ACCUMULATION OF AN INHIBITOR OF PROTEIN SYNTHESIS AFTER VALINE STARVATION[1]

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Received July 11, 1966

Streptococcus faecalis 9790 is a gram positive microorganism with an absolute requirement for at least ten amino acids[2]. Whenever an essential amino acid is withdrawn from the synthetic growth medium (Shockman, 1962), the culture shifts from an exponential growth rate of 1.8 hr^{-1}, to a slow post-exponential rise in turbidity (Toennies and Shockman, 1958). Since the organism has a natural requirement for a variety of amino acids, it may be considered equivalent to a multiple amino acid auxotroph. As with amino acid auxotrophs of certain gram negative enterobacteria (Pardee and Prestidge, 1956; Gros and Gros, 1958), deprival of an essential amino acid in cultures of S. faecalis leads to severe inhibition of RNA, as well as protein synthesis (Toennies, Bakay and Shockman, 1960). We have now found that deprival of the essential amino acid valine results in the accumulation of a protein inhibitor of cell free amino acid incorporation.

Cells were grown in a complete, chemically defined medium (Shockman, 1962), or in the same medium containing limiting amounts of valine (5 μg/ml). In valine limited cultures growth ceased at an O.D. (675 mμ) of 0.60 to 0.65. Thirty to sixty minutes following valine exhaustion, cells were harvested from the valine starved cultures (-VAL), and at the same time, from a control

[1]This work was supported in part by U.S.P.H.S. General Research Support Grant 5-501-FR-05417; L.D.M. is a Postdoctoral Fellow of the Arthritis Foundation; G.D.S. is a career development awardee of the U.S.P.H.S. (1K3-AI-4792).

[2]Arginine, glutamic, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine.

culture in the late exponential phase (LOG), at an O.D. (675 mμ) of 1.4 to 2.5 Protoplasts of both were prepared by a lysozyme procedure described elsewhere (Moore and Umbreit, 1965), in 0.5 M sucrose. Washed protoplasts were suspended in two volumes of buffer (Tris-HCl, 10^{-2} M, pH 7.6; magnesium acetate, 10^{-2} M; β-mercaptoethanol, 10^{-2} M; and KCl, 1.2×10^{-1} M) containing DNase (100 μg/ml), and were disrupted by three passages through a large bore hypodermic syringe needle. The extracts were cleared by two centrifugations at 4,600 x g and were fractionated by centrifugation at 105,000 x g for two hours into a soluble and a particulate fraction. The particulate fraction was resuspended in a volume of buffer equal to the volume of the supernate decanted. Extract fractions retained full activity for at least four weeks when stored at -20° C. Crude unfractionated extracts were stable on storage at -20° C for at least six months.

Fig. 1A shows the kinetics of C^{14} threonine incorporation into protein in a cell free system containing limiting amounts of LOG or -VAL particulate fractions, supplemented with LOG or -VAL supernates. Essentially the same results are obtained when extracts were supplemented with a mixture of C^{14} amino acids. As reported previously (Moore and Umbreit, 1965), the incorporation of amino acids into protein in the cell free system of S. faecalis is linear over a period of several hours. In contrast to a system derived from Escherichia coli (Nisman and Pelmont, 1964), the incorporation reaction of S. faecalis is not affected by Actinomycin D (12.5 to 125 μg/ml), indicating that it depends on preformed messenger RNA.

Fig. 1A also shows that the capacity for cell free protein synthesis is essentially the same when LOG or -VAL particulate fractions are supplemented with LOG supernates. Equal capacities for protein synthesis were obtained when: (1) the ratio of particulate to soluble fraction was close to that found in the intact cells, and (2) the cell free systems contained particulate fractions from equivalent quantities of cells. The latter is of some importance, since analysis of the two extracts shows that the -VAL particulate

fractions lost 20 to 35% of their RNA during the 30 to 60 minute period of valine starvation. Loss of RNA in valine starved particulate fractions is accompanied by an approximately two fold rise in the RNA and protein content of the -VAL 105,000 x g supernates. The degradation of RNA in -VAL particulate fractions does not appear to affect the structures which are active in

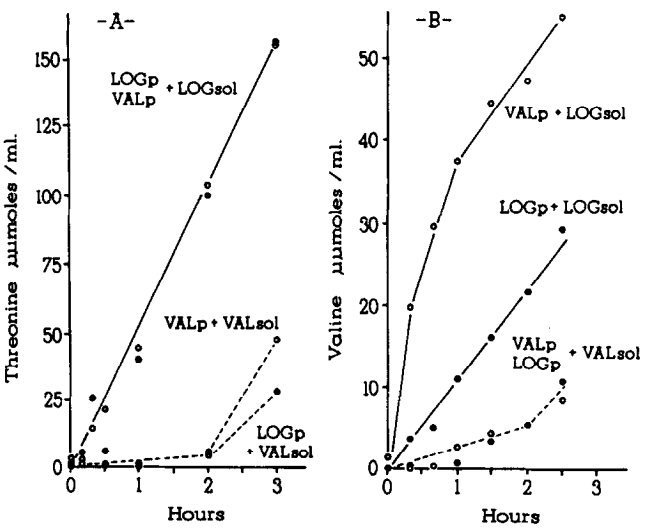

Figure 1. A. Incorporation of C^{14} threonine (6.75 mµmoles = 3.07 x 10^5 cpm) + 19 C^{12} amino acids (100 mµmoles each). RNA added to the reaction mixtures was: LOG particulate, 1.69 mg; -VAL particulate, 1.44 mg; LOG soluble, 0.44 mg; -VAL soluble, 0.76 mg. B. Incorporation of C^{14} valine (10.82 mµmoles = 1.47 x 10^6 cpm) + 19 C^{12} amino acids (100 mµmoles each). RNA added to the reaction mixtures was: LOG particulate, 1.98 mg; -VAL particulate, 1.74 mg. Note the different scales used to indicate incorporation of threonine and valine. ●——● LOG particulate + LOG soluble; o——o -VAL particulate + LOG soluble; ●- -● LOG particulate + -VAL soluble; o- -o -VAL particulate + -VAL soluble.

The reaction mixture contained, per ml: ATP, 8 µmoles; GTP, 0.4 µmoles; β-mercaptoethanol, 8 µmoles; creatine phosphate, 60 µmoles; Tris-HCl, pH 7.6, 10 µmoles; magnesium acetate, 10 µmoles; KCl, 60 µmoles; creatine phosphokinase, 22 µg; and DNase, 10 µg. Extracts or extract fractions (0.05 to 0.1 ml) were added to the incubation mixture in a total volume of 0.8 ml, and were preincubated at room temperature for 30 to 45 minutes prior to the addition of a C^{14} labeled + 19 unlabeled amino acids and then incubated at 37° C. At various times 0.1 ml aliquots were withdrawn from the incubation mixtures, precipitated into cold 7% perchloric acid and hydrolyzed in a steam bath for 30 minutes. Precipitates were collected by centrifugation, dissolved in 0.5 ml of 1 N NaOH, and reprecipitated with cold 10% trichloroacetic acid. Precipitates were collected on membrane filters, washed several times with 10% trichloroacetic acid, glued to planchets, and counted in a Nuclear-Chicago thin window gas flow counter. RNA and protein analyses of extracts were performed using the orcinol and Folin procedures, or estimates based on absorption at appropriate wavelengths (Layne, 1957).

cell free protein synthesis, as indicated by a capacity for threonine incorporation identical to that of LOG particulate fractions (Fig. 1A).

In some extracts, a "burst" of C^{14} valine incorporation during the first hour of cell free incorporation could be demonstrated when -VAL particulate fractions were supplemented with LOG supernates (Fig. 1B). In two separate experiments, using different extracts, the "burst" was 0.016 and 0.019 valine residues per ribosome[3]. The phenomenon was not observed consistently, and requires further study. Consistently the system from LOG cells incorporated between 2 and 3 times more threonine than valine.

When LOG or -VAL particulate fractions were supplemented with -VAL supernates, protein synthesis was reduced markedly (Figs. 1A and B). The reduction in synthetic activity is transient, and is followed by a resumption of protein synthesis after two to three hours. The lag in incorporation observed in extracts supplemented with -VAL supernates is analogous to that observed with amino acid starved intact cells (Moore and Shockman, unpublished data). However, the preliminary fractionation described below indicates that the lag and the subsequent resumption of protein synthesis are due to different constituents of -VAL supernates.

Fig. 2 shows that increasing amounts of -VAL supernates added to a crude extract from LOG cells (containing both a LOG particulate and a LOG soluble fraction) progressively inhibit protein synthesis. Addition of 0.5 ml LOG supernate did not give this effect. Therefore, rather than a deficiency of constituents required for protein synthesis, the -VAL supernates contain

[3] This unexpectedly low figure must be viewed within the context of the number of "active" ribosomes in the cell free system. Such a figure has not been obtained for extracts of -VAL or LOG cells of S. faecalis 9790. A "high" estimate was obtained for extracts of LOG cells of S. faecalis 10C1 (Moore and Umbreit, manuscript in preparation) by assay of fractions isolated after sucrose gradient centrifugation. According to the estimate, no more than 1 to 5% of the ribosomes are "active" in the cell free system. As a second approach one might consider the fact that the "burst" of valine uptake in -VAL fractions during the first hour of incubation is equivalent to the total valine uptake of LOG fractions over 2.5 hours of incubation.

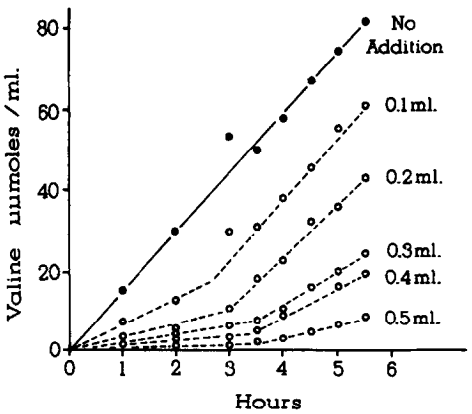

Figure 2. Effect of -VAL supernate on C^{14} valine incorporation in crude LOG extracts. System supplemented with C^{14} valine (1.54×10^5 cpm = 5.40 mμmoles) + 19 C^{12} amino acids. ●—● crude LOG cell extract (1.15 mg particulate RNA/ml reaction); o- -o reaction in the presence of -VAL soluble (0.84 mg protein/0.1 ml; 0.41 mg RNA/0.1 ml).

material inhibitory to protein synthesis in LOG extracts. The addition of unfractionated -VAL supernates to a crude LOG cell extract produced the "two step" effect observed in Fig. 1, e.g., inhibition followed by a rise in incorporation activity. The duration of the lag was not altered by increasing concentrations of -VAL supernates.

Substantial recovery of inhibitory activity was obtained on batch elution of -VAL supernates from DEAE sephadex with 0.01 M Tris-HCl, pH 8.3 (Table I). The fractionation resulted in a ten fold purification of the inhibitory material, and suggests that it is basic in nature. When assayed on crude LOG extracts, the 0.01 M Tris fraction produced a typical transient inhibition, followed by resumption of protein synthesis two hours later. The inhibition obtained from the 0.3 and 0.5 M Tris fractions continues throughout the incorporation reaction and appears to be due to low molecular weight nucleic acid derivatives.

DEAE sephadex column fractionation of -VAL supernates (to be described in detail elsewhere) yielded four basic protein peaks containing inhibitor. All four peaks inhibited protein synthesis over the entire four hour reaction,

TABLE I. BATCH FRACTIONATION OF -VAL SUPERNATE ON DEAE-SEPHADEX A-50

Tris-HCl, pH 8.3 molarity	mg RNA	mg Protein	Total Units	Units/mg Protein
0.01	0.189	0.915	10.35	11.32
0.05	0.154	0.935	5.41	5.78
0.10	0.174	0.705	None	-
0.30	0.968	6.050	0.49	0.08
0.50	1.160	4.825	1.16	0.24
Unfractionated	4.100	16.200	18.40	1.10
Recovered	2.645	13.430	17.41	-

Five ml of a -VAL supernate were added to 10 ml of DEAE sephadex previously equilibrated with 0.01 M Tris HCl, pH 8.3, and stirred at 4° C for 2 hrs. The gel was centrifuged at 4,600 x g, the supernate decanted, and an equal volume of Tris HCl, 0.05 M, was added. The procedure was repeated for the entire series of buffers, the eluates were precipitated with 70% ammonium sulfate, the precipitates were suspended in 2 ml of Tris HCl, 0.01 M, and dialyzed overnight against water. The extracts were lyophilized and assayed for activity, protein content (Folin) and RNA (O.D. 260 mμ). A unit of inhibitory activity is defined as that amount of material inhibiting the rate of protein synthesis by 50%, during the first two to three hours of incorporation, in a fully supplemented LOG cell crude extract.

TABLE II. EFFECT OF DEAE SEPHADEX FRACTIONS ON CELL FREE PROTEIN SYNTHESIS BY CRUDE LOG EXTRACTS

	Fraction No.	Peak	O.D. 260	O.D. 280	$\frac{280}{260}$	cpm/ml 2 hrs	cpm/ml 4 hrs
	No addition		-	-	-	1,083	2,240
a.	7	I	0.112	0.163	1.455	100	358
	11	II	0.087	0.123	1.420	430	618
b.	25	V	0.265	0.223	0.838	2,869	10,149
c.	23 + 7	V + I	0.123	0.094	0.740	100	2,134
	27 + 11	V + II	0.220	0.141	0.642	520	4,000

Five ml fractions were collected from a 1.5 x 42 cm DEAE-sephadex column eluted with 0.01 M Tris HCl, pH 7.6. The fractions were lyophilized and suspended in 1 ml of water and 0.2 ml aliquots were added to the standard assay system, supplemented with C^{14} valine (5.29×10^5 cpm/ml). Fractions 7 and 11 were obtained at 35 and 55 ml respectively. Fractions 23 to 27 were collected at 115 and 135 ml, respectively.

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as shown for Peaks I and II in Table II.

A nucleic acid fraction, presumably complexed to basic material

(Hoagland and Askonas, 1963) was eluted from the DEAE sephadex column with 0.01 M Tris-HCl (Table II). The fraction contained less than 5% of the total RNA in -VAL supernates, and had a low molecular weight, estimated at 8,000-10,000 by gel filtration. This fraction strikingly stimulated amino acid incorporation (Table II,b). When mixed, the nucleic acid peak and the purified inhibitory fractions produced the "two step" reaction (Table II,c) originally observed with unfractionated -VAL supernates (Fig. 2), or with the 0.01 M Tris DEAE sephadex batch eluate. A detailed study of the DEAE sephadex column fractions and of their effects on cell free protein synthesis is in progress.

Preliminary data on the inhibitory material indicate the following: (1) chromatographic behavior and electrophoresis in acrilamide gels (Reisfeld, Lewis and Williams, 1962) indicate that it is basic; (2) DEAE sephadex purified material is relatively homogeneous on gel filtration and sucrose gradient centrifugation (Martin and Ames, 1961), where it exhibits a molecular weight between 15,000 and 30,000; (3) inhibitory activity of -VAL supernates is not affected by DNase or RNase, is stable for at least two months at 4° C, and is not inactivated by heating at 50° C for 20 minutes; (4) supernates obtained from valine and threonine starved cells, but not from LOG cells, inhibit markedly polyphenylalanine synthesis in a polyuridylic acid directed cell free system (Nirenberg and Matthaei, 1961) derived from E. coli. Consequently an opportunity exists for studies of mode of action in a well characterized, relatively simple cell free system.

Attempts to relate the accumulation of inhibitory basic protein(s) in supernates from amino acid starved cells to a breakdown of ribosomes into constituent proteins and low molecular weight nucleic acids appear promising.

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