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# Amino acid and vitamin requirements in mammalian cultured cells

*Review Article*

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**Summary.** The development of the tissue culture technique has enabled us to cultivate mammalian cells in a way which is similar to that in use with bacterial cells. As such, the nutritional requirements of mammalian cells in culture have been studied with simplicity and exactness. According to Eagle's extensive works it is accepted that cultured cells generally require 13 amino acids, 8 or 9 vitamins, glucose and 6 inorganic salts. However, although some cultured cells have a capacity for the biosynthesis of Eagle's essential nutrients and others require non-essential nutrients.

In this review we will discuss the amino acid and vitamin requirements of cultured cells, and a cell line (R-Y121B · cho) which propagates continuously in a chemically defined medium containing 11 amino acids, 7 vitamins, glucose and 6 ionic salts. Arginine, glutamine, tyrosine and choline are synthesized in the R-Y121B · cho cells.

**Keywords:** Amino acids – Vitamin – Cultured cell – Nutritional requirement

Harrison employed frog lymph as supporting and nutrient media to study living nerve fibers. In his paper he described that the medullary tube from frog embryos survived and nerve fibers grew out into the lymph clot (Harrison, 1907). This showed that cells could survive and develop in tissue culture and could be used effectively to tackle biological questions. In the early studies of tissue culture, cells were usually embedded in biological materials such as plasma or fibrin clot. Single cells could not be kept alive at all and the culture remained in "tissue culture".

In 1948, Earle and his colleagues reported that they were able to grow a single cell isolated from carcinogen-treated mice and to obtain from them cultures which were designated L-cell (Sanford et al., 1948). To establish cultures from single cells it is necessary to dissociate tissues into viable single cells.

Rous and Jones had achieved the release of cells from the outgrowth of plasma clot cultures by digesting the clot and culture with trypsin (Rous and Jones, 1916). The trypsinization technique prepared viable dispersed cells for cell cultures from whole embryos, organs, tissues, or other cultures.

For many years, fluid-medium cultures had been prepared from minced and chopped tissues, originally for virus studies (Maitland and Maitland, 1928; Parker and Hollender, 1945a; Parker and Hollender, 1945b). These medium usually consisted of a balanced salt solution enriched with naturally occurring materials such as serum, body fluids and exudates, and extracts of tissues and organs. But the complexity and variability of these biological materials made it difficult to examine the metabolic processes of cultured cells under controlled conditions.

A major advance for nutritional requirements of cultured cells was introduced by Eagle and his colleagues (Eagle, 1955a; Eagle, 1955b; Eagle, 1959). They employed cell lines which adhered to the surface of the glass containers or grew in suspension. These cell lines propagated in a medium consisting of an arbitrary mixture of amino acids, vitamins, cofactors, and serum protein. In such a system the specific nutrients could be determined by the omission of a single essential component resulting in the early death of the culture. Eagle and his colleagues used this system to study the essential nutritional requirements for cultured cells. Although there were specific requirements for some animal cell lines, a relatively limited number of metabolites were essential for apparently indefinite propagation of cell cultures, whether human or animal in origin, and whether deriving from normal or malignant tissue. The minimal medium contained 13 amino acids, 8 vitamins, 6 ionic species, glucose and serum protein. This medium is called Eagle's minimum essential medium (Eagle's MEM) and is still in use at many laboratories (Eagle, 1959).

Although Eagle's MEM contained minimal components for growth and multiplication of cultured mammalian cells, some components were not essential in feeding experiments in animals. The fundamental point of our research is to explain the fact that culture cells require some nutrients which are not required for growth *in vivo*. In this review we outline the amino acid and vitamin requirements in cultured cells and also cell lines which required less than 28 nutrient factors.

## Amino acid requirements in mammalian cell cultures

It is known that proteins are composed of twenty amino acids. These amino acids are classified into those which are indispensable and those which are dispensable, with respect to their growth effects. Humans need only 8 amino acids, that is, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. In addition to the above eight amino acids, rats require histidine (Table 1). Although amino acid requirements of cultured cells differ from one cell culture to another, it is widely accepted that the 13 amino acids in Eagle's MEM were essential for the survival and growth of cultured cells. Eagle pointed out three possibilities to explain this fact; 1) loss of biosynthetic mechanisms, 2) lack of appropriate precursors or cofactors, 3) limited biosyn-

**Table 1.** Essential amino acids in man and rat[1] and amino acids composition in Eagle's MEM[2]

| Subjects | *Homo sapiens* | | | *Rattus norvergicus* | | MEM |
|---|---|---|---|---|---|---|
| | <1 yr old | >1 yr old | Adult ♂ | Immature | Adult | |
| Requirement for | Growth | Growth | Maintenance | Growth | Maintenance | Growth |
| Unit of measurement | mg·kg body $wt^{-1}\cdot da^{-1}$ | $mg\cdot da^{-1}$ | | % of diet | | $mg\cdot l^{-1}$ |
| L-Arginine | – | – | – | 0.60 | – | 105 |
| L-Asparagine | – | – | – | 0.40 | – | – |
| L-Cystine | + | + | + | + | + | 24 |
| L-Glutamine | – | – | – | 4.00 | – | 292 |
| L-Histidine* | 34 | – | – | 0.30 | 0.07 | 31 |
| L-Isoleucine* | 126 | 1000 | 700 | 0.55 | 0.43 | 52 |
| L-Leucine* | 150 | 1500 | 1100 | 0.75 | 0.25 | 52 |
| L-Lysine* | 103 | 1600 | 800 | 0.90 | 0.14 | 58 |
| L-Methionine* | 45[4] | 800[4] | 1100[6] | 0.60[6] | 0.23[6] | 15 |
| L-Phenylalanine* | 90[5] | 800[5] | 1100[7] | 0.80[7] | 0.19[7] | 32 |
| L-Proline | – | – | – | 0.40 | – | – |
| L-Tyrosine | + | + | + | + | + | 36 |
| L-Threonine* | 87 | 1000 | 500 | 0.50 | 0.17 | 48 |
| L-Tryptophan* | 22 | 250 | 250 | 0.15 | 0.07 | 10 |
| L-Valine* | 105 | 900 | 800 | 0.60 | 0.31 | 46 |
| DAAN[3] | – | – | – | 0.55 | 0.42 | – |

[1] Altmann P. L. and Dittmer D. S. ed.: Biology data book 2nd ed. Vol. III, Federation of American Societies for Experimental Biology, Bethesda, MD, USA, pp1473, 1974.
[2] Eagle H.: Science 130: 432, 1959.
[3] dispensable amino acid nitrogen.
[4] In presence of adequate L-cystine.
[5] In presence of adequate L-tyrosine.
[6] 30–50% of total requirement may be furnished by L-cystine.
[7] 30–50% of total requirement may be furnished by L-tyrosine.

thetic capacity, sufficient for survival but for growth (Eagle, 1959). Cell lines which do not require some amino acids in Eagle's MEM, have been reported. These cells shows why these amino acids are usually essential for most cultured cells. We will describe individual amino acids in the following sections.

## Arginine

The pathway of arginine biosynthesis in animal tissues is illustrated in Fig. 1. Arginine is synthesized from ornithine through citrulline and arginosuccinate. The enzymes for the conversion of citrulline to arginine are detectable in most tissues (Ratner, 1973). Most cell lines can utilize citrulline as a precursor for arginine (Levintow and Eagle, 1961; Naylor et al., 1976; Sun et al., 1979). But while citrulline synthesis from ornithine is unique in liver cells *in vivo*, most cultured cells can not utilize ornithine regardless of whether the cells were derived from a normal liver or hepatoma (Naylor et al., 1976). In a few cases, the cell growth in the arginine-free medium containing ornithine was reported

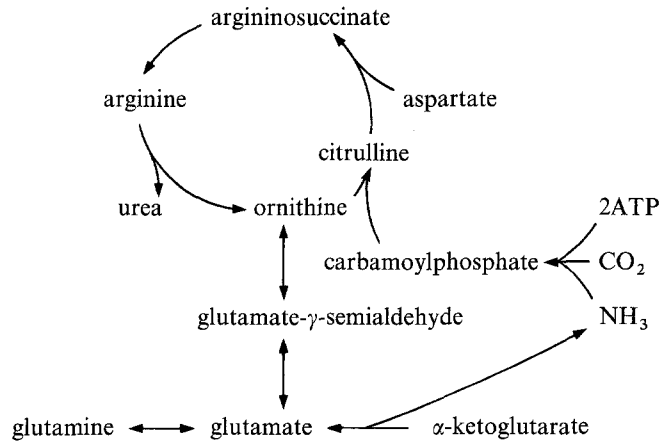


**Fig. 1.** Pathways of arginine and glutamine biosynthesis in mammals

in rat hepatoma cultures (Niwa et al.,1979; Niwa et al.,1980; Goss, 1984; Delers et al.,1984) and hepatocyte-hepatoma hybrid cells (Widman et al., 1979; Farmer and Goss, 1991). These cell lines and their ornithine carbamoyltransferase activities are shown in Table 2. Ornithine is made by transamination from glutamate-γ-semialdehyde in mammals. However, any cell lines have not been reported which grow in ornithine-free medium.

In primary culture of liver cells ornithine carbamoyltransferase activity usually decayed to an undetectable level within a month after the initiation of cultivation (Yasumura et al., 1978). Leffert and Paul used an arginine-free and ornithine-containing medium to selectively multiply fetal rat hepatocytes, which were able to form the arginine they needed (Leffert and Paul, 1973; Hasegawa et al., 1982). Even though hepatocytes were selected from arginine-required liver-derived cells in arginine-free and ornithine-containing media, continuous growth of adult hepatocytes has not been reported as yet.

## Glutamine

Glutamine is found in relatively high concentrations in many mammalian cells where it serves as an ammonia scavenger and a nitrogen donor for the biosynthesis of a number of important compounds such as nucleotides, amino acids and amino sugars. Furthermore, glutamine was used as the major source of energy for cultured human carcinoma cells (HeLa cell, Reitzer et al., 1979) and mouse macrophage (Newsholme and Newsholwe, 1989). Glutamine is synthesized from glutamate by glutamine synthetase, and glutamate is produced from α-ketoglutarate by the catalytic action of glutamate dehydrogenase (Fig. 1).

The necessity of glutamine in the amino acid requirements of cultured cells was first observed by Ehrensvärd et al. (1949). Since then, studies with several cell lines have demonstrated that glutamine is an essential requirement for optimal proliferation of cultured cells. Eagle and his colleagues reported that both L cells and HeLa cells degenerated and died in the glutamine-free medium. The maximally effective concentration was 0.2 to 0.5 mM for L cells, and 1 to 2 mM for HeLa cells (Eagle et al., 1956a).

**Table 2.** Ornithine carbamoyltransferase (OCT) activity and cellular growth in an arginine-deprived medium of various cell lines

| Cell line (origine) | Serum[1] in medium | Growth in −Arg + Orn | OCT activity nmol. Cit/min/mg Protein | Reference |
|---|---|---|---|---|
| 7800Ci (rat Morris hepatoma 7800) | 5% HS + 5% FBS | − | 1 | Richardson et al., 1974 |
| R-Y117-21B (rat Reuber hepatoma) | 2% DCS | + | 30 | Niwa et al., 1979 |
| Clone 119 (rat hepatocyte-hepatoma hybrid) | 10% FBS | + | 43 | Widman et al., 1979 |
| R-Y121B (rat Reuber hepatoma) | 0 | + | 122 | Niwa et al., 1980 |
| 777orn (rat Morris hepatoma 777) | 10% FBS | + | 120 | Goss, 1984 |
| Fa32A50 (rat Reuber hepatoma) | 10% FBS | + | 65 | Delers et al., 1984 |
| BWsft hybrid (mouse hepatoma-hepatocyte hybrid) | 5% FBS | + | 6 | Farmer and Goss, 1991 |

[1] *HS* Horse serum, *FBS* Fetal bovine serum, *DCS* Dialysed calf serum

In media supporting growth of L cells, glutamine could not be effectively replaced by any concentration of glutamic acid, although, in the case of HeLa cells, glutamic acid at the optimal concentration of 20 mM was an effective substitute for glutamine (10–20 times the glutamine concentration). Several investigators (DeMars, 1958; Paul and Fottrell, 1963; Goetz et al., 1973; Tiemeier and Milman, 1972; Yasumura et al., 1978) have reported that various cell lines also could grow in a glutamine-free and glutamate-supplemented medium due to the ability to produce glutamine synthetase, though the enzyme was usually induced under a condition of high concentration of glutamate. Although glutamine and/or glutamic acid is essential for the great majority of cell lines, some types of cells can synthesize enough glutamine to satisfy their needs (Table 3). Green monkey kidney cells (VERO-300 cells ) have a glutamate dehydrogenase and grow in protein-free medium lacking glutamine and glutamate (Yasumura et al., 1978). Rat hepatoma cells (R-Y121B cells) can also grow in glutamine-, glutamate- and protein-free medium (Niwa et al., 1980).

## Tyrosine

Tyrosine is formed from an essential amino acid, phenylalanine. It is known that three enzymes (phenylalanine hydroxylase, tyrosine hydroxylase and tryptophan hydroxylase) are responsible for the conversion of phenylalanine to tyrosine (Breakefield and Nirenberg, 1974). The phenylalanine hydroxylase activity has been found in only three mammalian tissues; the liver, kidney and pancreas (Tourian et al., 1969). In the case of cell culture a variant strain of HeLa cells was able to convert phenylalanine to tyrosine, though not at a rate sufficient enough to support growth (Eagle et al., 1957). Haggerty and his colleagues found the presence of phenylalanine hydroxylase as to be a constitutive enzyme in two rat hepatoma cell lines (H4-II-E-$C_3$, $MH_1C_1$, Haggerty et al., 1973). These two cell lines grew in medium lacking tyrosine, when phenylalanine was present (Haggerty et al., 1975). A pigmented murine melanoma line (PS1, Breakefield et al., 1978), a human hepatoma cell line (H–H1, Choo et al., 1980) and a mouse hepatoma cell line (BWTG3, Farmer and Goss, 1991) had phenylalanine hydroxylase and grew in tyrosine-free medium.

The tyrosine hydroxylase is found in the adrenal medulla, brain and other sympathetically innervated tissue (Nagatsu et al., 1964). Mouse neuroblastoma cells (N-115 cells) were able to grow in the absence of tyrosine due to the presence of tyrosine hydroxylase in the cell (Breakefield and Nirenberg, 1974).

Tryptophan hydroxylase is found in the brain (Graham-Smith, 1964). Mouse mastocytoma cells (P815-X2.1) possessing this enzyme were able to grow in a tyrosine-free medium but prolonged cultivation was not successful (Choo et al., 1976).

## Cystine and methionine

Cystine is synthesized from methionine by the pathway shown in Fig. 2. In the first step of this sequence, methionine loses the methyl group from its sulfur atom to become homocysteine. Homocysteine reacts with serine to yield cy-

**Table 3.** Serially cultured cells growing in an essential amino acid-free medium

| Amino acid | Enzyme involved | Cell line (origine) | Reference |
|---|---|---|---|
| Glutamine | Glutamic dehydrogenase | VERO-300 (normal monkey kidney) | Yasumurat et al., 1979 |
| | + Glutamine synthetase | R-Y121B (rat Reuber hepatoma) | Niwa et al., 1981 |
| Tyrosine | Phenylalanine hydroxylase | H4-II-EC3 (rat Reuber hepatoma) | Haggerty et al., 1975 |
| | | MH1C1 (rat Morris hepatoma) | Haggerty et al., 1975 |
| | | PS1 (mouse pigmented melanoma) | Breakefield et al., 1978 |
| | | H-H1 (humun hepatoma) | Choo et al., 1980 |
| | | BWTG3 (mouse hepatoma) | Famer and Goss, 1991 |
| | Tyrosine hydroxylase | N-115 (mouse neuroblastoma) | Breakefield and Nirenberg, 1974 |

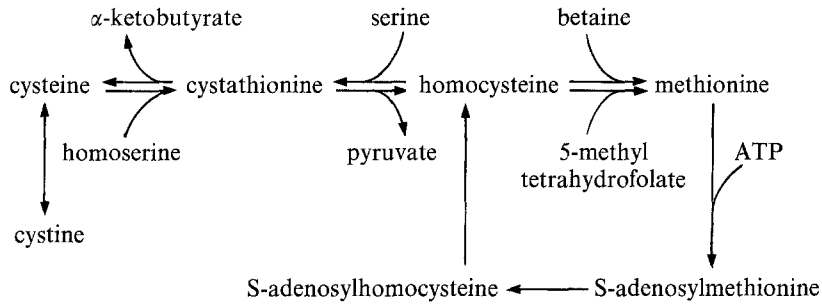


**Fig. 2.** Pathways of cysteine and methionine biosynthesis in mammals

stathionine. Cystathionine is cleaved hydrolytically to yield cysteine. Although a number of heteroploid human cell lines were able to synthesize cystine from methionine, the cells in a cystine-free medium died unless the population density was maintained in excess of 200,000 to 500,000 cells/ml. Even if the cells were provided with homocystine or cystathionine, growth was observed only if the initial population density was in excess of 10,000 to 60,000 cells/ml. A similar population-dependent requirement for metabolites such as asparagine, glutamine, serine, inositol and pyruvate was reported (Eagle et al., 1961; Eagle and Piez, 1962). Further studies showed this to be the case for heteroploid cells, but human diploid cells required cystine (Eagle et al., 1966). Some variant human diploid fibroblast clones were able to grow in the medium containing homocysteine in place of cystine (Jacoby and Littlefield, 1971; Hankinson and Jacoby, 1975). In rats, the enzymes of cystine synthesis are predominantly in the liver, pancreas and kidney (Mudd et al., 1965). A most cell lines of mouse and rat cells could grow using cystathionine in place of cysteine (Naylor et al., 1976). However homocysteine could only be used by hepatoma cells (Goss, 1986; Farmer and Goss, 1991).

In mammals methionine is synthesized by at least two enzymes, vitamin $B_{12}$-dependent 5-methyltetrahydrofolate-homocysteine methyltransferase and betaine-homocysteine methyltransferase (Fig. 2).

The vitamin $B_{12}$-dependent methyltransferase activity was widely distributed in rat tissue (Finkelstein et al., 1971) and was detected in various cultured mammalian cells. These cultured cells grew in a methionine deficient medium supplemented with homocysteine and vitamin $B_{12}$ (Mangum and North, 1968; Mangum et al., 1969; Kamely et al., 1973; Halpern et al., 1974).

Betaine-homocysteine methyltransferase was detectable in only a few mammalian tissues (Finkelstein et al., 1971). Although this transferase was detected in cultured mouse cells (Grzelakowska-Sztabert and Balińska, 1980), they did not multiply in the methionine-deficient medium.

### α-Keto analogues of the essential amino acids and D-amino acids

Essential amino acids were able to be replaced by the corresponding α-keto acids (Eagle, 1959; Naylor et al., 1976). Because the essential parts of the indispensable amino acids are their carbon skeletons, not their amino groups, the α-keto

analogues of the essential amino acids could accept amino groups from excess nonessential amino acid by transaminase action.

D-amino acid can be converted to the L-form by D-amino acid oxidase and aminotransferases. Human oral carcinoma cells (KB cells) were able to utilize D-cystine (Naylor et al., 1976) . Green monkey kidney cells (VERO-317 and VERO-300 cells) propagated continuously in the media in which L-amino acid was replaced by D-isomer of leucine, phenylalanine and/or valine (Yasumura et al., 1978).

## Non-essential amino acids

The individual requirements for non-essential amino acids will vary from one cell line to another. In some situations there is a specific requirement for an amino acid normally considered non-essential. Asparagine was required by certain malignant cells, including a number of lines of leukemia (McCoy et al., 1956; Haley et al., 1961; Broome, 1963; Summers and Handschumacher, 1971; Ohnuma et al., 1971; Schrek et al., 1973) . In addition, there was an unusual requirement of glycine by Rhesus monkey testicular cells (Tytell et al., 1958) and monkey kidney cells (Eagle et al., 1958) . Requirement of serine by rabbit fibroblast cells (Haff and Swim, 1957) and leukemic cells (Regan et al., 1969), that of combination of glycine and serine by Novikoff hepatoma cells (McCoy et al., 1959) and that of alanine and serine by mouse fibroblast cells (Nagle and Brown, 1971) were reported.

## Vitamin requirements in mammalian cell cultures

Eagle (1955a) reported the HeLa and L cells required 8 vitamins (biotin choline, folic acid, nicotinamide, pantothenate, pyridoxal, riboflavin, and thiamine). In this case Eagle's medium was supplemented with serum, which would supply biotin, vitamin $B_{12}$, and fat-soluble vitamins. Sanford et al. (1963) showed that cells required 8 vitamins (Eagle's 8 vitamins), after they examined the effects of the 16 vitamins, in a serum-free chemically defined medium on cell survival and proliferation rates. Eight vitamins such as A, D, K, E, C, p-aminobenzoic acid, $B_{12}$, and inositol appeared to have no significant effects on the cell growth. The fat-soluble vitamins were necessary for specialized organs, but did not play a necessary role in the metabolism of cultured cells (Smith, 1981). From these results, it was determined that Eagle's 8 vitamins are essential to sustain cell growth. However it was reported that some cell lines required vitamin $B_{12}$ (Higuchi and Robinson, 1973; Kan and Yamane, 1982) and inositol (Eagle, 1956b; Jackson and Shin, 1982).

Choline is necessary for a substrate of the biosynthesis of phospholipid rather than as cofactor. All of the nitrogen-containing phospholipids can be derived from phosphatidylserine. Decarboxylation of the serine residue gives phosphatidylethanolamine, and successive methylations yield phosphatidylcholine. There is also some direct incorporation of choline into phospholipids (Fig. 3). In cultured cells, the capacity to synthesize choline *de novo* is inadequate to meet the demand for phospholipid synthesis. Therefore, choline is essential in cultured

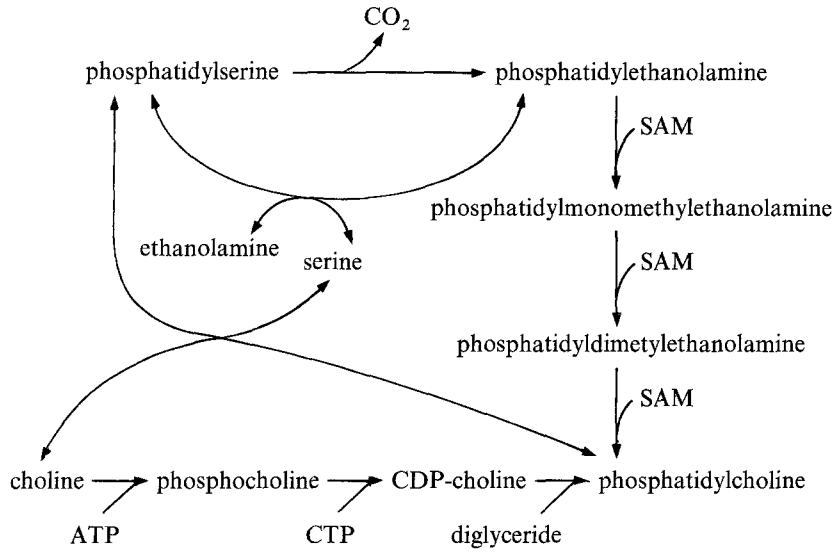


**Fig. 3.** Pathways of choline and phosphatidylcholine biosynthesis in mammals

cells. Phosphatidylcholine can be synthesized by the methylation of phosphatidylethanolamine in liver cells. Some cell lines were able to utilize monomethylethanolamine and dimethylethanolamine as a precursor for choline (Glaser et al., 1974, Schroeder et al., 1976). Rat hepatoma R-Y121B · cho cells were able to grow in choline-free medium (Yamamoto et al., 1985).

The vitamins act as the cofactor and coenzyme associated with many enzyme systems. Co-enzymes can generally be substituted for the parent vitamins. The active form of pyridoxal is pyridoxal phosphate. Pyridoxal phosphate serves as the tightly bound prosthetic group of a number of enzymes catalyzing reactions of amino acids. The most common and best known example of these is transamination. When cells were cultured in the absence of pyridoxal, all the amino acids had to be supplied in the culture medium (Swim and Parker, 1958; Sanford et al., 1963).

## Auxotrophic mutants

An auxotrophic mutant is considered here to be a genetically altered cells having at least one more nutritional requirements than the parent cells. Auxotrophic mutants are useful in studying the various problems in mammalian cellular genetics and metabolism of cultured cells. A method of selecting for auxotrophic mutants was first described by DeMars and Hooper (1960). It utilized the fact that aminopterin is more toxic for growing than for nongrowing cells. Glutamine-requiring cells were selected from HeLa cells. Puck and Kao developed a technique to obtain auxotrophic mutants. They treated Chinese hamster ovary and Chinese hamster lung cells with mutagenic agents and exposed them to BrdU followed by illumination with near-visible light (Puck and Kao, 1967). Then they isolated the auxotrophs for proline; glycine; inositol (Kao and Puck, 1968; Kao and Puck, 1972). Auxotrophs for serine (Jones and Puck,

1973) and tyrosine (Choo and Cotton, 1977) were also isolated using the same technique.

## Establishment of cultured cells that minimize the requirement for organic nutrient

Among Eagle's 13 amino acids and 8 vitamins, 4 of the amino acids (arginine, cystine, glutamine, and tyrosine) and choline are not essential for rats, however, they are essential for cells in culture (Table 1). Because these amino acids and choline are synthesized in liver *in vivo*, liver cell cultures might be capable of synthesizing these nutrients at a rate sufficient enough to sustain growth. Therefore we have tried to establish prototrophs to these nutrients by a multi-step selection procedure using rat hepatoma cells.

The cell line chosen as a parental line was H4-II-E which was established from Reuber hepatoma H-35 (Pitot et al., 1964). H4-II-E cells were maintained in Eagle's MEM supplemented with biotin (EM#303) and 10% of fetal bovine serum. Since drug-resistant cells are useful in cell hybridization experiments, the parental cells were adapted to grow in EM#303 including 5-bromodeoxy-

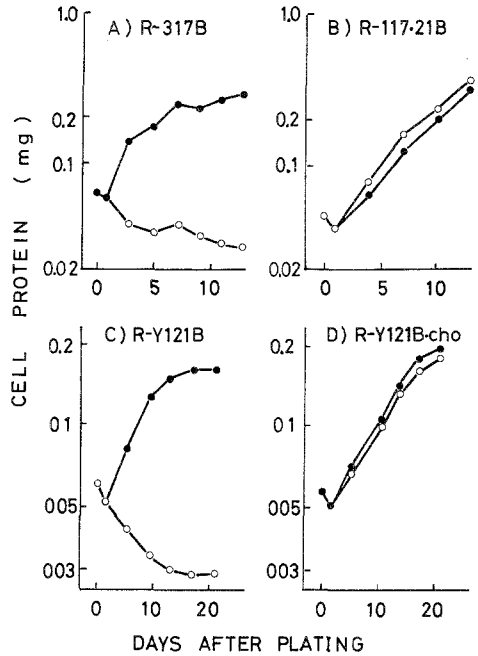


**Fig. 4.** The growth curves of R-317B, R-117·21B, R-Y121B and R-Y121B·cho cells. **A** R-317B cells were tested in glutamine-deprived and glutamate-supplemented MEM (EM#317, ●) or arginine-deprived and ornithine-supplemented EM#317 (○). Media were supplemented with 1% of DCS. **B** R-117·21B cells tested in EM#317 (●) or arginine-deprived and ornithine-supplemented EM#317 (○). Media were supplemented with 1% of DCS. **C** R-Y121B cells were tested in arginine-, glutamine-, and tyrosine-deprived and ornithine-supplemented MEM (EM#Y121, ●) or choline-deprived EM#Y121 (○). **D** R-Y121B·cho cells were tested in choline-deprived EM#Y121 (○) or choline- and inositol-deprived EM#Y121 (●)

uridine (BrdU). The BrdU-resistant line was next adapted to the medium containing glutamic acid in place of glutamine (EM#317). The concentration of serum in the medium was decreased gradually to 1% dialyzed calf serum (DCS) during 2 to 6 months of serial subculturing. The cells cultivated in EM#317 containing BrdU (50 μg/ml) and DCS (1%) were designated R-317B (Fig. 4A).

To establish a cell line that had complete set of the urea cycle enzymes, R-317B cells were maintained in an arginine-deprived, ornithine-supplementated medium (EM#117·21) containing 1% DCS. Thus, we established a cell line, R-117·21B, which grew continuously in EM#117·21 containing 1% DCS and 50 μg/ml BrdU (Fig. 4B). R-117·21B cells, like H4-II-E cells, preserved phenylalanine hydroxylase, and propagated in tyrosine-free medium without any lag period.

Although cells in culture usually require some components of serum, serum contained amino acids as well as many other unknown substances. Even if we performed serum dialysis exhaustively, amino acids do not dialyze out completely from serum, and moreover, during the course of culture, serum protein would yield free amino acids by proteolysis (Piez et al., 1960; Niwa et al., 1979). Therefore, studies in amino acid requirements of cultured cells are best conducted in a totally synthetic medium. Thus we tried to adapt R-117·21B cells in serum-free medium. Then the cells were adapted in a glutamic acid-deprived medium. The cells were designated R-Y121B cells, which grew continuously in fully an autoclavable synthetic medium (EM#Y121) which contained 11 amino acids (cystine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, and ornithine) (Fig. 4C). R-Y121B cells have a complete set of the urea cycle enzymes, phenylalanine hydroxylase, and synthesize urea via the urea cycle pathway (Table 4, Niwa et al., 1980; Yamamoto et al., 1981).

**Table 4.** Enzyme activities of normal rat liver and R-Y121B cells

| Enzyme | R-Y121B | Normal rat liver |
|---|---|---|
| | nmol product/min per mg cell protein | |
| Carbamoylphosphate synthetase (ammonia) | 7 | 36 |
| Ornithine carbamoyltransferase | 122 | 821 |
| Argininosuccinate synthetase | 5 | 17 |
| Argininosuccinate lyase | 16 | 23 |
| Arginase | 860 | 10800 |
| Phenylalanine hydroxylase | 10 | 245 |
| Tyrosine aminotransferase | 18 | 25 |

From the R-Y121B cells, a choline- and inositol-prototroph was also established. R-Y121B cells were cultivated in EM#Y121 with ethanolamine in place of choline. After 15 subcultures in this medium, the cells were subcultured in a choline- and ethanolamine-deprived medium. The cells cultivated in choline-free medium were named R-Y121B·cho cells which proliferated readily in inositol- and choline-free EM#Y121 (Fig. 4D).

The establishment of R-Y121B · cho cells indicate that the cells have not only biosynthetic mechanisms for some of Eagle's essential nutrients but also the products were sufficient for survival and growth. Further effort to establish an ornithine and/or cysteine non-required cells is continued.

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