Differential Effects of Amino Acid Deficiencies on Bacterial Cytochemistry*

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The effect of the depletion of different essential amino acids on the composition of the subsequently formed cellular substance of *Streptococcus faecalis* has been studied. Although *de novo* protein synthesis is prevented, formation of wall substance, membrane substance, and nucleic acids can continue. Depletion of valine is followed by synthesis of wall and membrane substance, with little or no nucleic acid formation. When the depleted amino acid is threonine, major amounts of DNA and RNA are formed, and there is more wall synthesis than in the case of valine depletion, while the amount of membrane substance formed is smaller. The data suggest that diverse shifts in cell morphology (cytoplasm-membrane-wall ratio) can be induced in a bacterial organism by withdrawal of different single nutrients. Bacterial membrane content was determined on the basis of its lipid content. The petroleum ether-soluble fraction of the methanol-solubilized lipid, as well as lipid phosphorus, was found to be absent in wall or cytoplasmic substance, and determinations of these two parameters in an isolated specimen of membrane substance and in whole cell substance gave concordant data for the membrane content of the cell types studied.

Observations on differences in cellular morphology related to bacterial culture conditions are numerous, but little is known of mechanisms or the role of specific variables (Wilkinson and Duguid, 1960). When *Streptococcus faecalis* grows in a buffered synthetic medium, the depletion of single essential amino acids has different consequences. Their range may be shown by three examples (Toennies and Shockman, 1959). When the medium contains a limited amount of lysine, its depletion is marked by termination of the exponential growth phase, followed by the prompt onset of bacterial lysis, which runs to completion. When the limiting amino acid is valine, rapid exponential growth also ceases upon depletion. However, it is followed not by lysis but by a slow and protracted increase in the dry weight of the culture, which attains a maximum of nearly 50% after about 20 hours of incubation. When the limiting amino acid is threonine, its depletion, and cessation of exponential growth, are followed in about 40 hours by a weight gain of nearly 100%. Phenomena intermediate in character between the three mentioned are seen after depletion of other essential amino acids.

These observations found a partial explanation when it became evident that lysine is a component of cell wall substance, while valine or threonine are not, and that major quantities of wall substance are being synthesized subsequent to the depletion of valine or threonine. When lysine is depleted, protein synthesis and wall synthesis both become impossible, and the prevalence of degradative processes, which during growth are counterbalanced by synthetic processes, may explain the occurrence of lysis. Among the points remaining obscure was the question of why in the absence of one protein building block (threonine) the postexponential gain is twice as large as in the absence of another (valine). In both cases about 70% of the total weight gain was accounted for by new wall substance (Shockman *et al.*, 1958; Shockman, 1959). After valine depletion there was no significant gain in nonwall nitrogen, while there was a 20% gain, largely accountable by nucleic acid formation, after threonine depletion (Toennies *et al.*, 1959). Apparent analytical inconsistencies led to a consideration of the role of the bacterial membrane in postexponential growth (Toennies *et al.*, 1959).

The amount of wall substance could be determined by quantitative physical isolation (Shockman *et al.*, 1957), but since quantitative isolation of the membrane fraction appeared not to be feasible (Shockman *et al.*, 1962) an indirect method had to be developed for its estimation. Determination of lipid phosphorus proved to furnish a relative index of membrane substance, since phospholipid, a major membrane component, was not found in either wall substance or cytoplasm (Kolb *et al.*, 1962). We have now established (Table I) that the petroleum ether–soluble fraction of the methanol-solubilized lipoprotein lipid (membrane lipid) is also specific for cell membrane, thereby providing another quantitative index. To obtain the absolute amount of membrane substance in a given crop of cells it is necessary to isolate a membrane specimen of sufficient purity and to determine its content of membrane lipid and of lipid phosphorus. When the corresponding percentage values (membrane lipid and lipid phosphorus) are obtained independently for the whole cell substance, its membrane content can be calculated from either the gravimetric membrane lipid data or the colorimetric lipid phosphorus data. In the culture types under study both approaches gave concordant results, as shown in Table I. According to these results, the three different culture types differ greatly in membrane content, just as they do in wall content, but there is no parallelism between the two fractions.

In order to obtain a more complete picture than heretofore available of the distribution of the different cytochemical and morphologic fractions, other data have been utilized. DNA was determined by the diphenylamine procedure according to Burton (1956), and an independent check was obtained by determining thymine microbiologically with the aid of the thymineless mutant of *E. coli* (Miller, 1958) on hot $HClO_4$ hydrolysates (Marshak and Vogel, 1951) of material preextracted with cold $HClO_4$ (Ogur and Rosen, 1950). Total nucleic acid was estimated from the ultraviolet spectra of the cell fractions solubilized by mechanical disruption (Toennies *et al.*, 1959). Nitrogen partition was estimated from total cellular N, wall N (Shockman *et al.*, 1958; Shockman, 1959), membrane N (Shockman *et al.*, 1962), and nucleic acid N (attributing 16% N to the spectrophotometric nucleic acid values). The nitrogen not accounted for by wall, membrane, or nucleic

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TABLE I

LIPID[a] AND LIPID PHOSPHORUS[b] IN DIFFERENT CELL PRODUCTS

	Cell Type	Exponential Phase[c]	Valine Depletion[c] (20 hr. post-exponential)	Threonine Depletion[c] (40 hr. post-exponential)
1	Lipid, as % of dry cells	4.93	10.96	4.22
2	Lipid, as % of membrane	28.3	40.2	36.0
3	Lipid P, as % of dry cells	0.151	0.311	0.111
4	Lipid P, as % of membrane	0.85	1.17	1.00
5	Lipid P, as % of membrane lipid	3.00	2.91	2.78
6	Lipid P, as % of cell lipid	3.06	2.84	2.63
	Membrane as % of cell			
7	calculated from lines 1 & 2	17.4	27.3	11.7
8	calculated from lines 3 & 4	17.8	26.6	11.1

[a] Lipid was determined by refluxing the bacterial product with 95% methanol in order to break lipoprotein complexes, evaporating the methanol extract, dissolving the residue in petroleum ether, extracting the solution with water for removal of nonlipid contaminants (Kolb *et al.*, 1963), and weighing the petroleum ether residue. In order to obtain correct results, the ratio of petroleum ether to lipid (ml/mg) should be above 12, and that of water to petroleum ether (v/v) above 4. [b] Lipid P was determined as described (Kolb *et al.*, 1963). [c] The dry cells were preparations earlier described for exponential and valine-depleted (Shockman *et al.*, 1958) and threonine-depleted (Shockman, 1959; Toennies *et al.*, 1959) postexponential cultures. The membranes were prepared by the lysis of protoplasts obtained by the action of lysozyme on freshly grown cultures suspended in sucrose solution (Shockman *et al.*, 1963).

acid was considered to be cytoplasmic protein nitrogen. The low-molecular-weight precursors of nucleic acid and protein, which represent minor fractions (Toennies and Shockman, 1959; Shockman *et al.*, 1961), were not evaluated separately. Thus, partition of total cellular substance was obtained as follows: DNA: by direct determination (DNA, thymine); RNA: from spectrophotometrically determined nucleic acid minus DNA; cytoplasmic protein: from nitrogen partition; membrane substance: from membrane lipid and lipid phosphorus; wall substance: by quantitative isolation, nitrogen partition, and rhamnose determination.

The results are shown in Table II. Proportional adjustment of each set of values to 100% recovery yields the pattern shown in Table III for the changes in composition and amounts which occur when 100 mg of exponential phase cells develop into about 145 mg of valine-deprived cells or about 190 mg of threonine-deprived cells.

It is apparent that formation of valine- and threonine-deprived cells is accompanied by much wall synthesis. Total (membrane and cytoplasm) protein remains nearly constant, as is to be expected in the absence of one amino acid essential for the formation of protein. Formation of valine-deprived cells is distinguished by absence of DNA synthesis and production of much membrane substance, which appears to involve conversion of cytoplasmic protein to membrane protein. The genesis of threonine-deprived cells is specifically marked by DNA synthesis. RNA synthesis is more pronounced in threonine depletion than in valine depletion. Membrane gain is greater in valine-deprived cells, while wall gain is greater in threonine-deprived cells.

TABLE II

COMPOSITION OF THREE TYPES OF CELLS

	100 mg Exponential Phase Cells Contain (%)	100 mg Exponential Phase Cells Grown in the Absence of: Valine in 20 hr. to 145 mg Substance Containing (%)	100 mg Exponential Phase Cells Grown in the Absence of: Threonine in 40 hr. to 190 mg Substance Containing (%)
Wall	25.5	38.0	44.0
Membrane	17.6	27.0	11.4
Membrane protein	(9.7)	(12.2)	(4.9)
Cytoplasmic protein	30.5	13.4	16.5
RNA	30.2	23.8	20.0
DNA	2.84	2.18	4.05
Thymine	(0.27)	(0.20)	(0.365)
Recovery additive	106.6	104.4	96.0

TABLE III

ESTIMATED CHANGES IN THE CONVERSION OF EXPONENTIAL-PHASE CELLS TO VALINE-DEPRIVED OR THREONINE-DEPRIVED POSTEXPONENTIAL CELLS

	Cell Type: Exponential Phase (mg)	Valine-Deprived (mg)	Threonine-Deprived (mg)
Wall	24	53	87
Membrane	16	37	23
Membrane protein	(9)	(17)	(10)
Cytoplasmic protein	29	19	32
RNA	28	33	40
DNA	2.7	3.1	8.0
Thymine	(0.25)	(0.28)	(0.72)
Total	100	145	190

Some meaning can be given to these data with the aid of two reasonably well-founded assumptions: (*a*) that the average amount of DNA per cell remains constant during the changes under study (Vendrely, 1959), and (*b*) that the average cell of *Streptococcus faecalis* is spherical (Knaysi, 1941).

The thymine values (Table II) confirm the DNA values. The molar ratio of (adenine + thymine)/(guanine + cytosine) = 0.88 ± 0.02, calculated from the thymine and DNA values, resembles the ratio found in some other bacteria (Chargaff, 1959). The DNA values (Table III) suggest that the valine-depleted culture contains 3.1/2.7 = 1.15 times as many cells, and the threonine-depleted culture 8.0/2.7 = 2.96 times as many, as were present at the point of depletion of the respective components. Accordingly, the average unit of the valine-deprived cells is 145/1.15 × 100 = 1.26 times as large (by weight) as the average exponential phase cell, and that of the threonine-deprived cells is 190/2.96 × 100 = 0.64 times as large. Studies with the Coulter counter (Toennies *et al.*, 1961) yield an esti-

mate of 0.50 μ^3 for the volume of the cellular unit of exponentially growing *S. faecalis*. Using this value and the estimates (above) of 1.00, 1.26, and 0.64 for the relative cellular volumes of the three cell types studied, we obtain the spherical diameters shown in Table IV. These diameters and the percentage values for wall and membrane (Table II) permit the calculation of the thickness of wall and membrane as the two outer layers of ideal spheres. The results, shown in Table IV, suggest as a first approximation (no attempt has been made to evaluate relative densities of the components) that wall thickness has doubled in both the valine- and threonine-deprived cells, while membrane thickness has doubled only in the valine-deprived cells. Obviously, other more complex explanations involving "polynuclear" cells or folded membrane structures (Edwards, 1962) cannot be disregarded at this stage. Verification of the conclusions regarding cell size and cell number is complicated by chain formation (Toennies *et al.*, 1961), and new techniques must be devised. The electron-microscopic study of cellular anatomy is also being explored.

TABLE IV
ESTIMATED CELL DIMENSIONS (mμ)

	Cell Type		
	Exponential Phase	Valine-Deprived	Threonine-Deprived
Diameter of cell	984	1072	846
Thickness of outer layer (wall)	43	76	78
Thickness of second layer (membrane)	35	73	27

Quantitative determination of bacterial membrane substance has not so far been possible. The present work presents two procedures, applicable to the organism studied, for this purpose, and indicates that withdrawals of single bacterial nutrients may produce major and diverse shifts in cell morphology. Additional observations seem desirable before concepts of enzyme repression and feedback inhibition (Davis, 1961) or other theoretical tools are applied.

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Accumulation of L-Homolanthionine by an *Escherichia coli* Mutant*

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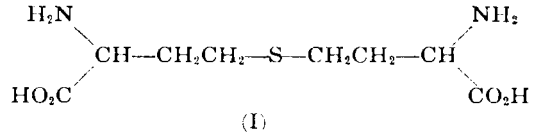
A methionine-requiring mutant of *E. coli* has been found to accumulate in submerged culture a sulfur-containing amino acid, which was isolated and identified as L-homolanthionine. This is the first record of the occurrence of homolanthionine in a living system.

Weiss and Stekol (1951) have reported the synthesis of the three diastereoisomers of *S*-bis(γ-amino-γ-carboxypropyl) sulfide or homolanthionine (I). They had established previously (Stekol and Weiss, 1948, 1949) that (I), without specifying which particular diastereoisomer, can be converted to cystine in the rat. So far, the occurrence of (I) in a living system has not been observed.



(I)

In the course of experiments on amino acid accumulation by mutants of *E. coli* (Huang, 1961), a methionine-requiring auxotroph 31–12 was developed and found to accumulate significant amounts of a sulfur-containing amino acid which resembled cystathionine in its behavior upon paper chromatography. The material was concentrated from a batch of fermentation broth by adsorption on IR-120 (H+) and elution with ammonium hydroxide. A pure product was obtained by chromatography on Permutit Q (H+) and recrystallizations from water-methanol. Elementary analysis gave an empirical formula of $C_8H_{16}O_4N_2S$. A Kuhn-Roth determination indicated that no C-methyl

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