JOURNAL OF BACTERIOLOGY, Oct. 1978, p. 209-218
0021-9193/78/0136-0209$02.00/0

Vol. 136, No. 1
Printed in U.S.A.

Isolation of Stable Ribosomal Subunits from Spores of *Bacillus cereus*

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Received for publication 29 June 1978

Analyses of ribosomes extracted from spores of *Bacillus cereus* T by a dry-spore disruption technique indicated that previously reported defects in ribosomes from spores may arise during the ribosome extraction process. The population of ribosomes from spores is shown to contain a variable quantity of free 50S subunits which are unstable, giving rise to slowly sedimenting particles in low-Mg^{2+} sucrose gradients and showing extremely low activity in in vitro protein synthesis. The majority of the ribosomal subunits in spores, obtained by dissociation of 70S ribosomes and polysomes, are shown to be as stable as subunits from vegetative cells, though the activity of spore polysomes was lower than that of vegetative ribosomes. In spite of the instability and inactivity of a fraction of the spore's ribosomal subunits, the activity of the total population obtained from spores by the dry disruption technique was 32% of vegetative ribosome activity, fivefold higher than previously obtained with this species. The improvement in activity and the observed variability of subunit destabilization are taken as evidence for partial degradation of spore ribosomes during extraction.

The processes of differentiation associated with bacterial sporulation are controlled by as yet unknown mechanisms. Also, the mechanisms responsible for preservation of the spore protoplast in the cryptobiotic state are still unknown. Some part of the explanation of these phenomena may involve ribosomal modifications or regulation of cellular metabolism at the level of translation of mRNA. One approach to the investigation of these possibilities has involved extraction of protein-synthetic systems from dormant spores to assess differences between the vegetative and spore systems in vitro (1-3, 6, 7, 10-14). Extractions of three different species of *Bacillus* have produced in vitro systems of differing characteristics. For example, the activity of spore ribosomes in synthesizing polyphenylalanine is reported at 5 to 100% of the corresponding activity of vegetative ribosomes (2, 6, 10, 13). Such variability may reflect species-specific differences in the spore translation apparatus, but conflicting results have been reported for a single species (6, 10).

In studies of the translation apparatus of *Bacillus cereus* T, the spore ribosomes appeared to be defective in several ways. In vitro polyphenylalanine synthesis by spore ribosomes was only 5% of vegetative ribosome activity, ribosomal subunits from spores appeared to be extensively degraded when analyzed on sucrose gradients, the ability of spore ribosomes to bind tRNA was severely impaired, and the protein content of spore ribosomes was abnormally low (11-13).

The existence of degradative proteases and ribonucleases (RNases) in spores and spore extracts (1, 16, 21), the dependence of degradative effects on details of the extraction procedure (1, 16), and the variability of the published descriptions of spore ribosomes are very suggestive that technical difficulty in the extraction of undegraded in vitro systems from *Bacillus* spores may be a limiting factor in assays of spore protein-synthetic capabilities. When we discovered that polysomes, not previously detected in this species, could be routinely isolated from *B. cereus* T spores, using a dry-spore disruption technique (7, 8), it seemed possible that the dry disruption technique might be generally useful in minimizing ribosomal damage during extraction. The re-examination of some properties of ribosomes extracted from spores of *B. cereus* T reported here supports that view.

MATERIALS AND METHODS

Bacterial strains. *B. cereus* T was obtained from H. O. Halvorson; stocks were maintained in 50% glycerol at −20°C.

Reagents. Tris(hydroxymethyl)aminomethane (Tris), agarose, polyuridylic acid (type II), GTP, ATP, phosphoenolpyruvate, and pyruvate kinase were from

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Sigma Chemical Co., St. Louis, Mo. "Ultrapure" sucrose and [^{14}C]phenylalanine were from Schwarz/Mann, Orangeburg, N.Y. *Escherichia coli* tRNA was from Grand Island Biological Co., Grand Island, N.Y. Reagents for polyacrylamide gel electrophoresis (except agarose) were from Eastman Organic Chemicals, Rochester, N.Y. Sand was "purified, washed, and ignited" from J. T. Baker Chemical Co., Phillipsburg, N.J. Brij 58 was from Emulsion Engineering, Inc., Elk Grove Village, Ill. RNase (EC 2.7.7.16) was from Sigma; RNase-free deoxyribonuclease (EC 3.1.4.5) was from Worthington Biochemicals Corp., Freehold, N.J. All other reagents were analytical reagent grade from various suppliers.

Cell growth. Vegetative cells of *B. cereus* T were grown in Difco medium (7) at 30°C to an undiluted 660-nm absorbance (A_{660}) of about 1.0, which corresponds to midlog phase. Cultures were cooled to 4°C with −20°C ice over a period of 10 to 20 min and then harvested in a continuous-flow rotor at 25,000 × *g*. After the cells were washed once with distilled water at 10,000 × *g* for 20 min, they were shell frozen in a dry-ice–acetone bath, lyophilized, and stored at −20°C. Spore production was as previously described (7).

Ribosome preparations. All operations were performed at 0 to 4°C, except spore grinding, which was at room temperature. Vegetative cells were extracted as follows. Lyophilized cells (1.5 g) were washed twice by suspension in buffer I (100 mM NH_4Cl, 20 mM Tris-hydrochloride, 10 mM magnesium acetate [MgOAc] except where specified otherwise, 4 mM mercaptoethanol, pH 7.5, at 20°C) and centrifugation at 10,000 × *g* for 20 min, suspended in 10 to 20 ml of buffer I, and passed once or twice through a French pressure cell at 4,000 lb/in^2. The lysate was incubated for 10 min with 1 μg of deoxyribonuclease per ml and centrifuged twice at 30,000 × *g* to remove debris, and the ribosomes were sedimented at 314,000 × *g* for 2 h. Ribosomal pellets were resuspended in buffer I, clarified by centrifugation at 25,000 × *g* for 10 min, and used immediately or stored at −70°C. Spores were suspended in buffer I after dry grinding with sand as previously described (7). The crude extract (S30) was used immediately for analytical sucrose gradients, or ribosomes were prepared as above and stored at −70°C. In some experiments (for example, see Fig. 2 and 4), spore S30 fractions were used directly, to avoid unnecessary incubation in the presence of possible degradative factors. Since this was not a problem with vegetative ribosomes, they were removed from S30 to facilitate assay by UV absorption. Preparation of a polysome-enriched fraction from spore extracts was by a method similar to Noll's (18). A 5-ml portion of spore S30 was layered over a step gradient of sucrose in buffer I, consisting of 2 ml of 2 M sucrose and 2 ml of 0.5 M sucrose in polycarbonate tubes for a type 65 rotor (Beckman Instruments, Palo Alto, Calif.). After centrifugation at 314,000 × *g* for 105 min, the S30 layer was discarded, the sucrose layers were pooled as nonpolysomes, and the pellet was resuspended in buffer I as the polysome fraction. Polysomes were clarified by 10-min centrifugation at 30,000 × *g* and then used immediately in analytical sucrose gradients.

Sucrose gradient analyses. Gradients were prepared by mixing-chamber methods described by Noll (18). Initially, gradients for subunit analyses were 5 to 30% concave exponential (C_r = 0, C_{mix} = 30%, V_{mix} = 7.4 ml). Later, all gradients were 10% top, convex exponential "isokinetic," for a particle density of 1.51 g/ml as described by McCarty et al. (15). Parameters for SW41 gradients were C_r = 30.1% (wt/wt), C_{mix} = 10% (wt/wt), V_{mix} = 9.75 ml; for the SW 50.1 rotor, C_r = 27.4% (wt/wt), C_{mix} = 10% (wt/wt), V_{mix} = 5.66 ml. All gradients were prepared at 4°C and used within a few hours of preparation. After centrifugation, tubes were punctured, and the contents were pumped at a rate of 2 ml/min through a flow cell in a Gilford spectrophotometer; A_{260} was recorded on a Heathkit recorder. Sedimentation coefficients are inferred from measurements made in a Beckman model E analytical ultracentrifuge and from the positions of peaks in isokinetic gradients based on the calculations of McCarty et al. (15). Estimates of the sizes of particle populations were obtained by planimetric analysis of peak areas.

Gel electrophoresis of RNA. A whole-ribosome technique was modified from Stutz and Noll (20) and Goodenough and Levine (9). Ribosomes at a concentration of 5 to 20 A_{260} units/ml in buffer II [89 mM Tris, 89 mM boric acid, 2.8 mM disodium(ethylenedinitrilo)tetraacetic acid, pH 8.3, without adjustment], plus 3% sodium dodecyl sulfate, 10% sucrose, and 0.01% bromophenol blue, were incubated for 2 min at 37°C, cooled rapidly on ice, and then layered in the sample slot of a vertical polyacrylamide slab gel (0.03 to 0.3 A_{260} unit of RNA in 5 to 50 μl, per slot) for electrophoresis. Gels were 2.5% acrylamide and 0.5% agarose (5% cross-linked), polymerized with 0.03% *N,N,N',N'*-tetramethylethylenediamine (TEMED) and 0.05% ammonium persulfate in a vertical slab apparatus (E-C Apparatus Corp., Philadelphia). Gels were allowed to polymerize overnight at 20°C and then were subjected to pre-electrophoresis with buffer II plus 0.2% sodium dodecyl sulfate for 2 h at 15°C. The buffer was replaced with fresh buffer II plus 0.2% sodium dodecyl sulfate prior to the actual electrophoresis, which was performed at 150 V, 38 mA, for 2 h. After electrophoresis, the slab was removed, incubated in 5 gallons (ca. 19 liters) of distilled water for several hours to reduce the background A_{260}, and then scanned at 260 nm in a linear gel transport (Gilford Instruments Inc., Oberlin, Ohio). Since it has been shown that 23S rRNA is subject to cleavage into two fragments of approximately 16S size (see reference 5 for discussion), the presence of "16S" RNA is not necessarily diagnostic of 30S ribosomal subunits. We observed such cleavage in preparations of RNA from degraded ribosomes of vegetative *B. cereus* T cells, with the fragments resolved at positions very slightly above and below 16S rRNA in our polyacrylamide gels. Under these circumstances, the presence of genuine 16S rRNA in an unknown sample was inferred from the additivity of a peak in the unknown with that of a genuine 16S rRNA reference.

Inhibition of protease activity during spore ribosome preparation. Surface proteases (21) were removed from spores by washing them in buffer III (60 mM NH_4Cl, 10 mM Tris, 10 mM $MgCl_2$, 5 mM 2-mercaptoethenol, 10% [wt/vol] glycerol, 5 mM Mg-

ethylenediaminetetraacetic acid [1:1] chelate, pH 7.5, at 20°C) which had been made 1 M in KCl. Spores were washed two more times with buffer III, which was made 0.2 mM in the protease inhibitor diisopropylfluorophosphate, but which lacked KCl. Washed spores were broken as previously described (7) and suspended in buffer III, and ribosomes were isolated and analyzed as described above.

In vitro protein synthesis assay. Two reaction mixtures were used for assay of incorporation of phenylalanine into trichloroacetic acid-insoluble protein. Reaction mixture A (total volume, 0.5 ml), used in early experiments, contained the following concentrations of components: 100 mM NH_4Cl; 20 mM Tris-hydrochloride, pH 7.5; 10 mM MgOAc; 5 mM spermidine; 4 mM 2-mercaptoethanol; 1 mM ATP; 0.2 mM GTP; 4 mM phosphoenolpyruvate; 40 mM each 20 amino acids; 0.66 μCi of [^{14}C]phenylalanine per ml; 0.6 mg of tRNA (*E. coli*) per ml; 0.2 mg of polyuridylic acid per ml; 20 μg of pyruvate kinase per ml; 6 to 10 A_{280} units of S100 proteins per ml; 16 A_{260} units of ribosomes per ml. Reaction mixture B (total volume, 0.5 ml), used in later experiments, contained the following concentrations of components: 100 mM NH_4Cl; 50 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES)-KOH, pH 7.0; 6 mM MgOAc; 4 mM spermidine; 4 mM 2-mercaptoethanol; 2 mM ATP; 0.2 mM GTP; 4 mM phosphoenolpyruvate; 50 mM each 21 amino acids; 0.76 μCi of [^{14}C]phenylalanine per ml; 0.6 mg of tRNA (*E. coli*) per ml; 0.2 mg of polyuridylic acid per ml; 40 μg of pyruvate kinase per ml; 1.6 mg of S100 protein per ml; 1.6 A_{260} units of ribosomes per ml. Reactions were incubated at 37°C, and samples were taken to follow the time course of phenylalanine incorporation. A detailed description of the preparation of S100 proteins, sample handling technique, and optimization of reaction mixture B will be published elsewhere (Douthit et al., manuscript in preparation).

RESULTS

Of the several defects ascribed to ribosomes from spores of *B. cereus* T, the presence of degraded subunits having abnormal sedimentation rates in low-Mg^{2+} sucrose gradients (11) seems most indicative of severe damage to ribosome structure. Lack of protein-synthetic activity or tRNA binding (13) could result from any number of minor injuries or specific inhibitions, and measurements of low protein content of spore ribosomes (11) are subject to ambiguities arising from specific and nonspecific adsorption to ribosomes of nonribosomal proteins. Since preliminary studies revealed that ribosomal particles having abnormal sedimentation behavior occur to some extent in extracts obtained from spores by the dry disruption technique, studies were undertaken to describe this phenomenon quantitatively and to attempt the isolation of stable ribosomal subunits from spores.

Subunit stability of ribosomes from vegetative cells. It has been established that bacterial ribosomes dissociate into 50S and 30S subunits in buffers of sufficiently low Mg^{2+} concentration and that the subunits themselves "unfold" in discrete steps at even lower Mg^{2+} levels (19, 22). The particular Mg^{2+} levels at which such transformations occur are influenced by many factors, including the functional state of the ribosome and the ionic properties of the suspending buffer aside from the Mg^{2+} concentration. To establish the Mg^{2+} levels required for such transformations in buffer I, we analyzed highly active ribosomes from vegetative *B. cereus* cells on sucrose gradients made in buffer I but containing various Mg^{2+} concentrations. Such ribosomes sedimented as 70S particles at 10 mM Mg^{2+}, were partially dissociated into subunits at 3 mM Mg^{2+}, and were completely dissociated at 1.0 mM Mg^{2+}. The subunits themselves were stable at Mg^{2+} levels as low as 0.3 mM Mg^{2+}, but unfolding of the 50S subunit was evident at 0.1 mM Mg^{2+} (Fig. 1).

Subunit stability of ribosomes from spores. When ribosomes were extracted from spores by the dry disruption technique and analyzed on gradients containing various Mg^{2+} concentrations, their behavior differed markedly from vegetative ribosomes. Gradients containing 10 mM Mg^{2+} reveal polysomal material as previously reported (7, 8), which accounts for 25 to 35% of the ribosomal 260-nm-absorbing material. In addition to the polysomes and 70S ribosomes, however, a large amount of material is found in the 50S region of the gradient, and a minor 30S component is present (Fig. 6A, control). Whereas the 30S peak is usually quite small, the 50S peak often exceeds the 70S peak in size and is quite variable from one ribosome preparation to the next, amounting to 20 to 40% of the ribosomal 260-nm-absorbing material.

When analyzed on gradients containing 1.0 mM Mg^{2+}, ribosomes from spores do not provide the simple dissociation into subunits shown by vegetative ribosomes. Depending on as yet unidentified factors in the extraction technique, two types of patterns have been observed. The first (Fig. 2A) is characterized by an apparent loss of 30S subunits, assuming that 30S and 50S subunits occur in equal numbers in the spore. The second (Fig. 2B) is characterized by the presence of variable amounts of material sedimenting at about 40S and 25S, in addition to the 50S and 30S peaks. The pattern containing particles of abnormal sedimentation rate is similar to the pattern reported by Kobayashi (11), the abnormal peaks of which were shown to be degradation products of spore ribosomes rather than ribosomal precursors. The pattern with the abnormally high ratio of 50S/30S peak areas (Fig.

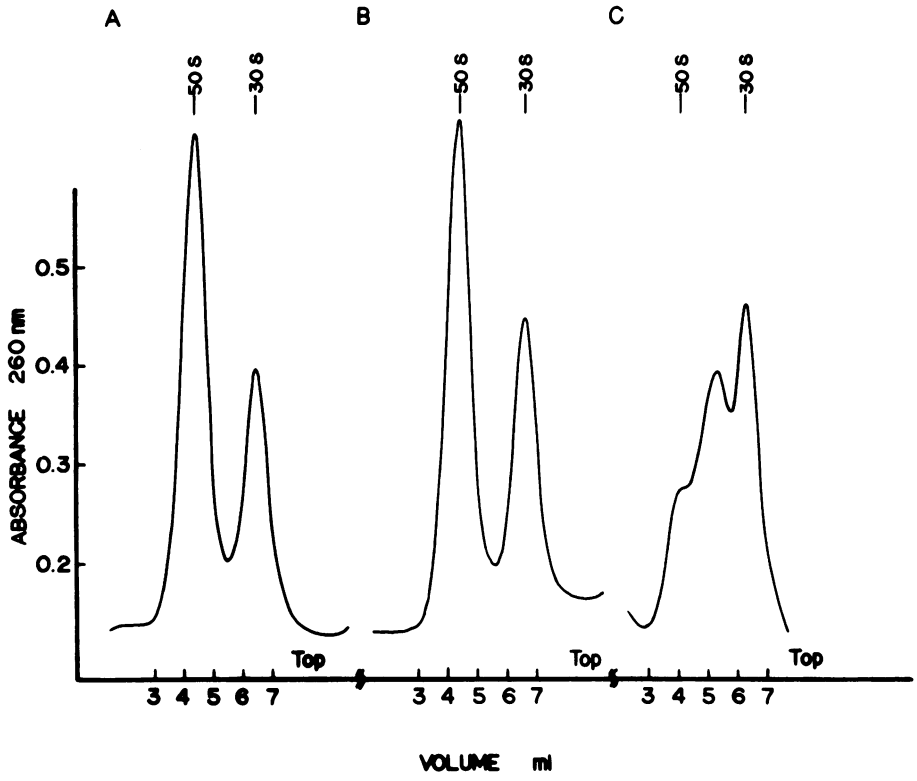

FIG. 1. *Sucrose gradient analysis of ribosomes from vegetative cells: SW41 rotor; 5 to 30% concave gradient; 40,000 rpm; 3.5 h; gradients in buffer I containing (A) 1.0 mM Mg^{2+}, (B) 0.3 mM Mg^{2+}, (C) 0.1 mM Mg^{2+}.*

2A) appears to result not from degradation of the 30S subunit, but from cosedimentation of aggregates of 30S subunits with the 50S subunit. This is suggested by the fact that 16S RNA, characteristic of the 30S subunit, could be isolated from the 50S peak (Fig. 3) and by the fact that mild treatment of spore extracts with RNase produced a shift of material from the 50S peak to the 30S peak (Fig. 4). It is also consistent with an empirical relationship which predicts that 30S dimers would sediment at 47S (19).

The abnormal pattern seen in Fig. 2B becomes more pronounced when ribosomes from spores are analyzed on sucrose gradients containing Mg^{2+} concentrations less than 1.0 mM. As the Mg^{2+} approaches 0.3 mM, the limit of stability of subunits from vegetative ribosomes, preparations from spores show an increasing loss of 50S subunits, with a parallel increase in the quantity of the abnormal 40S and 25S particles (Fig. 2C). There is considerable variability in the patterns at any one Mg^{2+} concentration, in terms of the amounts of the abnormal particles and the extent of loss of the 50S subunit. On the assumption that subunits are equimolar in the spore, with the 50S subunit ideally accounting for 67% of the 260-nm-absorbing material, it appears that 30 to 60% of the 50S subunits remain intact as 50S entities at 0.3 mM Mg^{2+}, with the remainder sedimenting at the abnormally slow rate(s).

Although the conditions of centrifugation differ slightly in experiments reported in Fig. 1 and 2, the ionic conditions were identical (buffer I), and pressure effects were extremely similar (40,000 rpm in the SW41 rotor). The small differences in centrifuge times would have altered slightly the position to which peaks had migrated and would have been accompanied by small pressure differences from one gradient to the next. However, experiments designed to test whether this was a cause for concern have shown that such small pressure differences could not have caused detectable alteration in peak shape. Thus, a direct comparison can be made of these gradients.

Fractionation of the spore ribosome population. Some insight into the properties of the ribosome population in dormant spores was obtained by fractionating the population of polysomes and subunits observed in 10 mM Mg^{2+}, followed by tests of each fraction's stability in

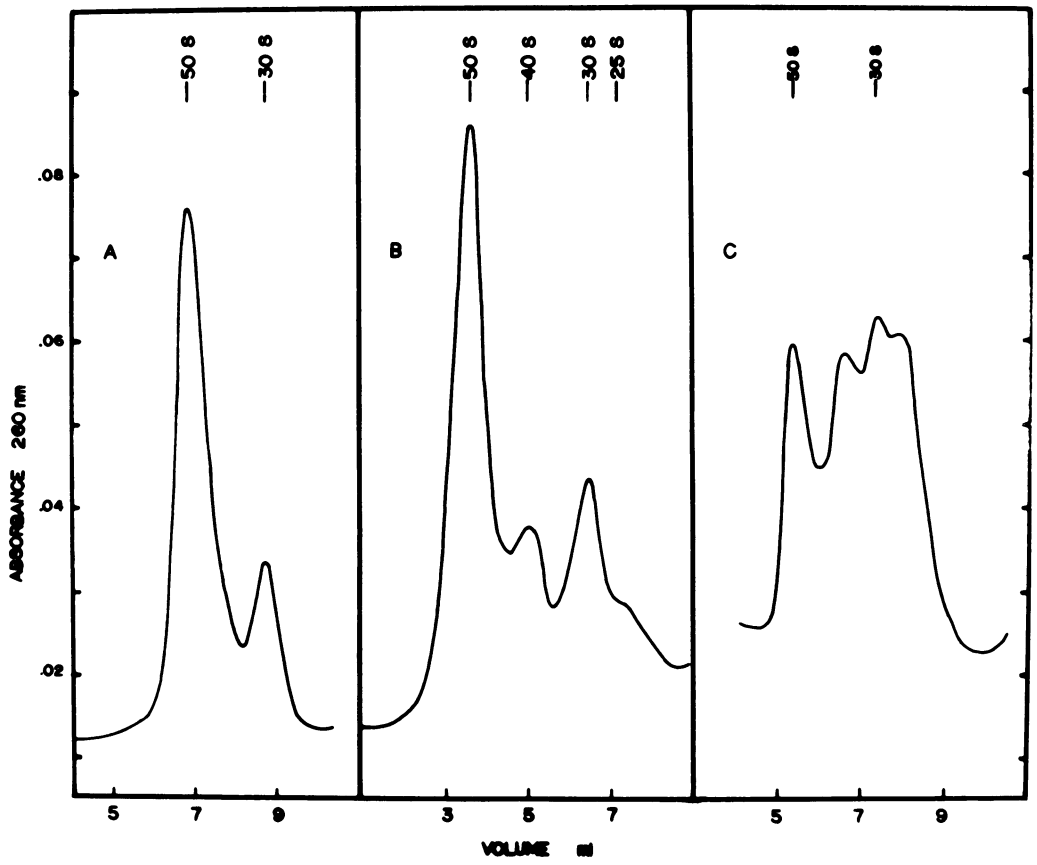

FIG. 2. *Sucrose gradient analysis of crude extract (S30) from spores: SW41 rotor; 10 to 27% isokinetic gradient; 40,000 rpm, gradients in buffer I containing 1.0 or 0.3 mM Mg^{2+}. (A) Preparation 6/30/75, centrifuged for 4 h in 1.0 mM Mg^{2+}; (B) preparation 2/6/76, centrifuged for 5 h in 1.0 mM Mg^{2+}; (C) preparation 7/10/75, centrifuged for 5 h in 0.3 mM Mg^{2+}.*

low-Mg^{2+} gradients. An enrichment for polysomes as described in Materials and Methods was used to produce a mixture of ribosomes containing 62% polysomes and 38% (70S + 50S + 30S) ribosomes. When this population was analyzed on gradients containing low Mg^{2+} levels, the particles of abnormal sedimentation rate characteristic of the total ribosome population were no longer detectable. In gradients containing 1.0 mM Mg^{2+}, the ratio of 50S to 30S peak areas was too high, as was often the case in analyses of the total population at this Mg^{2+} level. Again, this was a result of cosedimentation of 30S oligomers with 50S subunits, as evidenced by the extraction of 16S rRNA from the 50S subunit peak (data not shown).

When the polysome-enriched fraction was analyzed on gradients containing Mg^{2+} concentrations lower than 1.0 mM, dissociation of the 30S oligomers occurred, and subunit peaks of the proper size ratio were consistently obtained at 0.3 to 0.4 mM Mg^{2+}. This was the first indication that subunits of normal sedimentation rates could be isolated from spores of *B. cereus*. Fractions taken from the two peaks obtained at 0.3 mM Mg^{2+} and resedimented at 1.0 mM Mg^{2+} yielded single subunit peaks only slightly cross-contaminated with the other subunit. Confirmation of the identity of the isolated subunits followed from electrophoretic analysis of RNA extracted from the peaks, which revealed 23S and 16S rRNA characteristic of the respective 50S and 30S subunits (data not shown). When analyzed on gradients containing 0.1 mM Mg^{2+}, the polysome-enriched fraction revealed a pattern of subunit degradation similar to that seen with ribosomes from vegetative cells under the same conditions.

These results suggest that the stability of subunits obtained from polysomes is equal to that of subunits from vegetative cells. The source of the subunits responsible for the degraded pat-

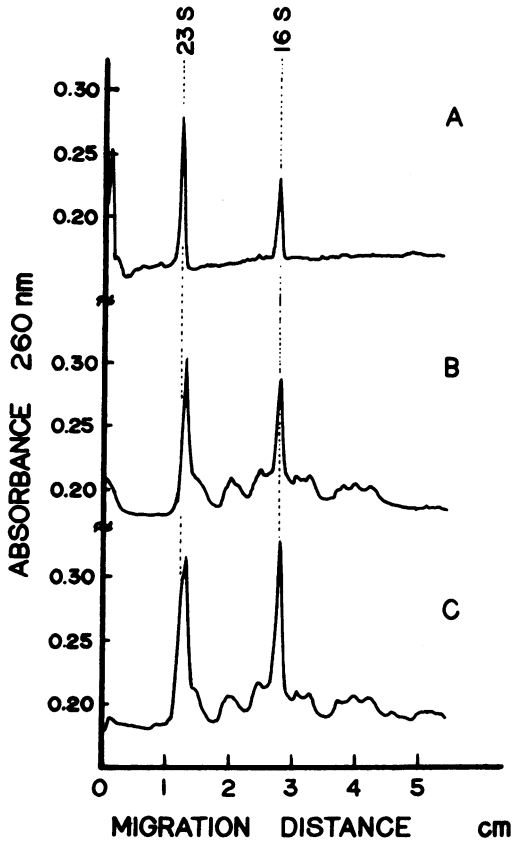

FIG. 3. *Polyacrylamide gel electrophoresis of rRNA from spore 50S fraction. Particles isolated from the 50S fraction of (A) were sedimented (314,000 × g, 8 h), resuspended in buffer II for dissociation with sodium dodecyl sulfate as described in the text, and then subjected to electrophoresis with E. coli D_{10} ribosomes as markers. (A) 0.03 A_{260} unit of E. coli ribosomes; (B) 0.09 A_{260} unit of 50S fraction from 1.0 mM Mg^{2+} gradient of B. cereus T spore ribosomes; (C) mixture of (A) and (B) subjected to electrophoresis together.*

terns obtained in low-Mg^{2+} analyses of the whole ribosome population appeared to be the fraction of spore ribosomes sedimenting at 70S or slower in 10 mM Mg^{2+} gradients. This was confirmed by isolating the 50S and 70S peaks from 10 mM Mg^{2+} gradients and analyzing them at low Mg^{2+} concentrations (free 30S subunits were not tested, since they are a minor component of the population, insufficient to account for the observed degradation). Whereas the 70S peak provided well-resolved subunits at both 1.0 and 0.3 mM Mg^{2+}, the 50S peak produced abnormal components sedimenting slower than 50S subunits (Fig. 5). At 0.3 mM Mg^{2+}, few intact 50S subunits remained in the fraction isolated as 50S at 10 mM Mg^{2+}. Thus, the majority of the subunits in the large 50S peak of spore extracts are unstable and account for the abnormal peaks found in low-Mg^{2+} analyses of the whole ribosome population. Oligomers of 30S subunits may contribute to the 50S peak at 10 mM Mg^{2+} in a manner similar to that seen in 1.0 mM Mg^{2+} gradients. However, the magnitude of this contribution is probably small, since RNase treatment of spore extracts results in only a minor reduction of 50S peak height at 10 mM Mg^{2+}.

Effects of protease inhibition. A possible cause of subunit instability is proteolytic degradation during ribosome extraction. To test this possibility, we washed spores with 1 M KCl to remove adsorbed proteases (21) and included the protease inhibitors Mg-ethylenediaminetetraacetate and diisopropylfluorophosphate during the ribosome extraction and analysis. The stabilities of polysomes and subunits were then examined on analytical sucrose gradients. Protected preparations contained more of the larger polysomes, and polysome breakdown in the unprotected control produced subunits rather than monomers (Fig. 6A). In low-magnesium gra-

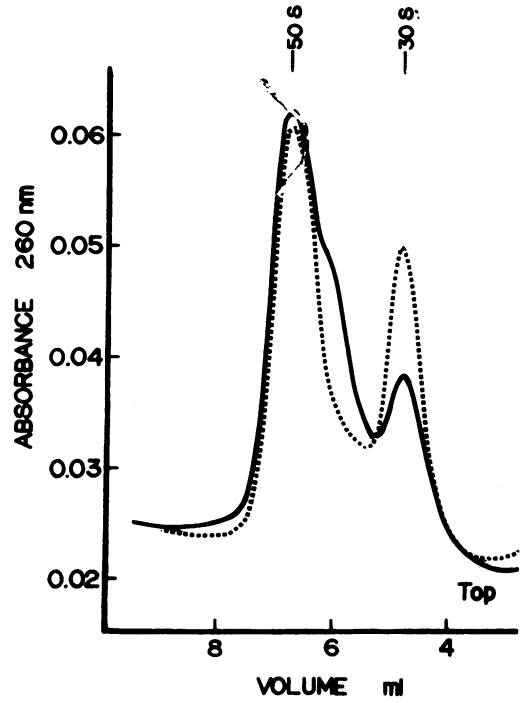

FIG. 4. *Sucrose gradient analysis of crude extract (S30) from spores: SW41 rotor; 10 to 27% isokinetic gradient; 40,000 rpm; 4.5 h, gradients in buffer I containing 1.0 mM Mg^{2+}. (Solid line) S30 control; (broken line) S30 incubated with 0.2 μg of RNase per ml for 10 min on ice prior to analysis.*

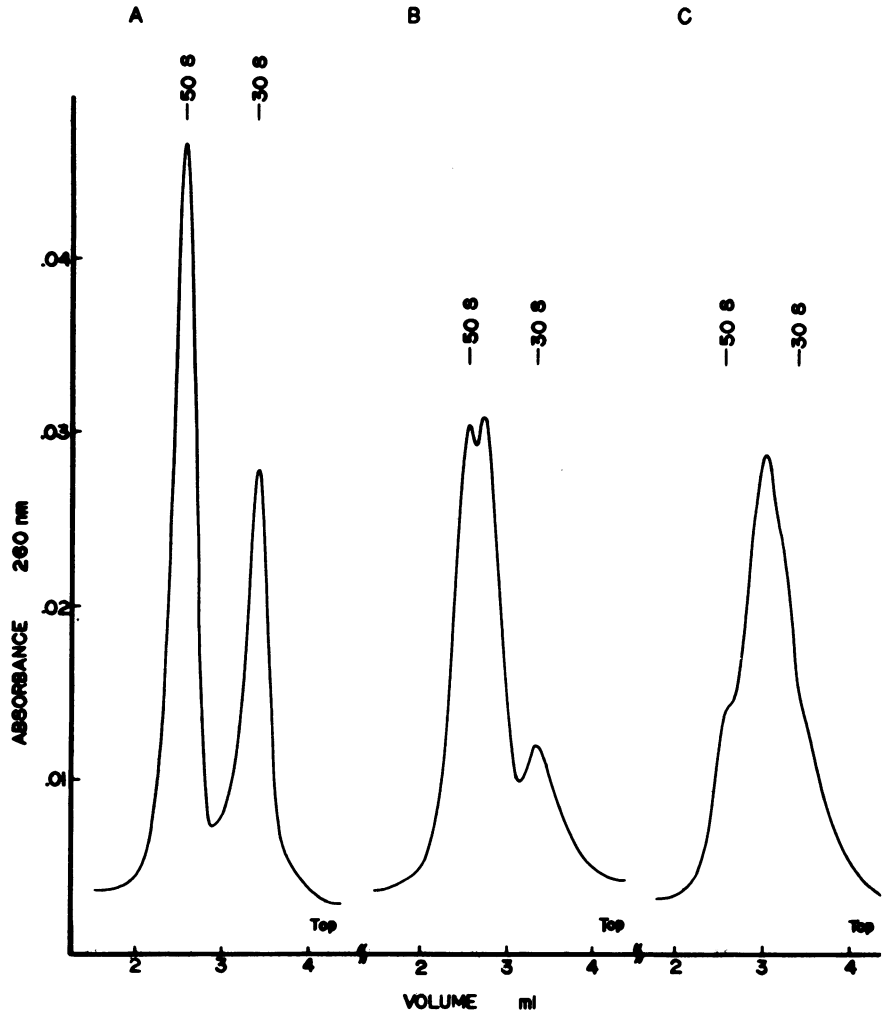

FIG. 5. *Sucrose gradient analysis of spore ribosomes: SW50.1 rotor; 10 to 30% isokinetic gradient; 45,000 rpm; 110 min. 70S and 50S fractions were collected from preparative gradients (containing 10 mM Mg^{2+}) of spore ribosomes. Fractions were sedimented (229,000 × g, 2 h), resuspended in buffer I, and analyzed on gradients containing 1.0 or 0.3 mM Mg^{2+}. (A) 70S fraction analyzed in 0.3 mM Mg^{2+}; (B) 50S fraction analyzed in 1.0 mM Mg^{2+}; (C) 50S fraction analyzed in 0.3 mM Mg^{2+}.*

dients, subunit breakdown was less severe in preparations protected from proteolysis (Fig. 6B).

In vitro protein-synthetic activities. To determine whether the dry-spore disruption technique would provide spore ribosomes of higher protein-synthetic activity than those obtained by other techniques, and to investigate the relationship between subunit instability and protein-synthetic activity, various fractions of the spore ribosomes were assayed for activity in a protein synthesis system. Representative results are shown in Table 1. The activity of unfractionated spore ribosomes is quite variable from one preparation to the next but averages about 32% of vegetative ribosome activity. This level of activity is about fivefold higher than previously reported for ribosomes from spores of *B. cereus* (11-13). The low activity observed appears to be partially related to the large population of unstable, free subunits in spores, which are almost totally inactive. However, the activity of the polysome-enriched fraction (containing stable subunits) is still only 35 to 53% as active as vegetative ribosomes, indicating the existence of abnormalities in spore ribosomes not necessarily associated with subunit instability in low-Mg^{2+} environments.

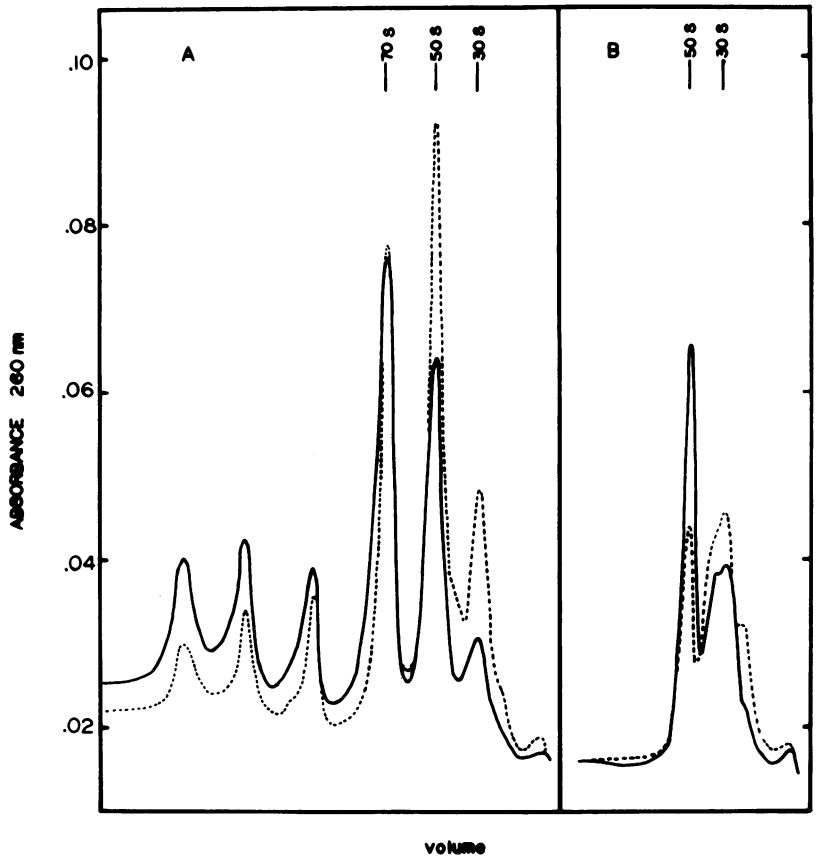

FIG. 6. *Effects of protease activity on sedimentation properties of spore ribosomes. Sucrose gradient analyses in 10 to 27% isokinetic gradients; SW41 rotor; 40,000 rpm; 110 min; 4°C. Gradient buffer was 100 mM NH_4Cl–20 mM Tris-hydrochloride (pH 7.5, at 4°C)–magnesium acetate as indicated–4 mM 2-mercaptoethanol. (A) Gradients containing 15 mM magnesium; (B) gradients containing 0.3 mM magnesium. (Broken line) Control; (solid line) protected from protease.*

DISCUSSION

The results of these experiments establish that many of the ribosomal subunits isolated from spores of *B. cereus* T by the dry disruption technique are stable in low-magnesium environments. Furthermore, the average in vitro activity of ribosomes isolated from spores by this technique is about fivefold higher than that observed using other techniques (11–13). In spite of these improvements, however, the present technique does not entirely eliminate the defects previously reported in ribosomes from *B. cereus* T spores. Our results confirm Kobayashi's reports (11, 12) of spore ribosomal particles having abnormal sedimentation coefficients and establish that the major source of such particles is the population of subunits which sediment as free 50S entities in sucrose gradients containing 10 mM Mg^{2+}. Subunits isolated from spore polysomes or 70S ribosomes, on the other hand, are as stable as vegetative subunits in low-Mg^{2+} environments. Thus, we can now prepare stable subunits from spores for further study of their protein-synthetic capabilities. Regardless of the stability of their subunits, however, polysomes isolated from spores still show impaired activity in protein synthesis.

The improved recovery of active, stable ribosomes from spores as a result of the dry-spore disruption technique makes it plausible that all of the defects of spore ribosomes could be artifacts of the extraction technique. The observations reported in this paper fit the simple hypothesis that, depending on still uncontrolled variables in the dry-spore disruption process, the 50S subunit of spore ribosomes is subject to varying degrees of degradation. Subunits only slightly damaged would be stable in low-Mg^{2+} sucrose gradients but would show reduced activ-

TABLE 1. *Activities of spore ribosomes*

Expt	Ribosomes	Activity[a] (pmol of phenylalanine/min per nmol of ribosomes)	% Vegetative
1-7[b]	Vegetative, unfractionated	50 ± 25	100
	Spore, unfractionated	16 ± 8	32
8[c]	Vegetative, unfractionated	410	100
	Spore, unfractionated	150	36
	Spore, polysomes	220	53
9[c]	Vegetative, unfractionated	310	100
	Spore, unfractionated	61	20
	Spore, polysomes	110	35
	Spore, free subunits[d]	13	4

[a] Activity calculated as initial rate of phenylalanine incorporation, based on the first 10 min of reaction, with the following assumptions: molecular weight of ribosome = 2.8×10^6; RNA = 50 $\mu g/A_{260}$ unit; rRNA/protein ratio = 1.7:1.

[b] Activity assay mixture A.

[c] Activity assay mixture B.

[d] Spore 50S and 30S ribosomal subunits collected from preparative sucrose gradients containing 10 mM Mg^{2+} in buffer I and present in assay mixture in an approximately equimolar ratio.

ity. Subunits more extensively damaged would be incapable of associating with 30S subunits and would occur as free 50S subunits in spore extracts at 10 mM Mg^{2+}. These free 50S subunits would be subject to unfolding at some reduced Mg^{2+} concentration determined by the extent and/or nature of the damage to the subunit. The source of the variation in survival of 50S subunits in these preparations is unknown. Hand grinding of dry spores with sand has been difficult to standardize and leads to variations in spore breakage and in the adsorptive properties of the colloidal abrasive. Both of these factors could lead to varying concentrations of degradative agents upon rehydration of ground spores; this is being tested with automatic grinding equipment and a variety of different types of abrasives.

The immediate cause of the degradation is also unknown. Proteolytic activity may be one source of degradation, since attempts to inhibit proteases did improve the sucrose gradient patterns of the ribosomes and subunits. The existence of proteolytic artifacts in *Bacillus* extracts has been documented (16, 21), and proteolytic attack would explain past observations of low protein content of *B. cereus* ribosomes (11) and stimulation of ribosome activity by added ribosomal protein (11). Another possible cause of ribosome degradation is related to the high calcium content of spores, which can amount to 3% of the spore dry weight (17) and is present in spore extracts in high concentrations. Calcium is known to be able to exchange freely with ribosomal magnesium in *E. coli* and to produce a specific set of ribosomal defects reminiscent of the properties of spore ribosomes described in this paper. These include partial loss of activity of the 50S subunit in protein synthesis, increased susceptibility of the 50S subunit to digestion by RNase, and changes in sedimentation rate of the 50S subunit involving the appearance of a particle sedimenting at about 40S (4, 23). In view of these findings, an analysis of possible calcium-induced alterations of *B. cereus* spore ribosomes has been initiated, including the possibility of synergistic calcium and proteolytic effects.

Further improvements in extraction methodology may lead to an increase in the activity of spore ribosomes, depending on the extent to which degradation during extraction is responsible for the defects of the ribosomes. We should point out, however, that attempts to induce changes of the sort mentioned above in vegetative ribosomes by incubating them with spore extracts, or by extracting spores and vegetative cells together, have not met with success. Therefore, we cannot rule out the possibility that spore ribosomes are subject to specific inhibitions having physiological significance for spore dormancy. For example, it remains possible that the low activity of our polysome-enriched fraction is not an artifact, as hypothesized above, but reflects a genuine spore-specific inhibition of ribosome function. The extent to which such an inhibition would influence ribosome properties cannot be predicted, but the identification and elimination of extraction artifacts is a necessary prerequisite to the detection of significant differences in the spore translation apparatus in vitro.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant 1R01-GM20296 from the National Institute of General Medical Sciences and by a grant (FRG Douthit) from the University of Michigan.

LITERATURE CITED

1. **Bishop, H. L., and R. H. Doi.** 1966. Isolation and characterization of ribosomes from *Bacillus subtilis* spores. J. Bacteriol. **91**:695-701.
2. **Bishop, H. L., L. K. Migita, and R. H. Doi.** 1969. Peptide synthesis by extracts from *Bacillus subtilis* spores. J. Bacteriol. **99**:771-778.
3. **Chambon, P., M. P. Deutscher, and A. Kornberg.** 1968. Biochemical studies of bacterial sporulation and germination. X. Ribosomes and nucleic acids of vegetative cells and spores of *Bacillus megaterium*. J. Biol Chem. **243**:5110-5116.
4. **Choi, Y. S., and C. W. Carr.** 1967. Ion-binding studies of ribonucleic acid and *Escherichia coli* ribosomes. J. Mol. Biol. **25**:331-345.
5. **Dalgarno, L., and J. Shine.** 1973. Ribosomal RNA, p. 115-116. *In* P. R. Stewart and D. S. Letham (ed.), The ribonucleic acids. Springer-Verlag, New York.

6. **Deutscher, M. P., P. Chambon, and A. Kornberg.** 1968. Biochemical studies of bacterial sporulation and germination. XI. Protein-synthesizing systems from vegetative cells and spores of *Bacillus megaterium*. J. Biol. Chem. **243**:5117-5125.
7. **Douthit, H. A., and R. M. Kieras.** 1972. Polyribosomes in spores of *Bacillus*, p. 264-268. *In* H. O. Halvorson, R. Hanson, and L. L. Campbell (ed.), Spores V. American Society for Microbiology, Washington, D.C.
8. **Feinsod, F. M., and H. A. Douthit.** 1970. Ribosomes from spores of *Bacillus cereus* T. Science **168**:991.
9. **Goodenough, V. W., and R. P. Levine.** 1970. Chloroplasts structure and function in ac-20, a mutant strain of *Chlamydomonas reinhardi*. J. Cell. Biol. **44**:547-562.
10. **Idriss, J. M., and H. O. Halvorson.** 1969. The nature of ribosomes of spores of *Bacillus cereus* T. and *Bacillus megaterium*. Arch. Biochem. Biophys. **133**:442-453.
11. **Kobayashi, Y.** 1972. Activation of dormant spore ribosomes during germination. II. Existence of defective ribosomal subunits in dormant spore ribosomes, p. 269-276. *In* H. O. Halvorson, R. Hanson, and L. L. Campbell (ed.), Spores V. American Society for Microbiology, Washington, D.C.
12. **Kobayashi, Y.** 1973. Characterization of ribosomes from dormant spores of *Bacillus cereus*. Agric. Biol. Chem. **37**:1929-1935.
13. **Kobayashi, Y., and H. O. Halvorson.** 1968. Evidence for a defective protein synthesizing system in dormant spores of *Bacillus cereus*. Arch. Biochem. Biophys. **123**:622-632.
14. **Kobayashi, Y., W. Steinberg, A. Higa, H. O. Halvorson, and C. Levinthal.** 1965. Sequential synthesis of macromolecules during outgrowth of bacterial spores, p. 200-212. *In* L. L. Campbell and H. O. Halvorson (ed.), Spores III. American Society for Microbiology, Ann Arbor, Mich.
15. **McCarty, K. S., Jr., R. T. Vollmer, and K. S. McCarty.** 1974. Improved computer program data for the resolution and fractionation of macromolecules by isokinetic sucrose density gradient sedimentation. Anal. Biochem. **61**:165-183.
16. **Maurizi, M. R., and R. L. Switzer.** 1975. Apparent inactivation of inosine 5'-monophosphate dehydrogenase in sporulating *Bacillus subtilis* is an artifact of in vitro proteolysis. J. Bacteriol. **123**:1269-1272.
17. **Murrell, W. G.** 1967. The biochemistry of the bacterial endospore. Adv. Microb. Physiol. **1**:133-251.
18. **Noll, H.** 1969. Polysomes: analysis of structure and function, p. 101-179. *In* P. N. Campbell and J. R. Sargent (ed.), Techniques in protein biosynthesis. Academic Press Inc., New York.
19. **Spirin, A. S., and L. P. Gavrilova.** 1969. The ribosome, p. 69-71. Springer-Verlag, New York.
20. **Stutz, E., and H. Noll.** 1967. Characterization of cytoplasmic and chloroplast polysomes in plants: evidence for three classes of ribosomal RNA in nature. Proc. Natl. Acad. Sci. U.S.A. **57**:774-781.
21. **Tesone, C., and A. Torriani.** 1975. Protease associated with spores of *Bacillus cereus*. J. Bacteriol. **124**: 593-594.
22. **Van Holde, K. E., and W. E. Hill.** 1974. General physical properties of ribosomes. p. 53-91. *In* M. Nomura, A. Tissieres, and P. Lengyel, (ed.), Ribosomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Weiss, R. L., B. W. Kimes, and D. R. Morris.** 1973. Cations and ribosome structure. III. Effects on the 30S and 50S subunits of replacing bound Mg^{2+} by inorganic cations. Biochemistry **12**:450-456.