cell, although glutamic acid did permit growth, approximately 10 to 20 times as much was required as of glutamine; and at the optimal level of 20 to 30m*M*, there was regularly less growth than with glutamine at 1m*M*. In this case also it is difficult to ascribe the relative inactivity of glutamic acid to the impermeability of the cell, since it was shown to be actively incorporated into protein from low concentrations. These data strongly suggest that glutamine plays an essential metabolic role which glutamic acid, NH_4^+, and ATP cannot fulfill in the case of the L cell; and with the HeLa cell glutamic acid may be active only by virtue of the fact that, at high concentrations, it can be transformed to glutamine in amounts adequate for growth (*10*).

Proline, ornithine, α-ketoglutaric acid, and asparagine, in any concentration tested, with or without NH_4^+ and ATP, failed to substitute for glutamine with either cell (*10*). Attempts to demonstrate glutamine synthetase activity, either with intact cells or with cell-free extracts, have to date been unsuccessful (*11*).

The parallelism in the relative amounts of the 13 amino acids required by the two cell lines is seen in

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Table 1. In both animal species, the concentrations present in the serum (*12, 13*), and thus available for distribution to the body fluids, were in most instances significantly in excess of those that suffice for the maximal growth of the cell line *in vitro*. However, with a few amino acids (arginine and isoleucine in the case of the mouse fibroblast; methionine, leucine, and isoleucine in the case of the HeLa cell), the requirement for optimum growth was of the same order of magnitude as the reported values for the concentration in the serum. If the requirements of these cells were the same *in vivo* as are now found after prolonged cultivation *in vitro*, then the availability of these compounds in the body fluids could have been a growth-limiting factor.

With both cell lines, a number of amino acids caused partial inhibition of growth in concentrations 2 to 5 times the maximally effective level. With both cell lines also, dipeptides were just as effective as the component amino acids in promoting growth (*14*). The degree to which these cells can use precursors of the essential amino acids is under study.

On the omission of a single amino acid from the medium, microscopic changes indicative of cell injury developed within 2 to 3 days, and the cells eventually died (Figs. 1 and 2). These changes differed significantly with the individual amino acids, perhaps reflecting their varying metabolic functions or differences in the amino acid composition and turnover rate of individual proteins. It is perhaps significant that, with many of the amino acid deficiencies, the cytopathogenic changes closely resembled those that result from viral infection.

Table 1. Amino acid requirements of a mouse fibroblast (strain L) and a human carcinoma cell (strain HeLa)

L-Amino acid	Optimum for growth of HeLa cell *in vitro*, (m*M*)	Optimum for growth of L-fibroblast *in vitro*, (m*M*)
Tryptophan	0.01	0.005
Histidine	0.02	0.01
Cystine	0.03	0.01
Tyrosine	0.03	0.02–0.05
Methionine	0.03	0.02–0.05
Phenylalanine	0.05	0.02
Arginine	0.05	0.05
Leucine	0.1	0.05
Threonine	0.1	0.05
Valine	0.1	0.05
Lysine	0.1	0.05
Isoleucine	0.1	0.1–0.2
Glutamine	1.0	0.2–0.5

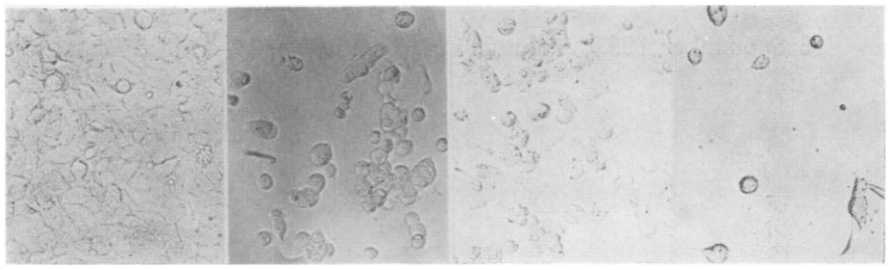

Fig. 1. Illustrative amino acid and vitamin deficiencies produced in the HeLa cell by the omission of a single nutrilite from the complete medium of Table 4. Left to right: normal control, 6 days; phenylalanine, 7 days; riboflavin, 8 days; glutamine, 5 days.

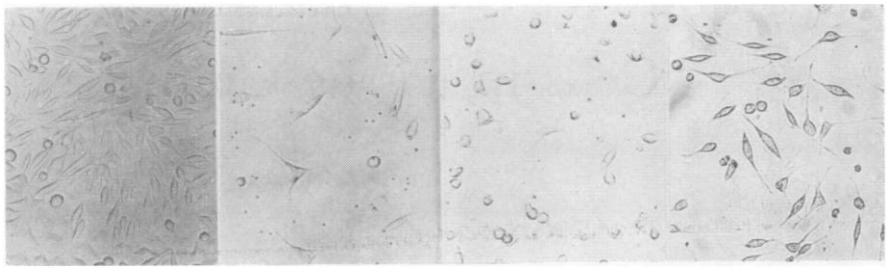

Fig. 2. Illustrative amino acid, vitamin, and salt deficiencies produced in the mouse fibroblast by the omission of a single nutrilite from the complete medium of Table 4. Left to right: normal control, 8 days; tyrosine, 9 days; choline, 4 days; Mg^{++}, 6 days.

In their early stages, the cytopathogenic effects of amino acid deficiencies were reversible. Cells that had been exposed to a medium deficient in a single amino acid and had largely degenerated in consequence could be revived on the restoration of the missing component (*3, 4*). The details of the microscopic changes produced by specific amino acid deficiencies and their reversal by the addition of the missing compound are now under study by phase and electron microscopy (*15*).

Minimum Vitamin Requirements

To date, seven vitamins have proved demonstrably essential for the growth of both the mouse fibroplast and the HeLa cell (choline, folic acid, nicotinamide, pantothenate, pyridoxal, riboflavin, and thiamine) (*16*). On the omission of any one of these from the medium, degenerative changes developed after 5 to 15 days, and the culture eventually died (Figs. 1 and 2). In their early stages, these specific vitamin deficiencies could be "cured" by the addition of the missing vitamin component to the medium. The minimum amounts of the individual vitamins and of the corresponding conjugates required for the maximal growth of the L-fibroblast are shown in Table 2. Nicotinic acid and nicotinamide proved interchangeable, as did pyridoxine, pyridoxamine, and pyridoxal; and vitamin conjugates regularly proved capable of substituting for the corresponding vitamin—for example, flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD) for riboflavin; diphosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide (TPN) for nicotinamide; coenzyme-A for pantothenic acid; cocarboxylase for thiamine).

It must be emphasized that the seven vitamins so far found to be essential are not necessarily the total vitamin requirement of these two cell lines. It is possible that a number of other vitamins are essential, but probably more prolonged cultivation in an appropriately deficient medium would be necessary in order to produce the specific deficiency. Further, a number of essential vitamins may well be present as trace contaminants in the other components of the medium and would, therefore, appear to be nonessential under the conditions of the present experiments.

Salt and Glucose Requirements

The ions demonstrably essential for the survival and growth of both the mouse fibroblast and the HeLa cell were Na^+, K^+, Mg^{++}, Ca^{++}, Cl^-, and $H_2PO_4^-$ (*17*). The concentration of each ionic species required for the optimal growth of both cell lines is shown in Table 3. No information is as yet available with respect to the need for trace elements.

Both the L-fibroblast and the HeLa cell grew well in a medium containing glucose as the only carbon source over and above the essential amino acids. A

number of carbohydrates could substitute for glucose. Some (galactose, mannose, and maltose) were almost as active as glucose, mole for mole; a few were only slightly less active; and a number were weakly effective in high concentrations. The degree to which these varying activities reflect (i) the varying permeability of the cell, or (ii) the varying ability of the cell to transform them to compounds that can then enter into the normal metabolic pathways remains to be determined.

Serum Protein

A medium containing the 13 amino acids and the seven vitamins found to be essential for the growth of the L and HeLa cells, each at the optimum concentration, and appropriately supplemented with glucose did not permit growth unless a small amount of serum protein was added, conveniently supplied as dialyzed serum. For the L cell, the concentration of protein that permitted maximal growth was 1 part in 1500, and 1 part in 5000 sufficed for limited growth. Approximately 3 to 4 times these concentrations were required for the HeLa cell.

The function of the serum protein is not yet clear. It is obviously not supplying the amino acids and vitamins already shown to be essential, at least in concentrations sufficient for survival and growth. Some of the serum fractions obtained by alcohol-salt precipitation proved inert (I, II, and III), while others (IV, V) were only weakly active, separately or in combination (*9*). Exhaustively dialyzed serum was similarly inactive. On the other hand, fractions obtained by simple salting out with $(NH_4)_2SO_4$, followed by 24-hour dialysis, were all more or less equally active. The possibility that the protein contributes trace elements or vitamins that are bound to the protein but slowly dissociate in the culture medium is under study.

Applications

Chemically defined medium. The initial objective of these studies was the identification of the specific metabolites required for the growth of various cell types rather than the development of a chemically defined medium for the cultivation of mammalian cells. Obviously, however, such studies may ultimately result in the provision of a completely defined medium in which every component has been shown to be essential for the growth of a specific cell, and each component is present in the concentration optimal for that cell.

To date, as is indicated in the foregoing sections, 27 factors have been identified as essential for the growth of a mouse fibroblast and a human carcinoma cell. The factors are listed in Table 4, together with the concentrations of each used for the propagation of the two cell lines under present consideration. There remains to be determined the function of the small amounts of serum protein that must be added over and above these essential components, and in the absence of which the cells degenerate and die.

Table 2. Minimum vitamin requirements of a mouse fibroblast

Vitamin, precursor, or conjugate	Optimal concentration range (mM)
Choline	10^{-2}
Acetylcholine	10^{-2}
Folic acid	10^{-5}
Citrovorum factor	10^{-6} ±
Nicotinamide	10^{-3}
Nicotinic acid	10^{-3}
DPN	10^{-3}
TPN	10^{-3}*
Pantothenic acid	10^{-5}
Coenzyme-A	10^{-4}
Pyridoxine	10^{-5} to 10^{-6}
Pyridoxal	10^{-5}
Pyridoxamine	10^{-4}
Pyridoxal phosphate	10^{-3}
Riboflavin	10^{-6}
Flavin mononucleotide	10^{-6}
Flavin adenine dinucleotide	10^{-5}
Thiamine	10^{-5}
Thiamine phosphate	10^{-5}*
Cocarboxylase	10^{-5}

* Significantly less growth than with vitamin.

Isolation of new cell lines in tissue culture. The striking similarity of the nutritional requirements of the HeLa cell and of the mouse fibroblast suggested that the optimum medium as so far defined for the growth of the HeLa cell might be similarly effective for other human cells. The medium of Table 4, containing the essential growth factors, all at the concentrations found optimal for the HeLa cell, and supplemented with 10-percent whole human serum was used for this purpose. A human epidermoid carcinoma of the floor of the mouth (strain KB) was cultured (*5*) by implantation of the tumor cells directly onto the glass surface, overlaid with the medium of Table 4; and initially promising results have been obtained with several other tumors.

Strain KB grows in this medium at a rapid rate, the generation time in the logarithmic phase of growth averaging 30 hours. Human liver and kidney cells cultured by Chang (*6*) and Henle (*7*), a human embryonic intestinal cell cultured by Henle (*7*), and a leukemia cell cultured by Osgood (*8*) have also been propagated in this medium, with average generation times of approximately 40 to 60 hours.

Nutritional deficiencies; antimetabolite assays. The fact that both cell types here studied degenerate and die on the omission of a single essential growth factor, whether that factor is a single vitamin, a single amino acid, or glucose, makes it possible to follow the cytopathogenic and biochemical changes that develop as a result of specific nutritional deficiencies and to follow also their reversal when the deficiency is cured by the restoration of the missing component to the medium (*15*).

It becomes possible also to determine by direct assay the growth-inhibitory activity of various antimetabolites on specific cell lines, and to determine also whether these inhibitors are competitive with normal metabolites.

The nutritional requirements for viral synthesis. With the identification of the specific metabolites required for the survival and growth of mammalian cells, it became possible to identify the components of the medium that are essential for the intracellular propagation of viruses. The amount of poliomyelitis virus released into the medium by HeLa cells was found to be quantitatively unaffected by the omission of serum protein, amino acids, or vitamins from the medium of Table 4 (*18*). In such a deficient medium the protein components of the virus are necessarily built up entirely at the expense either of the host cell protein or of its amino acid and peptide pool; and if cofactors are required for that synthesis, those already in the cell, or their precursors, suffice. On the other hand, the omission of both glucose and glutamine from the medium resulted in a marked decrease in virus production. The omission of each singly either had no effect or caused only a partial reduction.

Table 3. Minimum electrolyte requirements of a mouse fibroblast and a human carcinoma cell

Ionic species	Concn. (mM) permitting maximal growth* of	
	HeLa cell	L-fibroblast
Na^+	100	120
K^+	1	1
Ca^{++}		1
Mg^{++}		0.2
$H_2PO_4^-$		0.2 to 0.5

* In medium of Table 4, supplemented with dialyzed serum as there indicated.

Protein synthesis, turnover, and amino acid exchange in growing and resting cells. Given seven essential vitamins, the unidentified growth factors present in serum protein, and the necessary salts, both cell lines here studied could obtain their total requirements for energy and growth from 13 amino acids and glucose. On the omission of any one of the amino acids from the medium, the cells stopped multiplying, and the amount of cell protein then usually remained unchanged during a period of 48 to 72 hours or decreased slightly as the cells began to degenerate. There was, however, a continuing incorporation of labeled amino acids into cell protein during this entire period.

The amount of the individual amino acid incorporated was the same, whether one or six amino acids had been omitted from the medium, and in the case of L-phenylalanine approached as a limiting value approximately 35 percent of the total amount of that amino acid in the cell protein. When several labeled amino acids were used in conjunction, the amounts incorporated were additive. This incorporation of amino acids into protein from a medium in which there is no net synthesis could reflect protein turnover—that is, protein degradation and resynthesis—with total recapture of the essential amino acids that were not available from the medium. Alternatively, there may be an exchange of amino acid residues between the intact protein and the amino acid pool, similar to that described for bacteria by Gale and Folkes (*19*). These two possibilities are presently under study.

Studies are in progress on the metabolic pathways involved in the synthesis of nucleic acid and of the six nonessential amino acids from the 13 essential amino acids and glucose.

Table 4. Basal media for cultivation of the HeLa cell and mouse fibroblast (*10*)

L-Amino acids* (m*M*)			Vitamins‡ (m*M*)		Miscellaneous	
Arginine	0.1		Biotin	10^{-3}	Glucose	5m*M*§
Cystine	0.05	(0.02)†	Choline	10^{-3}	Penicillin	0.005%#
Glutamine	2.0	(1.0)‖	Folic acid	10^{-3}	Streptomycin	0.005%#
Histidine	0.05	(0.02)†	Nicotinamide	10^{-3}	Phenol red	0.0005%#
Isoleucine	0.2		Pantothenic acid	10^{-3}		
Leucine	0.2	(0.1)†	Pyridoxal	10^{-3}		
Lysine	0.2	(0.1)†	Thiamine	10^{-3}	For studies of cell nutrition	
Methionine	0.05		Riboflavin	10^{-4}	Dialyzed horse serum, 1%†	
Phenylalanine	0.1	(0.05)†			Dialyzed human serum, 5%	
Threonine	0.2	(0.1)†	Salts§ (m*M*)		For stock cultures	
Tryptophan	0.02	(0.01)†			Whole horse serum, 5%†	
Tyrosine	0.1				Whole human serum, 10%	
Valine	0.2	(0.1)†	NaCl	100		
			KCl	5		
			$NaH_2PO_4 \cdot H_2O$	1		
			$NaHCO_3$	20		
			$CaCl_2$	1		
			$MgCl_2$	0.5		

* Conveniently stored in the refrigerator as a single stock solution containing 20 times the indicated concentration of each amino acid.
† For mouse fibroblast.
‡ Conveniently stored as a single stock solution containing 100 or 1000 times the indicated concentration of each vitamin; kept frozen.
§ Conveniently stored in the refrigerator in two stock solutions, one containing NaCl, KCl, NaH_2PO_4, $NaHCO_3$, and glucose at 10 times the indicated concentration of each, and the second containing $CaCl_2$ and $MgCl_2$ at 20 times the indicated concentration.
‖ Conveniently stored as a 100m*M* stock solution; frozen when not in use.
Conveniently stored as a single stock solution containing 100 times the indicated concentrations of penicillin, streptomycin, and phenol red.

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"Fact," as I intend the term, can only be defined ostensively. Everything that there is in the world I call a "fact." The sun is a fact; Caesar's crossing of the Rubicon was a fact; if I have toothache, my toothache is a fact. If I make a statement, my making it is a fact, and if it is true there is a further fact in virtue of which it is true, but not if it is false. The butcher says: "I'm sold out, and that's a fact"; immediately afterwards, a favored customer arrives and gets a nice piece of lamb from under the counter. So the butcher told two lies, one in saying he was sold out, and the other in saying that his being sold out was a fact. Facts are what make statements true or false. I should like to confine the word "fact" to the minimum of what must be known in order that the truth or falsehood of any statement may follow analytically from those asserting that minimum. For example, if "Brutus was a Roman" and "Cassius was a Roman" each assert a fact, I should not say that "Brutus and Cassius were Romans" asserted a new fact. We have seen that the questions whether there are negative facts and general facts raise difficulties. These niceties, however, are largely linguistic.—BERTRAND RUSSELL, *Human Knowledge,* 1948.