Role of Amino Acids in Postexponential Growth

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ABSTRACT

TOENNIES, GERRIT (Temple University, and The Institute for Cancer Research, Philadelphia, Pa.). Role of amino acids in postexponential growth. *J. Bacteriol.* 90: 438-442. 1965.—It was shown earlier that depletion of different essential amino acids from the medium of growing *Streptococcus faecalis* leads to the formation of cell types of different compositions in terms of cytoplasm, wall, and membrane. The findings presented now indicate that these postexponential effects can be induced by very low concentrations of incomplete amino acid complements, that cystine and methionine have major and separate effects, and that these effects may be modulated differentially by the presence of different combinations of other amino acids.

Different amino acids essential for the growth of *Streptococcus faecalis* elicit different growth curves (Toennies and Gallant, 1949b). As long as all nutrients are available in nonlimiting concentrations, bacterial duplication proceeds at a constant rate (exponential phase). However, when a limited quantity of an essential amino acid has been used up, there is no cessation of growth but, instead, a protracted period of gradually subsiding postexponential growth (Toennies and Shockman, 1953). The principal products of this phase of growth are wall substance, membrane substance, and nucleic acids (Toennies, Shockman, and Kolb, 1963). The absolute and relative extent of these changes appears to depend on the composition of the incomplete complement of essential amino acids which are present in the medium during postexponential growth (Shockman, Kolb, and Toennies, 1958; Shockman, 1959). Although absence of one or several of the essential amino acids precludes net protein synthesis, protein turnover or transport during postexponential growth is probable: a large gain of membrane protein (in the case of valine-deprived cells) appears to be counterbalanced by a corresponding loss of cytoplasmic protein (Toennies et al., 1963). To illuminate further facets of the postexponential amino acid effect, I have examined quantitative requirements and the qualitative role of individual amino acids.

MATERIALS AND METHODS

S. faecalis ATCC 9790 was grown in a synthetic medium buffered at pH 6.5 with 0.3 M phosphate (Toennies and Shockman, 1953). In the current procedure, all ingredients (asparagine is no longer

included) were combined in solution at room temperature, and the basal medium was sterilized by passing it through a 0.45- μ Millipore filter. Growth of cultures was measured by determining turbidity spectrophotometrically (Coleman No. 14 spectrophotometer) at 675 m μ in calibrated round cuvettes (Toennies and Shockman, 1953), and the optical density readings were converted to AOD (adjusted optical density) values, which are proportional to bacterial concentration (Toennies and Gallant, 1949a). In the interest of easy interpretation of data, AOD values in the present studies were translated into concentrations of bacterial dry weight by assuming that one AOD unit, measured under the conditions specified, corresponds to 0.39 μ g/ml. This value is derived from previously reported (Shockman et al., 1958; Shockman, 1959) and additional unpublished measurements which gave values of 0.38 to 0.40 μ g for different cell types which, in composition, cover cytoplasm-wall ratios from 0.9 to 3.5, and wall-membrane ratios from 4.4 to 1.0 (Toennies et al., 1963; present paper, Table 3).

In studying the effect of nutrients under postexponential conditions, cells were grown at exponential rate (duplication time about 30 min) in nonlimited medium to dry weight levels of 400 to 800 μ g/ml. Growth was arrested by chilling, and, after centrifugation at 2 C, the cells were washed aseptically three or four times with iced wash or rump medium. This wash medium contained all normal ingredients except those which were experimental variables, i.e., in the present series, cystine and the ten essential amino acids not required for wall synthesis. This procedure was adopted to obtain thorough removal of those amino acids which were shown to be effective in minute concentrations (Table 1A), without otherwise impairing the responsiveness of the cells by excessive washing with cold water. Evidence of such impairment was seen in quantitative varia-

TABLE 1. *Postexponential amino acid effects*

Supplement	Amt ^a	Response ^b		
		43 $\mu\text{g}/\text{ml}^c$	146 $\mu\text{g}/\text{ml}^c$	143 $\mu\text{g}/\text{ml}^c$
A. Nine amino acids ^d	μg			
	400	48	168	
	200	45	161	
	100	41	150	
	25	43	156	
	6.25	42	150	
	1.56	45	146	
	0.78	39	152	
	0.39	39	143	
	0.20	41	112	
	0.10	36	76	
	0.050	38	60	
	0.025	37	58	
	0.013	35	60	
	0	28	56	
B. Methionine ^d	100			137
	10			159
	1			127
	0.1			70
	0			62

^a Amounts expressed as micrograms per milliliter of postexponential culture. The amounts of each of the nine amino acids used are shown.

^b The figures show estimated maximal postexponential gain, as micrograms (dry weight) per milliliter.

^c Estimated initial dry weight concentration of culture.

^d See text.

tions of response among separate experimental series and is supported by recent observations on removal of bacterial enzymes by cold-water washing (Neu and Heppel, 1964). All cells harvested for analysis were washed four times with iced water and lyophilized.

The analytical methods used are detailed in the footnotes to Table 3. Some of the data shown in Table 3 were obtained while methods suitable for the current investigation were being developed (Toennies and Kolb, 1964; Toennies et al., 1965), and are now presented within their physiological framework. [In Table 4 of the paper by Toennies and Kolb (1964), bacterial specimens I-VII refer to, respectively, Transfer cells A-D and log, valine-deprived, and threonine-deprived cells.]

RESULTS

When a growing culture of *S. faecalis* 9790 was arrested in exponential phase by chilling and, after washing, was transferred to a culture medium free from the essential amino acids not required for wall synthesis (arginine, histidine, isoleucine, leucine, methionine, phenylalanine,

threonine, tryptophan, tyrosine, and valine), further incubation led in about 24 hr to a nearly 100% gain in wall substance (Shockman et al., 1961). In the present study, a similar medium, containing all normal components except the above ten amino acids, was supplemented with a mixture of nine of these acids (omitting threonine) over a wide range of concentrations. Washed exponential cells were transferred into these media, and the resulting suspensions were incubated. Photometric turbidity readings taken periodically during the next 40 hr attained maxima, followed by slow decreases, after 10 to 30 hr of incubation. Table 1A shows the maximal dry weight gains which were attained by two initial levels of exponential cells in response to decreasing concentrations of the amino acid supplement. The postexponential gains which occur in the complete absence of the amino acid supplement were significantly increased by amino acid levels as low as 0.1 $\mu\text{g}/\text{ml}$ or less, depending on the initial culture density.

Further study showed that the postexponential effect varies with the composition as well as the quantity of the amino acid supplement, but that most (though not all) of the effect appears to be contributed by one single amino acid, methionine (Table 1B). At concentrations higher than 10 $\mu\text{g}/\text{ml}$, the methionine effect consistently tends to be lower.

A new question arising from the discovery of the methionine effect concerns cystine. A special postexponential role of cystine among the non-essential amino acids was noted (Shockman et al., 1961) before the dominant effect of methionine among the essential ones was recognized. It was found that a limited amount of wall synthesis can be achieved in a minimal "wall medium" that contains no amino acids other than those that are wall components (lysine, alanine, aspartic and glutamic acid) and, in addition, cystine. Cystine can be formed by *S. faecalis* from methionine in the presence of pyridoxamine (Speck and Pitt, 1947), and since this factor was present in our postexponential media, methionine, in addition to its own specific function, was expected to provide for the postexponential function of cystine, even though cystine would presumably not replace methionine. Experimental evidence did not conform to this expectation. Because the methionine-cystine conversion of *S. faecalis* appeared to be adaptive rather than constitutive, a crucial test was performed with a culture optimally adapted to growth in the absence of cystine (Table 2). The autolytic effect seen in the absence of cystine is little diminished by even 1,000 μg of methionine per ml, but strongly counteracted by 40 μg of cystine per ml.

TABLE 2. Postexponential effects of cystine and methionine in the absence of other essential nonwall amino acids*

Cystine $\mu\text{g/ml}$	No methionine			Methionine (10 $\mu\text{g/ml}$)			Methionine (100 $\mu\text{g/ml}$)			Methionine (1,000 $\mu\text{g/ml}$)		
	2 hr†	10 hr	20 hr	2 hr	10 hr	20 hr	2 hr	10 hr	20 hr	2 hr	10 hr	20 hr
0	24	-25	-47	34	-9	-34	36	0	-25	47	0	-22
40	33	54	36	47	91	76	58	98	89	62	107	109
200	36	60	49	45	91	85	45	96	103	65	130	145

* The primary exponential culture was grown in the absence of cystine, after prior adaptation to grow (duplication time, 40 min) under these conditions, and washed with rump medium. The data show estimated weight gains or losses, expressed as micrograms per milliliter, of postexponential cultures initially at 181 $\mu\text{g/ml}$.

† Hours of postexponential incubation at 38 C.

FIG. 1. Growth curves of transfer cells B, C, and D. The arrows indicate the harvesting points.

To study the results of the postexponential action of cystine and methionine, exponential cells (A) were grown in nonlimited medium to 800 $\mu\text{g/ml}$, washed as described, and samples were transferred to methionine- and cystine-free rump medium (B), rump medium containing 200 μg of cystine per ml (C), and to rump medium containing 200 μg of cystine and 10 μg of methionine per ml (D). The initial cell concentration in media B, C, and D was about 160 $\mu\text{g/ml}$. Each suspension was incubated until it reached its approximate maximal turbidity, as established by pilot experiment. After chilling and centrifugation, each crop was washed four times with iced water, a portion of the primary culture (A, which had previously been washed with rump medium) was also washed with water, and all four crops, designated as transfer cells A, B, C, D, were lyophilized for further study. The growth pattern of these cultures is shown in Fig. 1, and analytical characteristics of the crops are in Table 3. For comparison, the previously described three cell types (Toennies et al. 1963), with some new data,

have also been included. The estimates of overall cell composition are presumably improved because of better nucleate values (Toennies et al., 1965), but remain subject to difficulties connected with the measurement of cellular protein (Toennies and Feng, 1965) and possible variations in the composition of different wall and membrane fractions.

DISCUSSION

The new results show again (Shockman et al., 1961) that postexponential events represent a competition between synthetic and degradative (lytic) processes. During 3 hr, the B cells appear to achieve one-half as much postexponential wall synthesis as the C cells do in 22 hr; however, in the absence of cystine (B cells), the process is choked off by the ascendancy of lysis. The presence of cystine (C cells) delays overt lysis and permits the formation of much membrane substance, on the basis of lipid phosphorus data. The composition of the harvested culture resembles that resulting from valine depletion. The net effect of the presence of 10 μg of methionine per ml (D cells) is a further suppression of lytic tendencies and a resultant culture which resembles that formed after threonine depletion in that the postexponential gain of wall substance approaches 400%, while the gain in membrane substance does not exceed 50%. However, it must be noted in this connection that the valine and threonine depletion cultures do not differ in respect to environmental cystine and methionine (both of which are present in standard concentrations), even though in one case the cell composition resembles that produced by cystine alone, and in the other case that resulting when cystine and methionine are present. At the same time, the cystine culture (C) differs from the valine depletion culture by

TABLE 3 *Estimated changes in conversion of exponential to postexponential cells*

Determination	Cell type				Log ^a	-Val	-Thr
	A ^a	B	C	D			
Rump medium ^b supplemented with							
Cystine, $\mu\text{g}/\text{ml}$		—	200	200		200 ^c	200 ^d
Methionine, $\mu\text{g}/\text{ml}$		—	—	10		100	100
Harvesting time, hours postexponential.....	0	3	22	45	0	20	40
Whole substance ^e , μg /.....	160	198	249	301	160	232	304
Wall substance ^e , μg	37	69	102	178	30	73	144
Membrane substance ^h , μg	26	30	59	38	24	72	34
Nucleates ⁱ , μg	35	43	45	48	40	42	48
Cytoplasm ^j , μg	62	56	43	37	66	45	78
Cytoplasmic carbohydrate ^k , μg						2	13
Cytoplasmic protein ^l , μg	62	56	43	37	66	43	65
Membrane protein ^m , μg	13	15	30	19	12	36	17
Total protein ⁿ , μg	75	71	73	56	78	79	82
Nitrogen estimates							
5.5% of wall substance, % ^o	1.3	1.9	2.3	3.2	1.0	1.8	2.6
16.6% of nucleates, %.....	3.7	3.7	3.0	2.7	4.2	3.0	2.6
16.0% of protein, %.....	7.5	5.7	4.6	3.0	7.8	5.4	4.6
Total for cell substance, %.....	12.5	11.3	9.9	8.9	13.0	10.1	9.8
Nitrogen found ^p for whole cell substance, %.....	12.2	10.9	9.6	8.4	13.2	10.2	10.1

^a Grown in and harvested from complete medium.^b Complete medium, without cystine, arginine, histidine, isoleucine, leucine, methionine, phenylalanine, tryptophan, tyrosine, and valine.^c Complete medium without valine.^d Complete medium without threonine.^e Estimated from AOD.^f Belonging to, or having been formed from, 160 μg (dry wt) of exponential cells per ml at transfer (B, C, D) or at depletion point (-Val, -Thr).^g Estimates based on wall rhamnose values of 11 to 13%, found by anthrone (Toennies and Kolb, 1934).^h Estimates based on a lipid phosphorus content of membrane fractions of 1.0 to 1.2% (Toennies et al., 1963) and the following lipid phosphorus percentages found in the cells: A, 0.16; B, 0.18; C, 0.28; D, 0.16.ⁱ Nucleotides, ribonucleic acid and deoxyribonucleic acid (Toennies et al., 1965).^j By difference (whole substance — wall — membrane — nucleates).^k (Toennies and Kolb, 1964.)^l Cytoplasm — cytoplasmic carbohydrate.^m One-half of membrane (Shockman et al., 1963).ⁿ Protein of cytoplasm and membrane.^o Of whole substance, calculated for estimated composition.^p (Toennies et al., 1965.)

rapid lysis, and the cystine-plus-methionine culture (D) differs from the threonine depletion culture by an apparent loss in cellular protein, indicative of partial lysis.

The most essential point of the present observations is that they serve to confirm the previous evidence (Toennies et al., 1963) that in the absence of net protein synthesis not only wall substance but also membrane substance can be formed, and that the relationship between these two processes is flexible. The flexibility aspect is emphasized by the fact that major shifts in the wall-membrane ratio appear to be achieved under quite different nutritional circumstances. In view of these findings it may be useful to consider, as a

working hypothesis, the possibility that cystine and methionine play primary roles in the regulation of membrane and wall synthesis, that other essential amino acids can, under conditions not permissive of net protein synthesis, modulate the actions of cystine and methionine, and that these modulations can be effected by catalytic concentrations. Among the more immediate questions now under consideration are these. Why does the methionine-to-cystine process, after functioning efficiently during exponential growth, apparently fail to furnish cystine under postexponential conditions, as suggested by the experiments summarized in Table 2? Does the postexponential cystine effect

involve synthesis of coenzyme A from pantothenate, in view of the apparent stimulation by cystine of the synthesis of lipid-containing membrane substance, and in view of the essential functions of coenzyme A in lipid synthesis as well as in wall synthesis (acetylation of hexoseamines)? Is methionine involved in membrane synthesis by supplying the $-\text{CH}_2-$ group of lactobacillic acid (Chalk and Kodicek, 1961; this possibility was suggested by Marie Vorbeck)? Does methionine in its postexponential function act as a methyl donor in the synthesis of methylated nucleotides (Mandel and Borek, 1961)? Spermine and spermidine, potential components of the structural elements of *S. faecalis* (Guirard and Snell, 1964), may also depend on methionine for their biosynthesis (Tabor, Tabor, and Rosenthal, 1961). Another aspect demanding closer scrutiny is the apparent conversion of cytoplasmic protein to membrane protein.

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