Amino Acid Requirements of Some Continuous Cell Lines of Insects

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(Received March 22, 1978)

The essential amino acids for the growth of insect cells in cultures were determined by the use of single amino acid-deficient media. The continuous cell lines derived from a moth, *Mamestra brassicae*, and from two mosquito species, *Aedes aegypti* and *Culex molestus*, were cultured in the media from which one of amino acids had been omitted. The growth rate in 8-day cultures was examined, and further continuous subculturing was conducted up to 10th passage with amino acid-deficient media. Arginine, cystine, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine were found to be essential for the growth of all the cell lines examined. α -Alanine, β -alanine, asparagine, aspartic acid and glutamic acid seem to be non-essential for the growth of the cells. The role of glycine could not be determined.

INTRODUCTION

In order to improve or simplify the composition of culture media for insect cells, it is very important to know the amino acid requirements of insect cells cultured *in vitro*. The information on this problem has been limited (LANDUREAU and JOLLÈS, 1969; HINK et al, 1973; MITSUHASHI, 1976a,b), although there have been several reports on the utilization of free amino acids by insect cells in cultures (GRACE and BRZOSTOWSKI, 1966; LANDUREAU and JOLLÈS, 1969; HINK et al, 1973; CHAO and BALL, 1976; MITSUHASHI, 1976a,b, 1977; MITSUHASHI et al, 1978). The utilization of amino acids by the cells, however, does not necessarily indicate the amino acid requirements, because certain amino acids have been reported to be essential although they were not consumed in large quantities, and *vice versa* (MITSUHASHI, 1976a,b).

There is an obstacle to the study of the amino acid requirements of insect cells cultured *in vitro*. This is due to the fact that vertebrate serum or insect hemolymph is indispensable for cultured insect cells and these contain free amino acids. It is, therefore, extremely difficult to delete any amino acid entirely from a culture medium containing vertebrate serum or insect hemolymph. In the present study, the amounts of free amino acids derived from sera were reduced by decreasing the percentage of the serum added to the medium. By the use of such medium, the essential amino acids could be determined.

MATERIALS AND METHODS

Cell lines. Continuous cell lines from the embryos of the yellow fever mosquito, *Aedes aegypti* (PELEG, 1969; PELEG and SAHAR, 1969), from the adult ovaries of the Japa-

nese cellar mosquito, *Culex molestus* (KITAMURA, 1970), and from the pupal ovaries of the cabbage armyworm, *Mamestra brassicae* (MITSUHASHI, 1977), were used. These cells were maintained in MGM-431 medium (MITSUHASHI, 1972) by subculturing them once a week.

Experimental media In order to minimize contamination by free amino acids from the serum and the serum protein, fetuin was removed from the MGM-431 medium and the amount of fetal bovine serum was halved. Amino acids were omitted one at a time from such media.

Experimental culture. Cells were harvested from stock cultures and washed with sterile Ringer-Tyrode solution by means of centrifugation at $600\times g$. Then the cells were suspended in experimental media and distributed into culture vessels. Square MA-30 bottles were used for short-term cultures, and square MB-25 bottles for long-term cultures. For short-term cultures, the cells were cultured for 8 days at 25°C and the multiplication rate was recorded. Long-term cultures in amino acid-deficient media were also attempted in order to avoid the effects of carry-over amino acids. For this, cells were cultured in experimental media, and subcultured at least once a week with 1:2 split ratio. The cultures were continued until the 10th passage.

Cell enumeration. In short-term cultures, cell counts were made on days 0 and 8. For counting cells, a square grid having sides of $50\text{ }\mu\text{m}$ was projected onto a randomly selected field of a culture bottle by means of the data illumination system of a Nikon Inverted Microscope Type MD, and the cells enclosed in ten squares were counted. Five locations were chosen randomly for each bottle.

RESULTS

The results of short- and long-term cultures are summarized in Figs 1 and 2, respectively.

Growth rates were most impaired in cystine- and glutamine-deficient media. Omission of arginine, asparagine, isoleucine, leucine, lysine, methionine, serine, tyrosine and valine caused considerable decrease of growth rate in the cultures of all the cell lines examined. Deletion of aspartic acid, glutamic acid, glycine, phenylalanine, proline, tryptophan, and threonine had little effect on the growth rates of all the cell lines. Omission of β -alanine tended to promote cell growth.

Most of the amino acids were found to be necessary for the continuous subculturing of the cells. The amino acids apparently unnecessary for the growth of cells were α -alanine, β -alanine, asparagine, aspartic acid, and glutamic acid. Without them, the cells could be subcultured for more than 10 passages. In the absence of the other amino acids, the cells could be subcultured several times; however, the cells eventually died. The deficiency of these amino acids inhibited subculturing more than 5 times. Among them, the effect of cystine deficiency was very marked. Without cystine, cells immediately deteriorated. Glycine was an exceptional case. When glycine was not added to the medium, the *Aedes* cells could be subcultured more than 10 times, the *Mamestra* cells 8 times, and the *Culex* cells 7 times. For the growth of the *Aedes* cells, glycine seemed to be unnecessary but the growth was significantly retarded. In the cultures of the *Mamestra* and the *Culex* cells, growth was so retarded that subculturing became impossible after the 8th and 7th passages, respectively. However, many cells survived after the final subculturing. It might be, therefore, possible to continue subculturing these cells

Fig. 1. Growth rates of 3 insect cell lines in single amino acid deficient media. The growth rate is expressed as the ratio of cell density after 8-day culture to that of 0-day (mean $\pm$ standard deviation). Circles, NIAS-Mb-19 cell line derived from pupal ovaries of *M. brassicae*; triangles, a cell line derived from adult ovaries of *C. molestus*; squares, a cell line derived from embryonic tissues of *A. aegypti*.

beyond the 10th passages if the intervals of subculturing were prolonged.

DISCUSSION

The essential amino acids for the growth of the three insect cell lines examined were arginine, cystine, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. These contain five amino acids in addition to those essential for intact insects. For the growth of intact insects in general, ten amino acids, namely, arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine, were said to be essential (GILMOUR, 1961). Among *A. aegypti*, *C. molestus* and *M. brassicae*, only *A. aegypti* has been studied nutritionally. According to GOLBERG and DE MEILLON (1948), arginine, cystine, glycine, histidine, isoleucine, leucine, lysine, methionine, threonine and trypto-

Fig. 2. Passage numbers of 3 insect cell lines in single amino acid-deficient media. Solid bar, NIAS-Mb-19 cell line derived from pupal ovaries of *M. brassicae*; hollow bar, a cell line derived from adult ovaries of *C. molestus*; hatched bar, a cell line derived from embryonic tissues of *A. aegypti*.

phan are required for the larval growth of *A. aegypti*. This mosquito also requires either phenylalanine or tyrosine. The status of valine was not determined in their studies. When these results are compared with the requirements of amino acids by *A. aegypti* cells in cultures, it is evident that the cultured cells required qualitatively more amino acids than intact mosquitos.

The amino acids essential for the growth of mammals are the same as those for intact insects. In mammalian cell cultures, cultured cells also require qualitatively more amino acids than intact animals. Mouse fibroblast L cells and several human cells reportedly require 13 amino acids for their growth (EAGLE, 1955; EAGLE et al, 1956, 1957). These are cystine, glutamine and tyrosine in addition to the ten amino acids essential for intact animals.

The fact that cultured cells require more amino acids than intact animals may indicate active multiplication of the cells. The cultured insect cells might synthesize amino acids not essential for intact insects. However, even if the cells can synthesize these amino acids, the quantity of the products might not be enough to actively multiply the cells. On the other hand, in intact insects, cells usually do not multiply frequently, and, therefore, very low synthesizing activity might support the survival and growth of the cells *in vivo*.

An alternative possibility is that the cultured cells cannot synthesize some of the amino acids not essential for intact insects. For this several cases may be considered. The first is that the tissues or organs from which the cell line was established did not originally possess the ability to synthesize these amino acids. The second is that during the process of establishing cell lines, cells which cannot synthesize these non-essential amino acids were selected. The third is that mutation occurred during the process of establishing cell lines and the enzyme systems which are responsible for synthesizing these non-essential amino acids were lost.

At present, no evidence has been obtained to support either of these assumptions.

The determination of non-essential amino acids was not conclusive in the present study, since it was not possible to delete certain amino acids entirely from the culture medium. Sera totally lacking free amino acids can be prepared by dialyzing or ultrafiltrating the sera. However, even if such treated sera are used, it is still impossible to omit certain amino acids entirely. This is because free amino acids are produced from the treated sera, probably due to the degradation of serum protein. It is, therefore, necessary to use a protein-free medium for the determination of non-essential amino acids. Protein-free culture media which can support cell growth are not available at present and the development of such media is necessary. α -Alanine, β -alanine, asparagine, aspartic acid, and glutamic acid are, therefore, said to be non-essential or essential only in very small amounts for the growth of the cells.

The role of glycine could not be determined for insect cell lines in general, because each cell line showed different requirements of glycine.

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