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Regulatory Functions of Amino Acids and Their Combinations in Prokaryotes and Tissues of Higher Organisms

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Abstract—The influence of individual amino acids and their combinations on the growth of cultures of *Escherichia coli* O75 and the proliferation of the culture of organotypic tissues was studied. Based on their effect on the colony size, all amino acids were divided into neutral, stimulating, and inhibiting with regard to growth. The biological effect of amino acids was associated with the concentration of amino acids and was dependent on the heterogeneity of the bacterial population. The activity of two amino acids, which occur in pairs, was different from the activity of individual compounds. Some combinations of amino acids, which cause recognizable effects in bacterial cultures, also had an effect on the tissue cultures of mammals. It was shown that the combinations of amino acids that constitute peptides were less active than the original peptide.

Keywords: amino acids, peptides, *Escherichia coli*, organotypic tissue culture, growth regulation
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INTRODUCTION

The study of the mechanisms underlying the regulation of the major homeostatic functions of the body is one of the priority areas of modern biology and medicine. The manifestation of life is a constant process of the metabolism and reproduction of genetic information by means of various regulatory factors. The study of regulatory mechanisms in both unicellular and multicellular systems aids in the understanding of the genesis of the development of an individual organism, the mechanisms that govern cell differentiation and specialization, the regulation of specialized tissues, and the propagation of genetic information. In the last few decades, extensive evidence has showed that amino acids are not only monomeric building blocks of protein molecules, but also that amino acids alone can modify the expression of target genes and serve as signaling molecules.

In the 1950s, it was found that cultured tissues accumulate isotopically labeled amino acids to varying degrees depending on the type of tissue (Booth et al., 2005). It has been found that, in intestinal mucosa, testis, spleen, and kidney tissue culture, the inhibitory effects of neutral amino acids increased with the length of their hydrocarbon side chain. The effect of amino acids with basic radicals was different in various tissues. In the intestinal mucous membrane and testes, they caused the inhibition of development. Proline produced the significant inhibition of the cerebral cortex and spleen tissues (Makinoshima et al., 2002). Over the last two decades, there has been growing interest in studying the effect of encoded amino acids on cellular processes. Thus, during the study of specific and nonspecific resistance, it was revealed that lysine, arginine, glutamic and aspartic acids, and tryptophan have different immunostimulating, phagocytosis-stimulating, and detoxifying properties (Belokrylov et al., 1995). The results of the study of a preimplantation pig embryo showed that the accumulation of amino acids depends on the stage of embryo development (Booth et al., 2005). There is data available on the effect of arginine on the processes of cell proliferation and apoptosis (Philip et al., 2003). The apoptosis-inducing effect of arginine was confirmed for retinal cell cultures from postnatal rats. (Kim et al., 2004) Vascular smooth muscle cells also respond to arginine through an increase in the expression of the *fas* gene (Bing et al., 2002; Trulsson et al., 2004). In the study of amino acids with branched side chains, it was revealed that the concentration of leucine induced an increase in DNA synthesis in rat hepatocyte culture (Kimura et al., 2005). It has been shown in several papers that different groups of amino acids stimulate or inhibit (by apoptosis) cell proliferation in tissue cultures of various origins (Chalisova et al., 2001, 2002, 2011). It was revealed that some short peptides can also have a stimulating effect on proliferation (Khavinson et al., 2005, 2011a, 2011b; Anisimov et al., 2011; Fedoreyeva et al., 2011). When conducting a comparative analysis of incidence of effects of amino acids and peptides on cellular processes, we found that the most effective peptides have both stim-

ulating and inhibiting amino acids (Chalisova et al., 2007, 2011; Fedoreyeva et al., 2011). In recent years, due to advances in molecular biology, genes have been identified that are expressed under the following conditions of amino-acid deficiency:

(1) genes that encode the plasma-membrane transporters of amino acids;

(2) genes that encode transcription factors, including ATF3 (activating transcription factor), C/EBPa (CCAAT/enhancer-binding proteins), and c-jun;

(3) genes that encode ribosomal proteins or genes involved in signal transduction. The most studied of these include asparagine synthetase gene (ASNS), the gene of the CHOP (C/EBP homologous protein) nuclear protein from neutral amino acid transport System A mediated by products of gene SNAT2 (serotonine-*N*-acetyltransferase) that belong to the C/EBP family of transcription factors (Chalisova et al., 2011).

Much less is known about the regulatory functions of amino acids in microbial cultures, although new results have been obtained in recent years. It has been found that the amino acids have a variable effect on the growth of different species of bacteria, and even strains. For example, aspartic acid inhibited the development of *E. coli* M-17, but stimulated the growth of *E. coli* BL. Histidine inhibited the growth of both Escherichia, but had no effect or slightly stimulated the growth of *S. enteritidis*. Phenylalanine stimulated the growth of *E. coli* BL, but had no effect on the growth of *E. coli* M-17; valine stimulated the growth of *E. coli* M-17 and did not affect the growth of a number of other strains, but inhibited the growth of *E. coli* K-12; and tryptophan stimulated growth of *E. coli* BL, but had no effect on the growth of *E. coli* M-17 and *S. enteritidis* (Vakhitov et al., 2006). The differences in the effects of the amino acids on closely related strains of microorganisms may be due to strain-specific mutations. Thus, the valine inhibition of *E. coli* K-12 growth is associated with a lack of one of the three enzymes of acetohydroxy acid synthase (Lawther et al., 1981).

The regulatory effect of amino acids was indicated by the impact of cholera toxin on production by three strains of *Vibrio cholerae* (*cholerae* 569V, *eltor* 1310, *cholerae* O139 MO45). Surprisingly, it was found that the toxin production of each of these strains is determined by a set of individual amino acids (Ovsova et al., 2003). There are other examples of the effect of combinations of amino acids, but these issues have not yet been systematically explored.

The objective of this study was to examine the effect of amino acids and their combinations on the growth of bacterial colonies and organotypic cultures of mammalian tissues. It can be assumed that, during evolution, this regulation was the basis for the development of regulation that uses more complex molecules, i.e., proteins and peptides.

MATERIALS AND METHODS

Escherichia coli O75 were used in the experiments. The microorganism culture was grown to stationary phase in M-9 glucose-mineral medium in flasks on a shaker and plated in Petri dishes containing M-9 agar medium with the addition of amino acids at concentrations of 0.5, 5, 50 and 500 μM. For some amino acids, the concentration was 1000 and 2000 μM. The medium without the addition of amino acids was used as a control. Plated dishes were incubated at 37°C for 40 h. All experiments were repeated three times.

After incubation, inoculum was spread on the plates and individual colonies were counted. The size of the colonies was estimated from the photographs. Petri dishes were photographed by specialized digital camera MIKS-480, and the images were analyzed using software VideoTesT–Morpho 3.2. Then, the average diameter of colonies on the control plates and on the plates with the addition of amino acids was estimated. The area index (AI) was expressed in arbitrary units as the ratio of the area of the colonies on the plate with the amino acids over the area of the colonies on the control plate. The AI value was expressed as a percentage, with the AI value of the control taken as 100%. The effects of amino acids were evaluated based on these data.

The second series of experiments were carried out in an organotypic culture with 800 explants of heart, spleen, liver, and cerebral cortex of adult Wistar rats. The isolated organs were cut into small pieces of about 1 mm^3, then planted in Petri dishes with collagen-coated (bottom) surfaces. Growth medium consisted of Igla medium (35%), Hanks solution (35%), fetal calf serum (25%), and chicken embryo extract (5%). Glucose (0.6%), insulin (0.5 U/mL), and gentamicin (100 U/mL) were added into the medium. Studied compounds were added to the growth medium at concentrations of 0.5–2 μM.

Culture medium with the studied concentration of amino acids (3 mL) was added to Petri dishes with experimental explants. Culture medium (3 mL) was added to Petri dishes with control explants. Thus, explants from the experimental and control groups were developed in the same volume of growth medium. The Petri dishes were placed in an incubator at 37°C. After 3 days, the cultures were studied under a phase contrast microscope. Then, AI was calculated in arbitrary units as the ratio of the area of the explant (with the area of spread cells) to the area of the central zone of the explant. To visualize the explants, a microscope objective with a teleconverter (MTN-13 Alfa-Telecom, Model 10, Russia) was used. The software PhotoM 1.2 was used to calculate the area index of the explants. For each studied compound, 20–25 experimental and 20–23 control explants were analyzed. A Wilcoxon two-sample rank-sum test was used to perform a statistical analysis of the differences. Statistical analysis was conducted using Statistica 7.0 software.

RESULTS AND DISCUSSION

Effect of Individual Amino Acids on Growth of E. coli O75 Colonies

The experimental results (Table 1) showed that all amino acids can be divided into three groups based their effect on the size of the colonies, i.e., neutral amino acids, and stimulators and inhibitors of growth. The effect was manifested as an increase (decrease) in the diameter of Escherichia colonies and, therefore, the AI. In the case when AI was higher than 8%, the effect was considered to be stimulating and, when it was lower than 8%, it was considered to be inhibiting. In all other cases, it was considered neutral. Depending on the concentration, the effect of certain amino acids could be considered as stimulating, inhibiting, or neutral. The effect of other amino acids can be increased or decreased with a change in concentration without reversing its action.

In most cases, alanine (Fig. 1a) and valine (Table 1) were inhibitors of colony growth. The inhibitory effect of alanine increased with increasing concentration. The increase in the concentration of alanine in the culture medium from 0.5 to 2000 μM resulted in a decrease in AI by 26–69% compared to the control group. A slight increase in AI (6%) was only observed at an alanine concentration of 5 μM (Fig. 1a). Methionine, isoleucine, glutamic and aspartic acid, phenylalanine*, and threonine* demonstrated stimulating activity (AI was greater than 8%). The increase in the methionine concentration from 0.5 to 500 μM caused an increase in AI by 16–60% (Fig. 1b). The effect of other amino acids depended on their concentration. Similar to the effect of methionine, serine, lysine, and histidine* also stimulated the growth of the colonies at low concentrations and suppressed it at high levels. The opposite effect was observed for cysteine* and proline (amino acids marked with * are not included in the Table. 1). In low (0.5 μM) and high (500 μM) concentrations, leucine and tryptophan did not affect the size of the colonies (AI was within ±8% of initial area); however, in concentrations of 5 and 50 μM stimulated their growth. In general, the effect of arginine, in general was opposite to that of leucine and tryptophan.

A population of microorganisms is not homogeneous but is instead composed of subpopulations with different physiological properties (Makinoshima et al., 2002). For this reason, in some cases amino acids did not affect the whole population, but only a part (subset) of the population. They may act differently on different subpopulations, which resulted in the increased heterogeneity of the size of the colonies. As a result, stimulating (inhibiting) effects of amino acids in different experiments could vary depending on the initial ratio of subpopulations. For example, at a concentration of 0.5 μM, valine could manifest as either an inhibitor (AI = −11%) or stimulator of the growth (AI = +18%) depending on the initial structure of the population.

Table 1. Effect of different concentrations of amino acids and their combinations on the size of the *Escherichia coli* O75 colonies

Amino acids and their combinations	Concentration of amino acids, μM			
	0.5	5	50	500
Arg	+7	−10	−23	
Ser	+14	+33	−90	–
Arg+Ser	+53	+23	−62	–
Arg	+99	+71	+12	*+25*
Val	−11	−54	+17	*−4*
Arg+Val	−7	0	+4	*−10*
Asp	+10	+10	+13	+58
Met	+1	*+16*	+57	+60
Asp+Met	+14	*+66*	+75	+94
Asn	*−6*	+22	+10	+30
Val	*−6*	+14	+11	+6
Asn+Val	*+20*	+31	+30	+22
Asn	*−11*	−3	+19	+22
Leu	*−3*	+35	+71	−9
Asn+Leu	*+35*	+18	+53	+31
Lys	*+1*	−2	*−9*	*17*
Pro	*−22*	−8	*−9*	*−21*
Lys+Pro	*+11*	−1	*+35*	*+61*
Lys	+38	−8	−22	−14
Trp	−4	+19	+8	−7
Lys+Trp	−3	−4	−20	−7
Val	+18	*−53*	*−4*	+2
Ile	+14	*+24*	*+30*	+52
Val+Ile	+18	*+52*	*−28*	*+89*
Val	−25	−41	−7	*−9*
Glu	+5	−20	+15	*+52*
Val+Glu	−14	−14	+4	*+75*
Leu	+8	*+57*	+30	+2
Lys	+26	*−4*	−20	−10
Leu+Lys	−3	*−9*	−12	−29
Met	*+12*	+121	+59	+40
Glu	*+1*	+106	+160	+210
Met+Glu	*+69*	+93	+141	+130

Note: The effect of methionine and cysteine at concentrations of 2000 μM is not shown in the table.

Figure 2 shows the effect of valine on populations with different initial structure (Figs. 2a, 2b). In the first case (Fig. 2a), the initial population consisted of the cells that form large colonies (about 2 mm in diameter) and cells that form average-sized colonies (1.7 mm in diameter). The addition of valine to culture medium at low concentrations (0.5 μM) resulted in a significant change in the composition of the population (Fig. 2b), i.e., small (0.5 mm) and larger colonies (1.3 mm) were

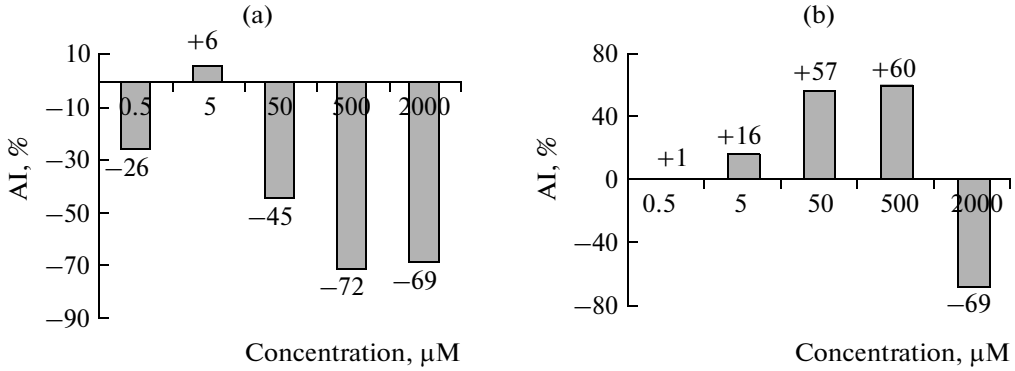

Fig. 1. Dependence of (a) alanine and (b) methionine effects on concentration in medium.

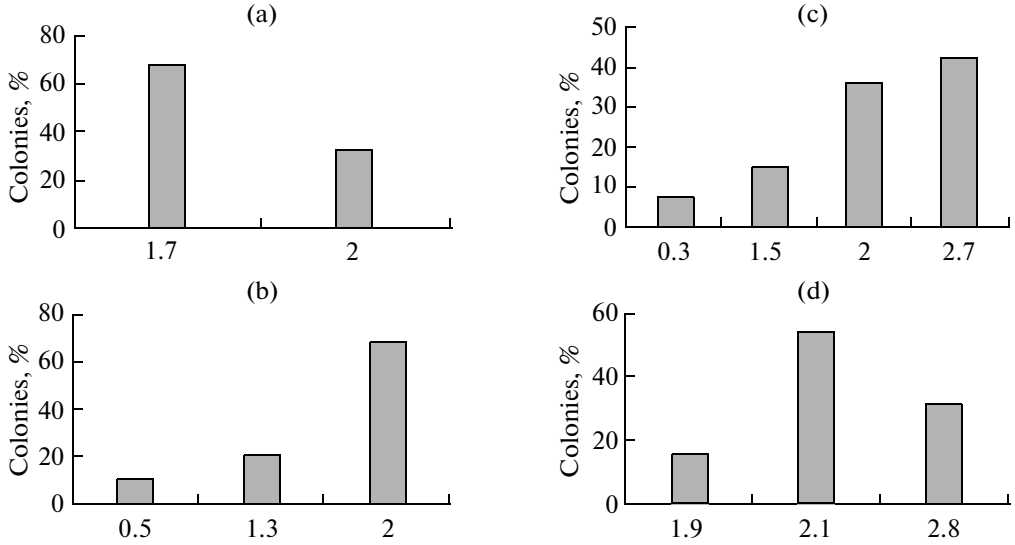

Fig. 2. Valine effect and heterogeneity of population of *E. coli* O75. Figure shows histogram of distribution of populations by size in the control (a, c) and in the medium with 0.5 μM valine (b, d).

observed. In addition, the percentage of colonies with diameters of 2 mm increased. In general, an inhibitory effect and an increase in the dispersion of the size of the colonies by a factor of 13 (compared to the controls) were observed.

In the case when valine at a concentration of 0.5 μM stimulated growth of *E. coli* (Figs. 2c, 2d), the dispersion decreased.

Effect of Combinations of Amino Acids on Growth of Bacterial Colonies

In cultures of bacteria and tissue fluids, an effective agent is usually not individual amino acids, but combinations of amino acids. As an approximation, these combinations can be considered as models of biologically active peptides that consist of the same amino acids. Table 1 shows that the effect of paired combinations of amino acids was different from that of the individual compounds. For example, when two neutral amino acids, asparagine and valine, were simultaneously added to the medium at a concentration of 0.5 μM, they stimulated the growth of the O75 strain. It is interesting to note that they also had a similarly pronounced stimulating effect on myocardial tissues (Table 2). The combination of inhibiting asparagine and neutral leucine (0.5 μM) led to an increase in AI by 35%. In the liver, however, asparagine stimulated and leucine inhibited proliferation, and their combination had marked inhibitory effect (Table 2). At concentrations of 50 μM, as well as methionine and cysteine (at 2000 μM), lysine and proline inhibited the growth of *E. coli* O75 colonies when applied alone, but together they had a stimulating effect.

The addition of neutral amino acid to stimulating amino acid could improve the stimulating effect. For example, at a concentration of 500 μM (AI = +2%), neutral valine with a stimulant isoleucine (AI =

+52%) increased the AI by 89%. In other cases, a similar combination had the opposite effect of inhibiting the growth of *E. coli* (e.g., arginine and valine at a concentration of 500 μM, as well as lysine with leucine at concentration of 5 μM). Combinations of two growth-stimulating amino acids, such as asparagine and methionine (5 μM), as well as methionine and glutamic acid (0.5 μM), had a greater stimulating effect than each of the individual amino acids. The increase in the stimulating effect when inhibitors (valine at concentration of 5 μM and lysine at concentration of 500 μM) and stimulants (isoleucine at concentration of 5 μM and proline at concentration of 500 μM) were added to the medium should also be noted.

Effect of Combinations of Amino Acids on Growth of Tissue Cultures

Experiments with organotypic tissue cultures demonstrated that the same amino acid combinations, which had effect on bacterial cultures, were efficient in the mammalian tissue cultures (Table 2). For myocardium, the active combinations were combinations of arginine, which stimulated proliferation (AI was 30% higher compared to the controls), and valine, which had no effect on the tissue (AI was similar to that of controls). The combined effect of these amino acids resulted in an increase in the AI of explants by 48%. A similar effect was observed for the combination of stimulants (asparagine and valine) with neutral amino acids (valine, glutamic acid, and isoleucine). In spleen tissue, a pronounced stimulating effect (AI of explants increased by 38%) was caused by a combination of amino acids, which stimulated (leucine) and inhibited (lysine) proliferation.

In liver tissues, the combination of stimulating and inhibiting amino acids showed an inhibitory effect on proliferation. For example, the stimulant asparagine with the inhibitor leucine lowered the AI by 55% compared to the controls. This may be explained by the fact that the high regenerative capacity of the liver tissue is associated with the rapid removal of many cells, which is critical for maintaining the cellular balance.

Thus, these results provide the basis for creating new effective dipeptides that may be used in the treatment of infectious diseases and may help to improve the regenerative capacity of the tissues. It should be noted that, according to our experiments, the combinations of amino acids that constitute peptides were less active than the original peptide. Thus, peptide cortagen facilitated an increase in AI by 27% relative to the controls, while a mixture of amino acids that constitute the peptide provided a less pronounced stimulatory effect on AI; this index increased by only 18%. Apparently, the peptide bond is necessary for more effective interaction, perhaps through the targeted delivery of the peptide to the tissue.

Table 2. Effect of individual amino acids and their combinations on growth of explants from rat tissues

Pairs of amino acids	0.5 μM		
Arg+Val	+30*		+2
(myocardium)		+48*	
Asn+Val	+20*		+2
(myocardium)		+47*	
Val+Glu	+32*		+2
(myocardium)		+59*	
Lys+Trp	+30*		−10
(myocardium)		+60*	
Val+Ile	+20*		+2
(myocardium)		+38*	
Leu+Lys	+8		+28*
(myocardium, spleen)		+70*	
	+25*		−32*
		+38*	
Asn+Leu	+30*		−20*
(liver)		−55*	

Note: * $p < 0.05$ compared to controls.

These results indicate that prokaryotes and eukaryotes have a similar regulation system. They also support the concept of the evolution of living matter before genes, i.e., when amino acids played an important role in regulatory mechanisms. The appearance of RNA, then DNA, which encode complex protein and peptide compounds, allowed living matter to step up to a new level of regulation. Thus, during evolution, peptide regulation apparently replaced less efficient regulation with amino acids and combinations thereof. However, it has not replaced it completely, but rather complemented it with other mechanisms to perform the most important functions.

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