Effects of Essential Amino Acid Starvation in *Streptococcus faecalis*: Structural Change in the 50S Ribosomal Subunit

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The 50S ribosomal subunits obtained from exponential-phase and valine-limited cultures of *Streptococcus faecalis* 9790 differed in protein content. Approximately 35% of the valine-limited subunits showed protein losses when analyzed by CsCl density centrifugation after formaldehyde fixation. Results of density gradient analysis and of acrylamide gel electrophoresis localized the protein to a "split" subunit fraction. No differences could be detected in the protein contents of 30S subunits from exponential-phase and valine-limited cultures.

In a study of the effect of amino acid starvation in *Streptococcus faecalis*, a basic protein fraction was recovered in the $105,000 \times g$ supernatant fraction of valine-limited cells (8). The appearance of soluble basic proteins was accompanied by a net loss in the protein content of the ribosomal fraction (9). These results suggested that limitation of an essential amino acid might lead to solubilization of ribosomal proteins.

In the present study, we examined valine-limited cultures of *S. faecalis* for ribosome alterations. The results confirm the preliminary observation of a ribosomal protein loss after amino acid limitation (8, 9). In addition, we find that the ribosomal protein loss is restricted primarily to a class of proteins in the CsCl "split" fraction of the 50S ribosomal subunit. As a result of protein loss in about 35% of the ribosome population, the 50S ribosomal subunits of amino acid-limited cells are physically heterogeneous.

MATERIALS AND METHODS

Cell growth and amino acid starvation. Exponentially growing cultures (LOG cells) of *S. faecalis* (ATCC 9790) were grown in a highly buffered synthetic medium described by Toennies and Shockman (18). Valine-limited cultures were grown in the same medium containing 6.0 μg of this amino acid per ml instead of the normal concentration of 100 $\mu\text{g}/\text{ml}$. This concentration of valine resulted in exhaustion at a turbidity equivalent to 0.24 mg (dry weight of cells per ml). Adjusted optical density units were used to determine cellular dry weight (one adjusted optical density unit at 675 nm = 0.39 $\mu\text{g}/\text{ml}$;

see reference 17). Valine-limited cells were harvested at 45 min after amino acid exhaustion by rapid chilling followed by centrifugation at 4 C ($10,000 \times g$ for 10 min). LOG cells were harvested in the same manner at an identical cell concentration.

Protein and ribonucleic acid determinations. Samples of cultures made 10% with respect to trichloroacetic acid were chilled at 4 C for 30 min. The precipitate was collected by centrifugation at $10,000 \times g$ for 10 min and washed twice with two volumes of 10% trichloroacetic acid. After a 30-min hydrolysis at 100 C, the samples were chilled at 4 C for 30 min, and the precipitates were collected by centrifugation. The protein content of the pellets was determined by the Folin procedure (5). Ribonucleic acid (RNA) was determined in the supernatants by the orcinol procedure (1).

Cell lysis and fractionation. Cell lysis was accomplished by combined enzymatic and mechanical treatment. Cell pellets (600 mg, dry weight) were washed at 4 C with 10.0 ml of a protoplast-forming medium consisting of 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.2), 0.01 M magnesium acetate, 0.5 M sucrose, and hen egg-white lysozyme (1.25 mg/ml). The washed cells were suspended in 2.2 ml of the same protoplast-forming medium, incubated at 37 C for 20 min, rapidly chilled in an ice bath, centrifuged at $10,000 \times g$ for 10 min, and then suspended in 2.2 ml 0.01 M Tris-hydrochloride buffer (pH 7.2) containing 0.01 M magnesium acetate. The resulting suspension was subjected to sonic treatment for 30 sec by using a model 60-w ultrasonic disintegrator (Measuring & Scientific Equipment, Ltd., London).

Sonic extracts were fractionated by centrifugation at $105,000 \times g$ for 2 hr into a soluble (S_{100}) and a particulate (P_{100}) fraction. P_{100} fractions were suspended in 2.2 ml of a solution containing 0.5 M NH_4Cl , 0.001 M magnesium acetate, and 0.01 M Tris-hydrochloride buffer (pH 7.2). The resulting suspension was slowly stirred at

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4°C for 30 min. The stirred suspension was further fractionated by $30,000 \times g$ centrifugation for 30 min at 4°C. This centrifugation resulted in a supernatant fraction (S_{30}) and a pellet (P_{30}) fraction.

Radioactive labeling of cell cultures. In labeling experiments, LOG cells were grown in a medium containing 0.07 μCi of ^{14}C -L-leucine (uniformly labeled, 0.5 mCi in 0.024 mg, New England Nuclear Corp.) and 30 μg of ^{12}C -L-leucine per ml. Valine-limited cells were grown in a medium containing the limiting concentration of valine and 2.0 μCi of ^3H -L-leucine (L-leucine-4,5- ^3H , 1.0 mCi in 0.026 mg, New England Nuclear Corp.) and 30 mg of ^3H -L-leucine per ml. Both cell cultures were inoculated at an initial cell concentration of 0.78 $\mu\text{g}/\text{ml}$. After approximately six mass doublings, when the cell density reached 39 $\mu\text{g}/\text{ml}$, a 50-fold excess of ^{12}C -L-leucine was added to the growth medium. Both LOG and valine-limited cells were harvested at a cell density of 236 $\mu\text{g}/\text{ml}$, which, in the case of the valine-limited cells, was 45 min after valine exhaustion.

In experiments involving the radioactive labeling of RNA and protein, the growth medium for the LOG and the valine-limited cells both contained 0.003 μCi of ^{14}C -L-arginine (uniformly labeled, 0.05 mCi in 0.0333 mg, New England Nuclear Corp.), 10.0 μg of ^{12}C -L-arginine plus 0.1 μCi of ^3H -uracil (1.0 mCi in 0.02 mg, New England Nuclear Corp.), and 0.5 μg of ^3H -uracil per ml. In addition, the valine-limited medium contained only 6.0 μg of valine per ml. Cultures were inoculated and harvested as described for the double label protein experiment. At a cell density of 39 $\mu\text{g}/\text{ml}$, a 100-fold excess of ^{12}C -L-arginine and ^3H -uracil was added to each culture.

Sucrose gradients and ribosomal subunit isolation. Twelve-milliliter 5 to 20% linear sucrose gradients containing 0.001 M magnesium acetate and 0.01 M Tris-hydrochloride buffer (pH 7.2) were prepared by the procedure of Britten and Roberts (2). Samples (100 μliters) containing 2.5 absorbancy units at 260 nm (A_{260} from the LOG S_{30} fraction and 2.5 A_{260} units from the valine-limited S_{30} fraction) were layered on the gradients. Separation of ribosomal subunits was achieved by a 3-hr centrifugation at 39,000 rev/min in a Beckman SW-41 Ti swinging bucket rotor. Sucrose gradients were fractionated into 0.25-ml volumes with the aid of an Isco model D density gradient fractionator and a model 220 fraction collector.

Preparative 12.0-ml 5 to 20% linear sucrose gradients for the isolation of ribosomal subunits were identical to gradients described above, except that 20 to 30 A_{260} units of the LOG S_{30} , valine-limited S_{30} , or mixtures of these two fractions were applied to these gradients. Fractions (0.5 ml) were collected and 25-liter fractions were removed from each fraction and diluted to 1.0 ml with water; the absorbance was read at 260 nm in 1.0-cm cuvettes. Fractions containing the same ribosomal subunit were pooled, diluted 1:2 with a solution of 0.001 M magnesium acetate and 0.01 M Tris-hydrochloride buffer (pH 7.2), and then centrifuged for 16 hr at 4°C and $105,000 \pm g$. Pellets were suspended in various solutions depending upon the intended use of the subunits. Cross-contamination between subunits was about 10%.

Formaldehyde fixation and CsCl density gradient analysis of ribosomal subunits. Radioactive ribosomal

subunit pellets were fixed in 6% formaldehyde by the method of Perry and Kelley (13) with one modification. An 0.2 M triethanolamine-hydrochloride buffer, pH 7.8, containing 0.5 M KCl and 0.01 M magnesium acetate was used during the fixation process. Twelve-milliliter CsCl gradients containing 2.0 to 2.5 A_{260} units of formaldehyde-fixed subunits were prepared by the procedure of Meselson et al. (7). However, these gradients contained 0.05 M magnesium acetate, 0.5 M KCl, and 0.02 M triethanolamine-hydrochloride buffer, pH 7.8. The density gradients were then centrifuged in the SW-41 Ti rotor at 39,000 rev/min for 64 hr and fractionated with the aid of the Isco model D density fractionator. The density of the various fractions was determined by weighing precisely 200-mm³ samples of each fraction with an H. Pederson 200-mm³ pipette on a Mettler type H-16 balance. The weight of each 200-mm³ sample was converted to the proper density (g/cm³).

CsCl density gradient analysis of unfixed radioactive ribosomal subunits was carried out somewhat differently. Solutions of 1.6 and 1.7 g of CsCl/ml in 0.05 M magnesium acetate and 0.02 M Tris-hydrochloride buffer, pH 7.2, were prepared as follows. The 1.6 g/ml solution contained: 10 g of CsCl, 0.123 g of magnesium acetate, 0.28 g of Tris, 0.25 ml of 1 N HCl, and water to 12.4 ml. The 1.7 g/ml solution contained: 12.4 g of CsCl, 0.13 g of magnesium acetate, 0.03 g of Tris, 0.28 ml of 1 N HCl, and water to 13.1 ml. Twelve-milliliter preformed CsCl gradients were made by using equal volumes of the CsCl solutions and Britten and Roberts' linear sucrose gradient mixing device. A_{260} units (2.0 to 2.5) in a 100- to 200- μliter volume of 0.001 M magnesium acetate and 0.01 M Tris-hydrochloride buffer, pH 7.2, were layered on top of each gradient. The gradients were centrifuged at 39,000 rev/min in the SW-41 Ti rotor for 48 hr.

Acrylamide gel electrophoresis of ribosomal proteins. Ribosomal proteins were extracted from subunit pellets by using 4 M urea to dissociate the protein and the RNA and 2 M LiCl to precipitate the RNA. The urea-LiCl solution also contained 500 μg of beef pancreatic ribonuclease per ml (Worthington Biochemical Corp.). Preparation of 10% acrylamide gels, electrophoresis, and staining of these gels was done in 0.07 M β -alanine buffer, pH 4.3, by the procedure of Gesteland and Staehelin (3). Glass columns (12 cm by 6 mm) were used for the separation of 10- to 40- μliter samples. Seventy to 100 μg of 50S protein, 50 μg of 30S protein, and 50 μg of 50S split and core protein were applied to the gels. Protein content was determined by the Folin procedure.

The 50S split and core proteins were prepared by dialyzing pooled tubes from CsCl gradients containing these proteins against three 3.0-liter volumes of 0.001 M magnesium acetate (11). The dialyzed samples were then lyophilized and subsequently treated with 4 M urea, 2 M LiCl, and ribonuclease, as described above. A 10-day treatment of subunits at 4°C was necessary to insure sufficient disaggregation of the proteins.

After electrophoresis, fixation, and staining, the gels were sliced with the aid of a single-edge razor blade and a piece of glass tubing (8-mm inside diameter) into which the gel could be pulled in a syringe-like manner with a 7-mm glass rod. Successive bands were extruded from the glass tube, sliced with the razor blade, and

placed in scintillation vials. These slices were solubilized in the vials with H_2O_2 and prepared for counting by the method of Moss and Ingram (10). After this preparation, 16.0 ml of Takanami's toluene scintillation solution (16) was added to each vial.

RESULTS

Gross changes after amino acid limitation. Figure 1 shows that in *S. faecalis* valine limitation results in the immediate cessation of RNA synthesis. A similar response of *S. faecalis* to amino acid starvation was also demonstrated by using ^{14}C - or 3H -uracil as the label during valine or threonine limitation (L. Daneo-Moore, *unpublished data*). An increase in the net protein content of the cultures was observed during the first 45 min of amino acid limitation (Fig. 1). This increase was in the order of 15% of the total cellular protein mass.

When cells were prelabeled with arginine and chased for 90 min before valine limitation, the protein content of the ribosomal fraction was reduced by 6.5%, when compared to the exponential (LOG) cell ribosomal fraction (a 4.4% reduction by difference, Table 1). In other experiments using arginine or leucine, a net loss in the

FIG. 1. Effect of valine starvation on cell mass, and RNA and protein synthesis. A 5.0-ml sample was removed at the indicated times from a culture of cells growing in valine-limited medium (6.0 $\mu g/ml$), and the protein content and RNA content were determined. Symbols: Δ , cell mass; $\bullet$, protein; $\blacksquare$, RNA.

total particulate fraction (ribosome plus membrane) was accompanied by an increase in the protein content of the $105,000 \times g$ supernatant fraction (Table 2). This increase was detected when the nonradioactive chase was for 90 min (approximately 3.0 mass doublings). In 15-min chase experiments, there was a smaller difference between the distribution of soluble and particulate counts in LOG and valine-limited cultures (Table 2). The alteration in the distribution of counts in a long but not in a short chase suggests removal from ribosomes of a ribosomal protein component, rather than of adsorbed, recently synthesized proteins.

The possible appearance of proteolytic activity accounting for loss of ribosomal counts was examined in turnover experiments with valine-lim-

TABLE 1. Fractionation of prelabeled LOG and valine-starved extracts^a

Fraction	LOG		Valine-limited	
	counts/min	Per cent of total counts/min	counts/min	Per cent of total counts/min
Ribosomal (S_{30})	146,957	66.9	136,492	62.5
Soluble (S_{100})	19,833	9.0	21,318	9.8
Membrane (P_{30})	36,575	13.9	36,025	16.5
Recovery	197,365	89.7	193,835	88.8

^a Two cultures, one containing a full complement of valine (LOG) the other with limiting valine (6 $\mu g/ml$; valine-starved), were labeled for six mass doublings and then chased for 90 min, when valine-limited cultures reached the valine depletion point (Fig. 1). The LOG culture was harvested at this time, whereas the valine-depleted culture was incubated for an additional 45 min. Cells were converted to protoplasts and fractionated. Total trichloroacetic acid-precipitable ^{14}C -arginine counts were 219,633 for LOG, and 218,365 for valine-starved unfractionated extracts.

TABLE 2. Distribution of radioactivity in soluble and particulate fractions

Expt	Label ^a	Counts/min		Per cent counts/min ^b
		Soluble	Particulate	
Expt A	^{14}C -arginine			
LOG		8,642	31,245	78.4
Valine-limited		6,961	23,052	76.7
Expt B	^{14}C -arginine			
LOG		6,910	34,180	81.9
Valine-limited		6,021	29,280	76.3
Expt C	Leucine ^c			
LOG		24,583	73,350	75.2
Valine-limited		272,150	646,312	70.4

^a Chase time, 15 min.

^b Results given are percentages of (particulate counts per minute) / (total counts per minute) $\times 100$.

^c ^{14}C -leucine label for LOG, 3H -leucine label for valine-limited cells.

ited cultures. In long- and short-term chases, protein turnover was of the order of 3 and 4% per hour, respectively (Table 3). In the presence of chloramphenicol, the turnover of a short-term chase was reduced from 3.79 to 2.99% per hour. In addition, ^{14}C -arginine and ^3H -uracil prelabeled cells were examined for loss of acid-precipitable counts under our conditions of fractionation (Table 4). No significant loss of counts was observed after incubation of valine-limited cell extracts for 3 hr at 37 C.

Physical studies of isolated ribosomal subunits.

Sucrose gradient analysis of LOG and valine-limited ribosomal fractions, density changes in formaldehyde-treated 50S ribosomal subunits, density gradient centrifugation of unfixed 50S subunits, and acrylamide gel electrophoresis of ribosomal proteins are described in the following paragraphs.

Sucrose gradient analysis of LOG and valine-limited ribosomal fractions. Figure 2 shows the distribution of labeled protein when a mixture of ^3H -leucine-labeled LOG extracts and ^{14}C -leucine-labeled valine-limited extracts was centrifuged in a 5 to 20% sucrose gradient. The counts in the gradient pellet and those in the 30S subunit peak of the two extracts were essentially superimposable. Valine starvation resulted in a loss of 8 to 10% protein in the 50S subunit region. There was no evidence of a "light" shoulder at the level of resolution obtainable with sucrose gradients.

Density changes in formaldehyde-treated 50S ribosomal subunits. Fixation of ribosomes followed by CsCl density gradient centrifugation separates ribonucleoprotein particles on the basis of

TABLE 3. *Turnover of proteins during valine starvation^a*

Conditions	0 min	240 min	
		LOG control	No valine
A. Chased at 39 $\mu\text{g}/\text{ml}$			
Counts per min per ml	6,898	6,806	6,080
Loss of counts per min per ml during 240-min incubation		92	818
Per cent change/hr		0.33	2.96
B. Chased at 390 $\mu\text{g}/\text{ml}$			
Counts per min per ml	20,131	19,880	17,080
Loss of counts per min per ml during 240-min incubation		251	3,051
Per cent change/hr		0.32	3.79
C. Chased at 390 $\mu\text{g}/\text{ml}$, CAP added			
Counts per min per ml	20,131	16,541	17,727
Loss of counts per min per ml during 240-min incubation		3,590	2,404
Per cent change/hr		4.46	2.99

^a Cells labeled with ^{14}C -leucine were chased at the mass indicated with a 100-fold excess of ^{12}C -leucine. At a cell density of 390 $\mu\text{g}/\text{ml}$ (0 time), each culture was diluted 1:10 into unlabeled medium and split into two; one-half was incubated in the presence (LOG control) and the other in the absence of valine. Samples were removed at 0 and at 240 min for determination of hot trichloroacetic acid-precipitable counts per min. In C, chloramphenicol was present (50 $\mu\text{g}/\text{ml}$) from 0 to 240 min.

TABLE 4A. *Determination of proteolytic activity in LOG and valine-limited extracts^a*

Fraction	LOG			Valine-limited		
	0 min (counts/min)	180 min		0 min (counts/min)	180 min	
		Counts/min	Per cent of 0 time		Counts/min	Per cent of 0 time
Soluble	2,571	2,297	89.3	2,720	2,496	91.8
Ribosomal	12,634	12,423	98.3	11,292	10,680	94.6
Membrane	2,937	2,887	98.3	4,374	4,000	91.4

TABLE 4B. *Determination of ribonuclease activity in LOG and valine-limited extracts^a*

Soluble	4,766	5,163	108.3	5,284	5,248	99.4
Ribosomal	179,782	191,762	106.7	229,528	219,913	95.8
Membrane	32,879	33,171	100.9	55,842	61,736	110.5

^a Portions (300 μl) of LOG and valine-starved extract fractions obtained from cells labeled with ^{14}C -arginine and with ^3H -uracil for three generations and chased for 90 min were incubated at room temperature for 180 min. Samples (15 μl) were removed from each fraction at the beginning and end of the incubation period. A 5.0-ml amount of 10% trichloroacetic acid was added to each. Samples were assayed for cold (RNA) and for hot (protein) acid-precipitable radioactivity.

their RNA/protein ratio (14). Consequently, the method permits unequivocal distinction between two alternate possibilities: (i) a net loss of ribosomes in valine-limited extracts, and (ii) a selective loss of protein from valine-limited ribosomes. Selective protein loss from ribosomal subunits would increase the RNA/protein ratio and result in an increase in particle density.

CsCl density gradient centrifugation was performed by using a mixture of formaldehyde-fixed LOG and valine-limited 50S subunits. Figure 3 shows a major peak for both particles, at a density of 1.622 g/cm³. The LOG subunit profile gave a relatively homogeneous peak. In contrast, the valine-limited subunits gave four reproducible peaks with densities of 1.622, 1.628, 1.634, and 1.640 g/cm³. A total of 38% of the counts in the valine-starved 50S subunit was not within the density region expected from the density profile of the LOG 50S subunit. A duplicate experiment not shown here gave a total area shift of 37% in another extract.

The density of normal or formaldehyde-fixed *Escherichia coli* ribosomes has been reported as

FIG. 2. Linear sucrose gradient analysis of mixed ribosomal fractions from LOG and valine-starved cells. Ribosomal fractions prepared as described from ¹⁴C-leucine-labeled LOG cells and ³H-leucine-labeled valine-starved cells. Radioactivity in the fractions was determined, and count data were corrected for isotope overlap and normalized by using the count data obtained from an analysis of a sample of the original ribosome mixture, which had been prepared for counting in the same manner as the gradient fractions. Symbols: ●, ¹⁴C-leucine (LOG) counts per minute; ▲, ³H-leucine (minus valine) normalized counts per minute.

1.64 (14), and as 1.61 g/cm³ (9). Discrepancy between the values apparently arises from the correction factor used to convert refractive indices to density values, using gradients containing buffer and various Mg²⁺ concentrations. An experimentally determined linear correction factor of 0.028 g/cm³ was used to correct for the Mg²⁺ concentration in the gradients.

It can be readily calculated that the density peaks obtained from valine-limited subunits (Fig. 3) represent particles with a protein content of 33.2, 32.3, 31.4, and 30.5%. [This calculation is based on weight per cent RNA = 100 × (ρ_{RNA}/ρ_{measured}) × (ρ_{measured} - ρ_{protein})/(ρ_{RNA} - ρ_{protein}) where ρ_m is the measured density and ρ_{RNA} and ρ_{protein} are the densities of RNA and protein, taken to be 1.900 and 1.250 g/ml, respectively (4).] The protein content of the LOG 50 subunit peak is 33.2%. Consequently, the dense peaks obtained from valine-limited subunits represent particles which lost 3, 6, and 9% of their normal protein content. None of the valine-limited subunit peaks appeared to correspond to "core" particle obtained when 50S subunits are centrifuged in CsCl (see section below). The corrected density of the 50S *S. faecalis* "core" was 1.652 g/cm³, corresponding to particles with 28.9% protein.

Density gradient centrifugation of formaldehyde-fixed 30S subunits gave two major peaks of corrected densities, 1.59 and 1.61 g/cm³ (Fig. 4). No significant difference could be seen between the buoyant density of peaks from LOG and valine-limited cells.

Density gradient centrifugation of unfixed 50S subunits. Centrifugation of *E. coli* ribosomes in CsCl gradients containing 0.05 M Mg²⁺ fractionates the particles into a split protein and a ribonucleoprotein core (7). LOG and valine-limited 50S subunits doubly labeled with ³H-uracil and ¹⁴C-arginine were isolated and analyzed for protein and RNA content after CsCl gradient centrifugation (Fig. 5). The particles from both extracts gave a core with a corrected density of 1.652 g/cm³ (Fig. 5B). When protein counts were normalized on the basis of input RNA, the following was inferred. (i) The cores of LOG and valine-limited extracts contain the same absolute amounts of protein; (ii) the split fractions of LOG subunits contain more protein than the corresponding valine-limited fractions. The split fraction of the LOG 50S subunit contained 43.2% of the counts. The valine-limited subunits had lost 19% of the split fraction protein, or 8.2% of the total subunit protein.

A similar experiment, utilizing a protein label only, is summarized in Table 5. The results indicate that, if the valine-limited 50S core is assumed unchanged in protein content when compared to

FIG. 3. CsCl density analysis of 6% formaldehyde-fixed mixed 50S ribosomal subunits from LOG and valine-starved cells. Two-hundred-microliter 2.5 A_{280} units of a mix of 6% formaldehyde-fixed ^{14}C -leucine (LOG) 50S and ^3H -leucine (minus valine) 50S subunits were prepared. Five-drop fractions (175 μl) were collected and analyzed for hot trichloroacetic acid precipitable radioactivity. ^3H counts were normalized by using count data obtained from a sample of the 50S mix. Density of fractions was determined by weighing 200-mm³ samples before the radioactive analysis. Symbols: $\circ$, ^{14}C -leucine (LOG) counts per minute; Δ , ^3H -leucine (minus valine) normalized counts per minute; $\square$, density.

the LOG subunit, then 23.2% of the split protein fraction was missing in the valine-limited subunits. In this experiment, the LOG 50S subunit had 37.6% of the protein in the meniscus fraction. Consequently, during valine starvation there was an 8.7% loss in total protein from 50S subunits. In two separate experiments, the net protein loss from valine-limited 50S subunits ranged between 8.2 and 8.7%. This value is in good agreement with the 8 to 10% 50S protein loss observed by the sucrose gradient technique (Fig. 2).

Acrylamide gel electrophoresis of ribosomal proteins. The results of polyacrylamide electrophoresis of 30S and 50S ribosomal proteins obtained from LOG cells are shown in Fig. 6. When 30S and 50S ribosomal proteins from LOG and valine-limited cells were analyzed in the same electrophoretic run, no gross qualitative differ-

ences appeared. However, it was impossible to eliminate quantitative losses in band components of either subunit, due to the large number of bands present in the gels, and to minor variations in the relative mobility of bands in individual experiments. Consequently, it became necessary to compare protein bands obtained from mixtures of LOG and valine-starved extracts each containing a different protein label.

Table 6 shows normalized counts obtained for 30S subunit proteins where the LOG extract was labeled with ^{14}C and the valine-limited extract with ^3H -leucine. The normalized $^3\text{H}/^{14}\text{C}$ ratios in Table 6 indicate that, aside from a 10 to 15% loss of protein in bands 14 and 15 of valine-starved subunits, there was no significant quantitative change in the major 30S proteins during valine limitation. This result agrees well with the obser-

FIG. 4. *CsCl* density gradient analysis of formaldehyde-fixed mixed 30S ribosomal subunits from LOG and valine-starved cells. Analyses were performed as described in Fig. 3, except that 2.0 A_{260} units (200 μ liters of mixed ^{14}C -leucine (LOG) and ^3H -leucine (valine-starved) 30S ribosomal subunits were used, and centrifugation was for 16 hr. Symbols: ●, ^{14}C -leucine (LOG) counts per minute; Δ, ^3H -leucine (minus valine) counts per minute; □, density.

vation that there was no loss of radioactivity from the 30S region of linear sucrose gradients during starvation.

Analysis of 50S proteins on polyacrylamide gel was considerably more difficult than that of 30S proteins. Ribosomal 50S subunits contain a larger number of protein bands than 30S subunits, and the individual neighboring proteins are very close to one another. Consequently, precise slicing of individual protein bands could not be achieved, as indicated by the fact that duplicate gels did not give identical results. It became necessary to fractionate the 50S ribosomal subunits into a split and a core fraction before protein extraction and polyacrylamide gel electrophoresis. Figure 7 shows the polyacrylamide gels of mixed LOG and valine-limited 50S split and core proteins. The fractionated 50S subunits gave reasonably well resolved protein bands. A total of 13 and 16 bands were found in the split and core fractions, respectively.

The majority of the split protein bands showed some change (Table 7). This confirms the finding that protein lost from ribosomes during 45-min valine limitation comes mainly from the split class of ribosomal proteins. All split proteins were not decreased to the same extent during valine starvation. Two split bands, 9 and 17, appeared unchanged, whereas the remaining bands gave normalized ratios ranging from 0.56 to 0.80. The

FIG. 5. Distribution of ^3H -uracil and ^{14}C -arginine in *CsCl* density gradients of 50S ribosomal subunits from LOG and valine-starved cells. Two-hundred-microliter 2.0 A_{260} units of LOG 50S ribosome subunits were layered on duplicate 12.0-ml preformed *CsCl* gradients. Two *CsCl* gradients containing 2.0 A_{260} units of valine-starved 50S subunits were also prepared. One gradient of each cell type was analyzed for cold trichloroacetic acid-precipitable counts (^3H -RNA); the other was analyzed for hot acid-precipitable radioactivity (^{14}C -protein). A, ^3H -RNA in the LOG (●) and the valine-starved (■) 50S ribosomal subunits. B, ^{14}C -protein from LOG (●) and valine-starved (■) 50S ribosomal subunits. These protein data were normalized by using the RNA content of the two 50S subunit preparations.

TABLE 5. Distribution of radioactivity in CsCl density gradients of 50S ribosomal subunits from LOG and valine-starved cells

Gradient	LOG 50S		Valine-starved 50S	
	¹⁴ C counts/min	Per cent of sum recovered	¹⁴ C counts/min	Per cent of sum recovered
Meniscus ^a	2,630	37.6	1,626	29
Peak density of 1.652 g/cm ³ . . .	4,045	56.6	3,526	63.2
Remainder of gradient	484	6.7	427	7.8

^a Meniscus refers to the first 2.0 ml of two gradients prepared and analyzed essentially as in Fig. 5.

altered normalized ratios could be classified into three groups: group 1 with ratios of 0.78 and 0.80 (bands 5 and 10–11), group 2 with ratios of 0.66 to 0.69 (bands 7, 8, 20, and 22), and group 3 with ratios of 0.56 and 0.60 (bands 15 and 24). The core proteins were virtually unchanged.

DISCUSSION

In cells approaching valine starvation through valine limitation in the growth medium, alterations occur which include a loss of protein content in the ribosomal fraction, and a gain of protein content in the 100,000 × g supernatant fraction (9). These changes occur during the first 45 min after cessation of exponential growth, when net RNA synthesis has come to a halt and net protein synthesis is occurring at a constantly decreasing rate. Thus, amino acid starvation of *S. faecalis* appears to result in a continuum of physiological shift-downs.

The loss in protein content of the 50S ribosomal subunit reported here does not appear to be due to direct proteolysis. Two proteolytic activities have been observed in *E. coli* deprived of the essential amino acids threonine, arginine, and histidine (15). An endogenous proteolysis directed to a “fast turnover” fraction occurs immediately after amino acid starvation in *E. coli*. Subsequent to amino acid starvation, an apparently new proteolytic activity affects all cellular proteins. The combination of these two proteolytic activities results in a 4% degradation of bulk protein in the first hour after starvation and a linear rate of 3% per hour for several hours thereafter.

In *S. faecalis*, extracts starved for valine for 45 min, we have been unable to obtain evidence for any significant proteolytic activity (Table 4) or for a very high turnover (Table 3). The observed turnover is in the order of 2.96% per hour. The ribosomal proteins affected by the amino acid starvation are not in a “fast turnover” fraction, since the experiments shown in Tables 1 and 2 in-

dicate that the protein loss in the ribosomal fraction affects stable proteins. In addition, the labeling conditions selected here restrict analysis to ribosomal proteins surviving a chase of 2.5 mass doublings.

Three possibilities can be considered to account for restriction of the protein loss to certain 50S

FIG. 6. Photograph of polyacrylamide gels after electrophoresis of LOG 30S and 50S ribosomal proteins. The 30S and 50S ribosomal pellets were obtained from LOG cells harvested at a cell density of 195 µg/ml. The polyacrylamide gels were prepared and the electrophoresis was run.

TABLE 6. Isotope content of 30S ribosomal protein from ^{14}C -leucine LOG and ^3H -leucine valine-starved cells^a

Band no.	^3H counts/min	^{14}C counts/min	Normalized $^3\text{H}/^{14}\text{C}$
9	1,895	549	1.03
10	623	192	0.97
11	740	232	0.95
13	1,052	324	0.97
14	688	231	0.89
15	616	214	0.87
16	1,006	298	1.02
17	909	261	1.05
19	82	29	0.85
21	72	25	0.87

^a The 30S ribosomal subunits were obtained from radioactively labeled cells and electrophoresed. Band numbers refer to those assigned in Fig. 6 to the various 30S protein bands. Counts were corrected for isotope overlap. Normalized $^3\text{H}/^{14}\text{C}$ ratios were obtained by dividing $^3\text{H}/^{14}\text{C}$ ratios by the $^3\text{H}/^{14}\text{C}$ ratio obtained from a sample of the mixture of 30S proteins.

TABLE 7. Isotope content of 50S split and core protein from ^{14}C -leucine LOG and ^3H -leucine valine-starved cells^a

Split				Core			
Band no.	^3H counts/min	^{14}C counts/min	Normalized $^3\text{H}/^{14}\text{C}$	Band no.	^3H counts/min	^{14}C counts/min	Normalized $^3\text{H}/^{14}\text{C}$
5	659	468	0.78	5-7	1,473	270	1.00
7	347	292	0.66	8	1,125	208	0.98
8	423	341	0.69	9	3,190	575	1.00
9	1,021	568	1.00	10-11	3,140	575	1.00
10-11	578	406	0.80	12	4,660	780	1.09
12	668	522	0.71	13	2,840	50	0.96
15	342	317	0.60	14-15	1,390	252	1.00
17	435	226	1.07	16	420	77	1.00
19	500	394	0.70	17	1,167	222	0.96
20	301	251	0.67	18	866	163	0.96
22	155	126	0.69	19	1,088	207	0.96
24	269	269	0.56	20	658	114	1.05

^a The 50S ribosomal subunits were obtained from labeled cells and electrophoresed. Band numbers refer to those assigned in Fig. 7 to the various 50S protein bands. Protein bands seen in Fig. 8, but not listed here, were not consistent bands in duplicate polyacrylamide gel analyses. Counts have been corrected for isotope overlap. Normalized $^3\text{H}/^{14}\text{C}$ ratios were obtained as described in Table 6.

ribosomal proteins. First, the 50S subunit may adsorb pool proteins, readily solubilized after amino acid starvation. Excess nonfunctional proteins have been found in 50S and 30S subunits (19). The suggestion is that precursor pool proteins may adsorb to ribosomes either *in vivo* or during the preparation of extracts. Second, the 50S protein loss during amino acid starvation might represent ribosome factors such as Tu, Ts, and G (6). Such factors might be adsorbed to functional ribosomes but released from ribosomes

inactive in protein synthesis. This situation would be analogous to that of initiation factors adsorbed to native but not to polysome-derived 30S subunits (12). Finally, the change observed in the 50S subunit may represent some functional alteration of ribosomes exposed to essential amino acid limitation.

FIG. 7. Photograph of polyacrylamide gels after electrophoresis of 50S split and core proteins from ^{14}C -leucine LOG and ^3H -leucine valine-starved cells. The 50S ribosomal subunits were obtained from labeled cells. Mixed extracts of 50S split and core proteins were prepared and electrophoresed.

The data presented here do not eliminate any of these possibilities in a definitive way. Other experiments (Ziegler, Ph.D. Thesis) to be reported elsewhere tend to discount the first hypothesis since (i) the 50S subunit of valine- or threonine-limited cultures is functionally deficient, and (ii) protein-deficient subunits are "free" rather than polysome-associated.

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