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Ribosomal Complexes from an Extremely Halophilic Bacterium and the Role of Cations[1]

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Concentrated extracts of *Halobacterium cutirubrum* were prepared at 0 C by gently disrupting cells with a nonionic detergent in a medium containing 3.0 M KCl, 0.5 M NH_4Cl, and 0.04 M (or more) magnesium acetate and then treating the gelatinous mass with deoxyribonuclease. On KCl-sucrose gradients containing 0.5 M NH_4Cl and 0.04 M magnesium acetate, these extracts showed 30*S* and 50*S* ribosomal subunits plus a flat profile of faster-sedimenting material up to high *S* values. Only after frozen storage or brief incubation of the extract were 70*S* ribosomes and distinct classes of small polyribosomes detected. Digestion with ribonuclease converted faster-sedimenting material to 70*S* particles. The presence of chloramphenicol during preparation of the extracts did not affect these results. The evidence suggests that ribosomal particles exist in these cells as subunits or as polyribosomes but not as 70*S* ribosomes. To investigate the function of Mg^{++} and NH_4^+ ions in ribosomal complexes from this halophile, concentrated cell extracts and extracts incubated with ^{14}C-leucine were examined on KCl-sucrose gradients containing different concentrations of these ions. Polyribosomes and the bulk of 70*S* ribosomes dissociated reversibly to subunits at about 0.01 M Mg^{++}, whereas a small fraction of the 70*S* particles, including those which in vitro incorporated ^{14}C-leucine into nascent protein, dissociated only below 1 mM Mg^{++}. Below this concentration of Mg^{++}, nascent protein remained attached to the 50*S* subunit even at 0.04 mM Mg^{++} in the presence of 0.35 to 0.5 M NH_4Cl. Nascent protein, presumably as peptidyl-transfer ribonucleic acid, dissociated reversibly from 50*S* subunits only at 0.04 mM Mg^{++} and 0.1 M or less NH_4^+. Thus, the stability of polyribosomes from *H. cutirubrum* depends specifically on both Mg^{++} and NH_4^+ ions.

Two aspects of protein synthesis about which our understanding is limited are, firstly, the proportion of ribosomes in a cell that at any instant are associated with polyribosomes and are actively synthesizing protein, and, secondly, the nature of the forces binding components of the polyribosome together.

In general, density gradient analyses of homogenates from a variety of cells have shown polyribosomes and significant amounts of 70*S* ribosomes and ribosomal subunits (6, 9, 17, 22-24). In many of these preparations, the profile of polyribosomes showed a peak in the region of ribosomal pentamers to decamers. A qualitatively similar picture was obtained recently by Flessel, Ralph, and Rich (4) on homogenates of bacteria, except that these authors found a higher percentage (85 to 90) of the ribosomal material as polyribosomes than previous workers. Somewhat different results were obtained with easily lysed forms of *Escherichia coli* by Mangiarotti and Schlessinger (10). These authors found absorbance peaks for ribosomal subunits, but ahead of these on the gradients there was essentially uniform absorbance up to polyribosomes of very large size. Mangiarotti and Schlessinger (10) concluded that few if any 70*S* ribosomes exist in vivo and that about half the total ribosomes in the cells they studied exist as 30*S* and 50*S* ribosomal subunits. This and later evidence (11, 19) suggested that the cell contains a pool of 30*S* and

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50S subunits which associate with one another only during synthesis on the polyribosome. This concept has found further support from recent studies with *E. coli* systems (5, 13, 14) which show that, during chain initiation, 30S and 50S subunits enter the synthesizing complex independently.

Among the factors affecting the stability of polyribosomes, the importance of cations has been recognized for some time. In *E. coli*, Mg^{++} ions are involved not only in binding 30S and 50S subunits together to form active ribosomes (7, 18), but also in the binding to the ribosomes of messenger ribonucleic acid (mRNA; 15, 16) and of nascent protein, probably as peptidyl-transfer ribonucleic acid (tRNA; 18). Under the influence of mRNA, the attachment of specific amino acyl tRNA to ribosomes is dependent on K^+ or NH_4^+ ions (21). The precise function of these different ions has not been determined, and whether they provide ionic links between macromolecular components or act nonspecifically to neutralize negative charges, as suggested for mRNA binding (12), remains an open question.

Recently, a cell-free amino acid-incorporating system was developed from an extremely halophilic bacterium, *Halobacterium cutirubrum* (1; Griffiths and Bayley, *in preparation*). In this system, which functions only in saturated salt solutions, ionic interactions between the protein and nucleic acid components of the machinery for synthesizing protein must be reduced to a minimum by the abundant ions available. It was interesting, therefore, to compare the ionic requirements of the ribosomal complexes in this system with those in nonhalophilic systems.

This paper reports observations made with density gradients on the distribution of polyribosomes and ribosomal particles in gently prepared homogenates of *H. cutirubrum* and on the behavior of ribosomal complexes from this bacterium under different cationic conditions.

MATERIALS AND METHODS

Bacteria. H. cutirubrum was grown at 37 C in the medium described by Sehgal and Gibbons (20), except that 10 μg (per ml) of Fe^{++} (as $FeSO_4$) was added and the final *p*H was adjusted to 6.5. The principal inorganic salts in this medium were: 4.3 M NaCl, 0.027 M KCl, and 0.08 M $MgSO_4$. Cells were harvested during the early exponential growth phase at an absorbance at 660 nm (A_{660}) of 0.14 to 0.15.

Cell extracts. (i) Concentrated cell extracts were prepared in the cold by pouring 2 volumes of culture over 1 volume of frozen 4.5 M NaCl–0.03 M KCl–0.1 M $MgSO_4$ and sedimenting the cells at 6,800 × *g*. The cells were washed in cold 3.0 M KCl–0.5 M NH_4Cl–0.02 M tris(hydroxymethyl)aminomethane (Tris) chloride buffer (*p*H 8.05) with magnesium acetate as specified below. One gram (wet weight) of cells was suspended in the same solution to a total volume of 3 ml and was then mixed with 50 μliters of Cutscum (isooctylphenoxypolyoxyethanol; Fisher Scientific Co., Pittsburgh, Pa.) by drawing the suspension three times into a 10-ml syringe through a short, 2-mm diameter, Tygon tube. (The shearing of polyribosomes was negligible, as six subsequent passages of the cell extract through a tube one-fifth of this diameter produced no change in the ribosomal distribution.) Ten minutes later, the suspension was centrifuged in a Spinco no. 40 rotor at 30,000 rev/min for 30 min to yield a gelatinous mass beneath a liquid. This liquid was discarded and 500 μg of deoxyribonuclease (free of ribonuclease; Worthington Biochemical Corp., Freehold, N.J.), dissolved in a minimal volume of suspending salt solution, was added to the gelatinous sediment and mixed by five passes through the Tygon tubing syringe assembly. Thirty minutes later, the suspension was clarified for 10 min at 25,000 rev/min. The supernatant fraction constituted the concentrated cell extract.

(ii) Cell extracts for amino acid incorporation were prepared from washed cells broken with a Teflon-glass homogenizer as described by Bayley and Griffiths (1). The homogenate was centrifuged at 30,000 rev/min and the supernatant extract was dialyzed against 3.4 M KCl–0.1 M $MgCl_2$–0.01 M Tris chloride (*p*H 7.7). ^{14}C-leucine (30 to 34 mc/mmole; New England Nuclear Corp., Boston, Mass.) was incorporated into this extract by incubating it with sodium adenosine triphosphate (0.3 μmole); sodium phosphoenolpyruvate (1.2 μmole); sodium or lithium guanine triphosphate (0.18 μmole); and 19 other amino acids (0.008 μmole of each) in the presence of 3.8 M KCl; 0.89 M NH_4Cl; 0.11 M $(NH_4)_2SO_4$; 0.04 M magnesium acetate; 0.03 M Tris-chloride buffer, *p*H 8.05 [Mixture I, of Bayley and Griffiths (1)], for 40 min. The mixture was then chilled.

To remove free ^{14}C-leucine and also, where necessary, to alter the concentrations of monovalent and divalent cations, incubated extracts were diluted threefold with cold 3.0 M KCl in 0.02 M Tris-chloride buffer (*p*H 8.05) containing appropriate concentrations of Mg^{++}, NH_4^+, or Na^+, and then dialyzed against three changes of 125 ml of the same solution for a total of 5 to 6 hr.

Polyribosomal material was digested with ribonuclease by exposing one A_{258} unit of cell extract (i.e., the amount of material which in 1 ml has $A_{258} = 1$) to 2 μg of ribonuclease (Worthington Biochemical Corp.) for 10 min at 0 or 37 C.

Density gradients. The essential requirement in this work was to produce density gradients that were stable at 0 to 4 C and yet contained concentrations of salt as similar as possible to those in the amino acid incorporation system. It was found that satisfactory results could be obtained by using a sucrose gradient containing the required concentrations of NH_4Cl and magnesium acetate and nearly saturating concentrations of KCl.

Linear, 31-ml gradients were prepared in the cold with a Beckman gradient maker by mixing 2.75 M KCl (low density) with 2.5 M KCl and 35% (w/v)

sucrose (high density), both containing 0.1 M magnesium acetate, 0.5 M NH_4Cl, and 0.02 M Tris-chloride buffer (*p*H 8.05). The top of the resulting gradient contained 2.69 M KCl and 9% (w/v) sucrose, the bottom 2.54 M KCl and 29% (w/v) sucrose. At lower concentrations of magnesium acetate and NH_4Cl, the same gradient densities were maintained by adding extra sucrose; this amounted to 2% (w/v) at 0.04 mM magnesium acetate and 4% (w/v) in the absence of NH_4Cl. Reagent grade chemicals were used throughout and gradient solutions were filtered through washed 8 μm membrane filters (Millipore Corp., Bedford, Mass.), the smallest pore size that would pass the high-density sucrose solutions.

The ultraviolet (UV) absorption of blank density gradients was constant from the top down to the 25th ml, after which in some cases it increased (*see*, e.g., Fig. 2). This increased absorption was not eliminated by substituting analytical grade for reagent grade chemicals.

Density gradients were centrifuged in a Spinco SW 25.1 rotor at a bowl temperature of 10 F (12.2 C), after which they were floated out by pumping 70% (w/v) sucrose into the base of the centrifuge tube through a hypodermic needle. The A_{258} of the effluent was measured continuously in a 0.06-ml flow cell with a 2-mm path length on a Unicam spectrophotometer.

To examine the distribution of radioactivity through a gradient, 1-ml fractions of the effluent were collected and precipitated in the cold with 1 ml of 10% trichloroacetic acid, heated at 90 C for 15 min, and then cooled. The residues were collected on 1.2-μm Millipore filters and rinsed three times with 2 ml of 5% trichloroacetic acid. The filters were dried, placed in vials containing 13 ml of scintillation fluid [5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene per liter of toluene], and counted in a Beckman scintillation spectrometer.

The density gradients were calibrated with the heavier ribosomal subunit. Ribosomes were dissociated into subunits by lowering the Mg^{++} concentration to 4 mM and were then sedimented through a gradient containing the same Mg^{++} concentration. The portion of the gradient containing the heavier subunit was dialyzed against 3.4 M KCl, 0.1 M $MgCl_2$, 0.01 M Tris-chloride buffer (*p*H 7.7), and was then centrifuged at 50,000 rev/min for 2.5 hr. The pellet was suspended in a minimal volume of 4 M KCl, 0.1 M $MgCl_2$, and 0.01 M Tris-chloride buffer (*p*H 7.7). On gradients, this suspended material sedimented at the same rate as the heavier subunits in fresh ribosomal extracts, whereas, in the analytical ultracentrifuge, it behaved like 52*S* particles described earlier (3). From this calibration, the other peaks were readily identified with the nominal *S* values given in the figures.

RESULTS

Experiments with concentrated cell extracts. To obtain high concentrations of relatively undamaged polyribosomes, cells were disrupted gently and efficiently with a nonionic detergent (9), which dissolved the cell envelope. Unfortunately, these preparations were contaminated by the red pigment of the envelope. The pigment was not dialyzable, but it was partially removed in the centrifugation prior to deoxyribonuclease treatment; it remained at the top of the density gradients.

A cell extract, prepared in 3.0 M KCl, 0.04 M magnesium acetate, 0.5 M NH_4Cl, and Tris buffer, showed peaks of UV absorbance only for 30*S* and 50*S* ribosomal subunits along with UV-absorbing material spread uniformly to the bottom of density gradient (Fig. 1a). There was also a pellet which after suspension gave a UV absorption spectrum typical of nucleoprotein. After mild ribonuclease digestion and a further centrifugation, this material appeared as 70*S* ribosomes and ribosomal subunits (Fig. 1b). Cell extracts which had been frozen and stored under liquid nitrogen, stored for 24 hr at 2 C, or held at 37 C for 10 min, typically gave gradient profiles showing 70*S* ribosomes, dimers, trimers, and tetramers in addition to ribosomal subunits (Fig. 1c). The size of the pellets in these gradients was less than those in Fig. 1a. Mild ribonuclease digestion of cell extracts converted the dimers and faster-sedimenting polyribosomes into 70*S* particles (Fig. 1d).

To help stop protein synthesis rapidly during cell disruption, so as to prevent "run-off" of ribosomes from polysomes, extracts were prepared in the presence of 0.3 mM chloramphenicol, even though the amino acid-incorporating system showed almost no sensitivity to this compound (1). Results with these extracts were identical to those in Fig. 1.

The stability of polyribosomes at various Mg^{++} concentrations was studied with a cell extract stored under liquid nitrogen. Samples of this extract, which had been prepared in the presence of 100 mM magnesium acetate, were analyzed on density gradients containing 40, 4, or 0.4 mM magnesium acetate, respectively. In 40 mM magnesium acetate, monomers, dimers, and trimers were evident (Fig. 2a). As the Mg^{++} concentration was reduced, polyribosomes and then 70*S* ribosomes disappeared; only a few monomers remained at 4 mM Mg^{++} (Fig. 2b), and complete dissociation into 30*S* and 50*S* subunits occurred at 0.4 mM Mg^{++} (Fig. 2c). This dissociation was reversible since, on restoring the Mg^{++} concentration in an extract like that of Fig. 2c to 40 mM, monomers as well as dimers reappeared (Fig. 2d).

Experiments with cell extracts after ^{14}C-amino acid incorporation. Extracts were incubated with ^{14}C-leucine and then analyzed on density gradients to study the influence of cations on the compo-

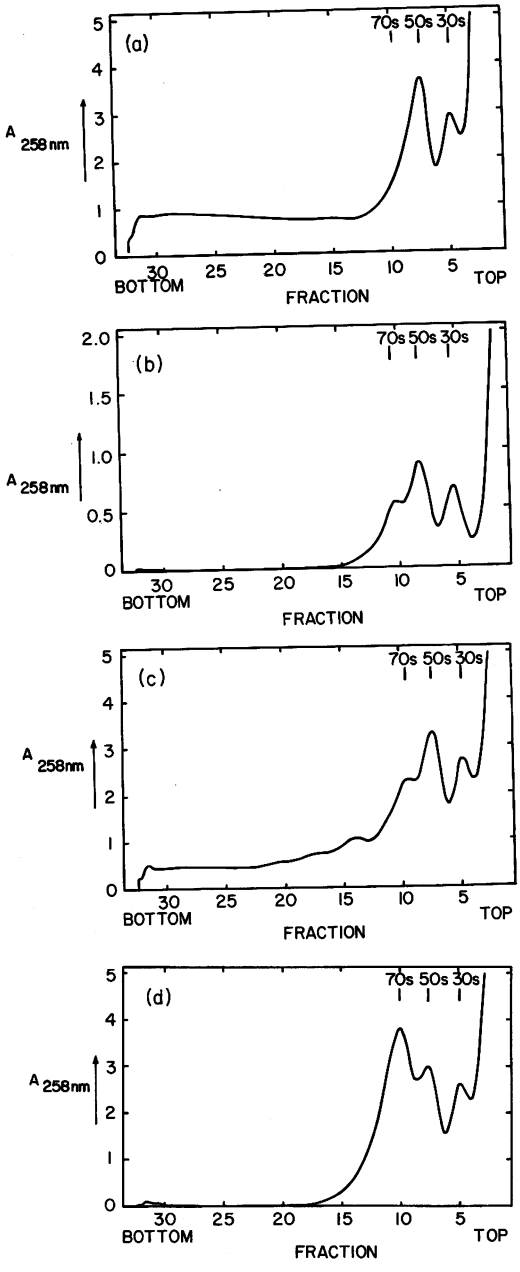

FIG. 1. *Density gradient analyses of concentrated cell extracts treated in different ways. A concentrated cell extract was prepared in 3.0 M KCl, 0.04 M magnesium acetate, 0.5 M NH_4Cl, and 0.02 M Tris-chloride buffer, (pH 8.05), and samples were analyzed on KCl-sucrose gradients containing the same concentrations of magnesium acetate and NH_4Cl as the extraction medium. Centrifugation was at 19,000 rev/min for 10 hr. Here and in Fig. 2, the amounts of sample analyzed were between 50 and 60 A_{258} units. (a) Extract maintained at 0 to 4 C throughout isolation. (b) The pellet from (a) after suspension in 1 ml of the extraction medium and digestion with ribonuclease for 10 min at 37 C. (c) Extract after incubation at 37 C for 10 min. (d) Extract after digestion with ribonuclease at 37 C for 10 min.*

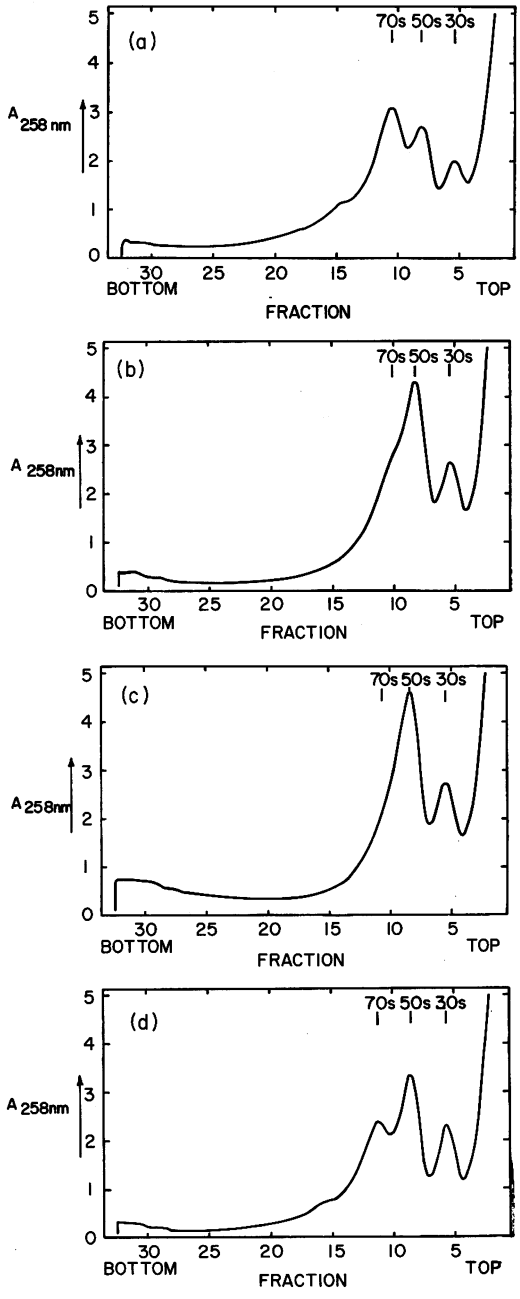

FIG. 2. *Effect of different Mg^{++} concentrations on polyribosomes and ribosomes. Samples of a concentrated cell extract, prepared in 3.0 M KCl, 0.1 M magnesium acetate, 0.5 M NH_4Cl, and 0.02 M Tris-chloride (pH 8.05) and stored under liquid nitrogen, were analyzed on KCl-sucrose gradients containing 0.5 M NH_4Cl and different concentrations of magnesium acetate. Extract in (a) 40 mM, (b) 4 mM, and (c) 0.4 mM magnesium acetate. In (d), the Mg^{++} concentration was lowered to 0.4 mM to dissociate ribosomes as in (c) and then raised by dialysis to 40 mM as in (a). All centrifugations were at 20,000 rev/min for 10 hr.*

nents in the protein-synthesizing complex that carried the label.

In a KCl-sucrose gradient containing 0.5 M NH_4Cl and 0.1 M magnesium acetate, the radioactivity profile showed slowly sedimenting material, monomer and dimer peaks, and a broad region of faster-sedimenting material (Fig. 3a). In a parallel incubation mixture treated with ribonuclease before centrifugation (Fig. 3b), the faster-sedimenting material had largely disappeared and there was a concomitant accumulation of radioactivity in the monomer region. No radioactivity peak was associated with either the 30*S* or 50*S* ribosomal subunits.

In studies paralleling those with concentrated cell extracts already described, incubated extracts were analyzed on gradients at different Mg^{++} concentrations. At 40 mM magnesium acetate, monomers, dimers, and faster-sedimenting material were evident in the distribution of radioactivity but were scarcely detectable in the UV profile (Fig. 4a). At 4 mM Mg^{++}, the only

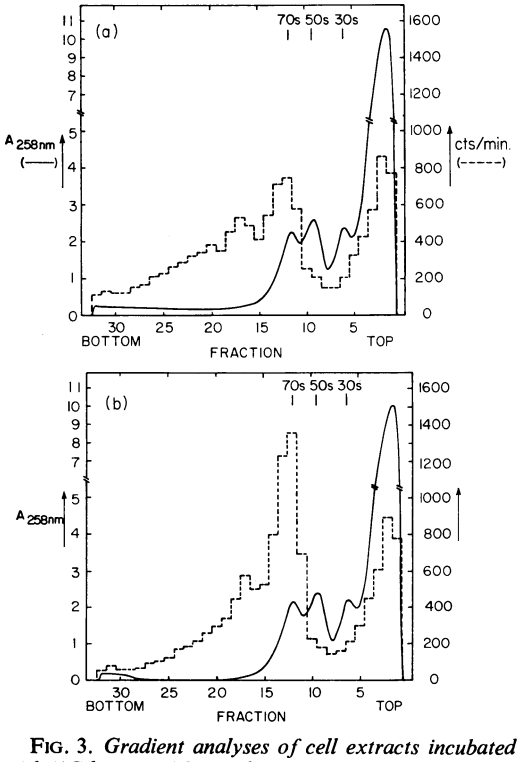

FIG. 3. *Gradient analyses of cell extracts incubated with ^{14}C-leucine. The KCl-sucrose gradients contained 0.5 M NH_4Cl and 0.1 M magnesium acetate. The incubated extract was (a) untreated; (b) digested with ribonuclease for 10 min at 0 C. In both cases, centrifugation was at 20,000 rev/min for 11.6 hr. In Fig. 3–6, samples of 32 to 35 A_{258} units were used.*

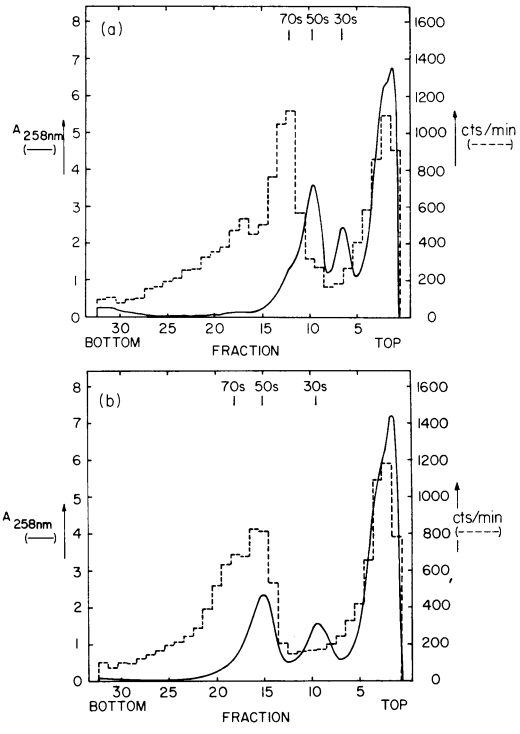

FIG. 4. *Effect of different Mg^{++} concentrations on cell extracts after ^{14}C-leucine incorporation. The KCl-sucrose gradients contained 0.5 M NH_4Cl and (a) 40 mM Mg^{++} and (b) 0.04 mM Mg^{++}. They were centrifuged at (a) 20,000 rev/min for 11.6 hr and (b) 22,000 rev/min for 15 hr.*

detected components sedimenting faster than ribosomal subunits were 70*S* ribosomes in the radioactivity profile alone; in the UV profile, only subunits were evident. On decreasing the Mg^{++} concentration to 0.4 mM, radioactivity became associated with the 50*S* subunits, and this association increased at the expense of the 70*S* peak at 0.04 mM Mg^{++} (Fig. 4b).

As a measure of the release of ^{14}C label from ribosomes, the radioactivity (counts/minute) from the top of the gradient down to the minimum UV absorbance between 30*S* and 50*S* ribosomal subunits was calculated as a percentage of the total counts/minute in the gradient. In gradients containing saturating amounts of KCl and concentrations of NH_4Cl and magnesium acetate at least equal to those in the reaction mixture, the radioactivity at the top of the gradient probably represented polypeptide chains completed and released during incubation. Any increase in this activity as the cationic conditions in the gradient were altered should then represent nascent protein released from ribosomal particles

as peptidyl-tRNA. In the series of gradients containing 0.5 M NH_4Cl (Table 1; Fig. 3a, 4a, 4b), there was only a 1.4-fold increase in this radioactivity on lowering the Mg^{++} concentration from 100 to 0.04 mM.

Since NH_4^+ ions are known to be involved in binding tRNA to *E. coli* ribosomes (21), their role in the halophile system was investigated. In 0.1 M NH_4Cl and 0.04 mM Mg^{++}, 79% of the radioactivity appeared at the top of the gradient (Table 1); only a small amount was associated with the 50*S* to 70*S* region. When NH_4Cl was omitted, 50% of the label appeared at the top of the gradient at 0.4 mM Mg^{++} (Fig. 5a, Table 1) but all of it was released from ribosomal particles at 0.04 mM Mg^{++} (Fig. 5b, Table 1). The binding of label to ribosomes therefore depends on both the Mg^{++} and the NH_4^+ concentration.

To test whether released label would bind to ribosomes on raising the Mg^{++} and NH_4^+ concentrations, a preparation was treated to release all the label as in Fig. 5b. Magnesium acetate and NH_4Cl were then added to 0.4 and 0.7 M, respectively, and the preparation was dialyzed against 0.04 M Mg^{++} and 0.5 M NH_4^+. After this treatment, 75% of the released radioactivity had returned to the ribosomal region of the gradient, with approximately equal proportions associated with 50*S* subunits and 70*S* ribosomes (Fig. 5c).

The ability of a cell extract to incorporate amino acids after similar treatment was tested as follows. A sample of cell extract was dialyzed for 3 hr against 3.4 M KCl, 0.04 mM magnesium acetate, and 0.01 M Tris-chloride (*p*H 7.7). Mg^{++} was then added to 0.13 M, and the sample was dialyzed against 3.4 M KCl, 0.1 M magnesium acetate, and 0.01 M Tris buffer. In Mixture I of Bayley and Griffiths (1) containing 3.8 M KCl, 1.1 M NH_4 salts, and 0.04 M magnesium acetate, the dialyzed preparation incorporated ^{14}C-leucine (275 mc/mmole; New England Nuclear Corp.) to

TABLE 1. *Distribution of radioactive label[a] in density gradients of H. cutirubrum ribosomal preparations under various ionic conditions*

Magnesium acetate concn	NH_4Cl concn (M)		
	0.5	0.1	0
mM			
100	29, 31, 28		
40	33		
4	38		
0.4	36, 37	47	51
0.04	35, 44	79	91, 87

[a] Expressed as the percentage of slowly sedimenting material (counts/minute), taken as the percentage from the top of the gradient down to the minimum absorbance between 30*S* and 50*S* subunits. When 0.35 M cation was added as NH_4Cl, KCl, and NaCl (the KCl concentration normally present being increased by 0.35 M), at 0.04 M magnesium acetate, the results were 53, 81, and 77 percentage counts/min, respectively.

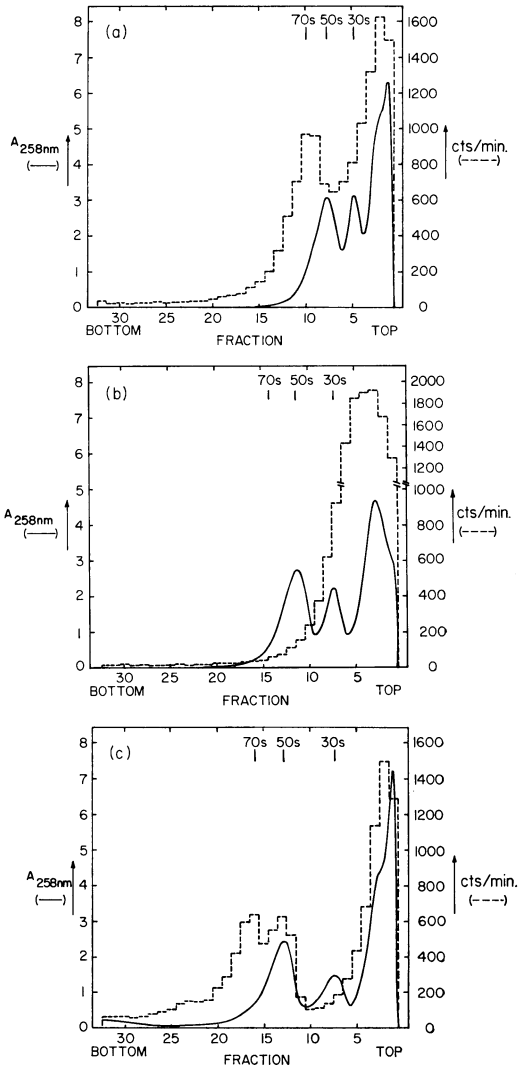

FIG. 5. *Reversible release of ^{14}C label from ribosomes. After incubation with ^{14}C-leucine, cell extracts were analyzed in KCl-sucrose gradients containing no NH_4Cl and (a) 0.4 mM and (b) 0.04 mM magnesium acetate. In (c), the sample was subjected to 0.04 mM magnesium acetate and no NH_4Cl as in (b) and then returned to 0.5 M NH_4Cl and 40 mM magnesium acetate for analysis. Centrifugations were (a) 20,000 rev/min for 11.6 hr; (b) 22,000 rev/min for 15 hr; and (c) 23,000 rev/min for 11 hr.*

the extent of 30% (3,995 counts/min) of a nondialyzed control (13,335 counts/min).

The binding of labeled material to 50*S* subunits at 0.04 mM magnesium acetate in the presence of 0.5 M NH_4Cl but not in its absence (Fig. 4b, 5b) may have been due either to a specific action of NH_4^+ ion or merely to the higher salt concentration in the gradients containing 0.5 M NH_4Cl. To distinguish between these alternatives, analyses were carried out on gradients containing 0.35 M NH_4Cl, NaCl, or KCl (Fig. 6); the 0.35 M KCl was in addition to that present in all gradients. (A concentration of 0.35 M rather than 0.5 M was chosen for these comparisons because this was the maximal additional amount of KCl which could be dissolved in the gradient solutions at 0.04 mM magnesium acetate.) With KCl or NaCl, there was no association of radioactivity with the 50*S* ribosomal subunit (Fig. 6a, 6b); this occurred only in the presence of NH_4Cl (Fig. 6c). Thus, the label appears to be bound specifically by NH_4^+ ions. The percentages shown in Table 1 reflect the same result but not as clearly as the gradients. This was because the calculations were affected by the rather high sedimentation rate, about 20*S*, of the released label in Fig. 6a and 6b.

DISCUSSION

Evidence from the present work and from studies on a cell-free amino acid-incorporating system (1; Griffiths and Bayley, *in preparation*) suggests that protein synthesis in *H. cutirubrum* follows a pattern similar to that in nonhalophilic systems. The present results will therefore be discussed in terms of the currently accepted model for protein synthesis.

Population of polyribosomes and ribosomal particles. In freshly prepared, concentrated cell extracts, UV measurements failed to detect distinct classes of polyribosomes or any 70*S* ribosomes (Fig. 1a). Only ribosomal subunits and a broader region of heavier material were found, despite repeated attempts to improve resolution on the gradients. Monomers, dimers, and trimers became apparent only after frozen storage (Fig. 2a) or after incubation (Fig. 1c; *see also* the radioactivity curve in Fig. 3a). Ribonuclease digestion destroyed heavier material to produce 70*S* particles (Fig. 1d). (Comparison of the UV profiles in Fig. 1b, 1d, and 3b shows that when polysomes were digested with ribonuclease, varying relative amounts of 70*S* ribosomes and ribosomal subunits were produced. The reason for this is not known; the variation may be due to the different treatments the samples of polysomes had received before digestion.)

The procedure adopted for preparing the concentrated cell extracts was mild and involved no mechanical disruption. The cells were broken in an ionic environment similar to that for amino acid incorporation (1) with a nonionic detergent to avoid upsetting the ionic balance. To prevent changes in the polyribosomal population due to continued synthesis during extraction, cells were cooled rapidly before harvesting; the addition of

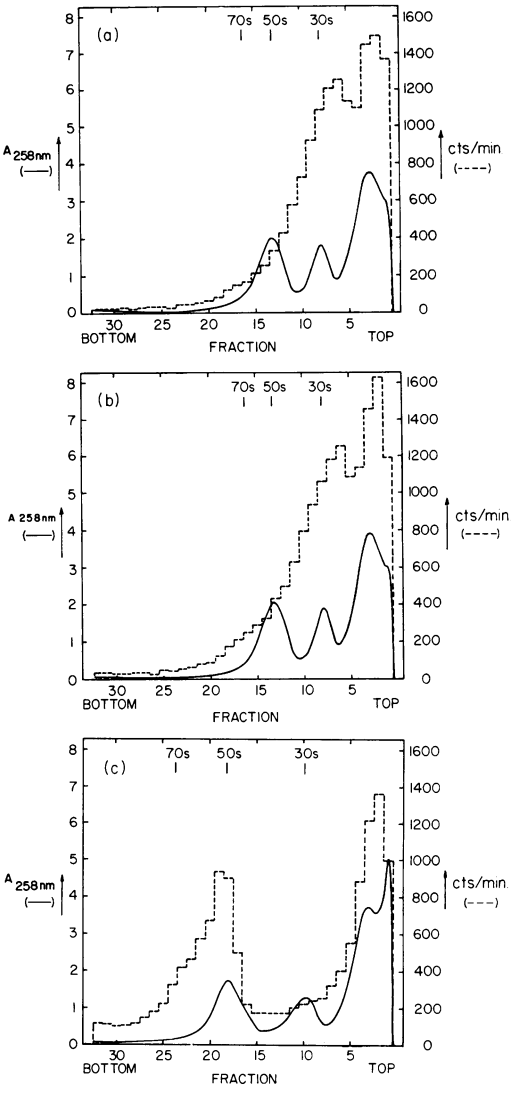

FIG. 6. *Influence of different monovalent cations on the binding of* ^{14}C *to 50S ribosomal subunits. Cell extracts incubated with* ^{14}C*-leucine were analyzed on KCl-sucrose gradients containing 0.04 mM magnesium acetate and (a) an additional 0.35 M KCl, (b) 0.35 M NaCl, or (c) 0.35 M* NH_4Cl*. Centrifugations were (a, b) 22,000 rev/min for 15 hr; (c) 21,500 rev/min for 14.5 hr.*

chloramphenicol had no effect on the results. In these cell extracts, digestion by cellular ribonuclease was probably slight, as the activity of this enzyme(s) is very low (D. J. Kushner, *private communication*).

It is likely, therefore, that the ribosomal population observed in these concentrated cell extracts reflects fairly well the population in the living cell. In extrapolating to conditions in vivo, however, allowance must be made for material discarded in the supernatant fraction before deoxyribonuclease digestion. The loss was probably greatest for the lighter ribosomal subunit. In a few experiments, the supernatant fraction was compared on gradients to the concentrated cell extract. Whenever 70*S* ribosomes as well as subunits were present in the extract, they were also found in the supernatant fraction. Thus, it seems unlikely that 70*S* monosomes had been preferentially discarded when these particles were not detected in the concentrated cell extract, as in Fig. 1a.

The evidence therefore suggests that, in vivo, the ribosomal population consists of subunits plus a wide range of sizes of polyribosomes, the heaviest of which penetrate the gradient to form a pellet (Fig. 1b). Only as these large polyribosomes "run down" on incubation or become degraded on storage are sufficient monomers, dimers, and trimers formed to be detectable. In contrast to *E. coli* ribosomes, there is no evidence that *H. cutirubrum* ribosomes alone will dimerize to form 100*S* particles at high Mg^{++} concentrations (3). The "native" 30*S* and 50*S* subunits found in fresh cell extracts (Fig. 1a) did not appear to bear nascent protein (Fig. 3), and the proportion of these subunits in an extract varied little with the treatment of the extract (Fig. 1a, 1c, 1d). Furthermore, many of these "native" subunits did not associate to form 70*S* particles at 0.1 M Mg^{++} (Fig. 3a; cf. Fig. 4a at 0.04 M Mg^{++}) or even at 0.4 M Mg^{++} (not shown).

Since all the cellular RNA was not recovered in the extracts, these studies are less accurate than those of Mangiarotti and Schlessinger (10) on fragile forms of *E. coli*. However, they lend support to these authors' conclusion that in vivo 70*S* particles are virtually absent, ribosomal subunits being either free in the cytoplasm or associated with one another as units of polyribosomes. Within the limits of accuracy of this work, absorbance measurements on gradients such as Fig. 1a suggest that a minimum of 40% of the ribosomal population exists as subunits. This is consistent with the 35 to 50% found by Mangiarotti and Schlessinger (10) but is significantly larger than the 10 to 15% of Flessel et al. (4). The uniform absorbance in the polyribosome region of Fig. 1a confirms a further conclusion of Mangiarotti and Schlessinger (10) that the number of polyribosomes of a given size in the population is inversely proportional to the number of ribosomes they contain. In this respect our results differ from those of Flessel et al. (4), who obtained a preponderance of smaller polyribosomes. Besides dissimilarities in the bacteria and extraction conditions used, this difference could be related to the metabolic state of the cells, since, unlike the other work, the present study was carried out on a wild-type strain growing in a complete medium.

Cation requirements of ribosomal complexes. The experiments at different cation concentrations were designed to examine the binding of both mRNA and peptidyl-tRNA to ribosomal particles.

It was hoped that the ionic conditions necessary for binding mRNA could be defined by using synthetic ^{14}C-labeled polyribonucleotides. A system from *H. cutirubrum* which is preincubated to destroy endogenous mRNA activity incorporates amino acids in response to added synthetic mRNA (2). Unfortunately, however, attempts to detect binding of preincubated ribosomes to ^{14}C-polyuridylic acid (Miles Chemical Co.) were unsuccessful, both on gradients and by filtering through Millipore filters.

Nevertheless, an estimate of the Mg^{++} concentration that is probably necessary for the attachment of ribosomes to mRNA was obtained from studies on native polyribosomes. It is clear from both the UV (Fig. 2) and the radioactivity measurements (Fig. 4a, 5c) that dimers of 70*S* dissociate reversibly at about 10 mM Mg^{++} to 70*S* and smaller particles. This is presumably due to reversible dissociation of ribosomes and mRNA, since, as mentioned above, there is no evidence that these ribosomes alone will dimerize at high Mg^{++} concentrations. At 10 mM Mg^{++}, the bulk of the 70*S* ribosomes also dissociate reversibly into subunits (cf. Fig. 2a, 2b, 2d). However, a small fraction of the 70*S* particles, including those which carry ^{14}C-labeled nascent protein and must therefore have been active during incubation of cell extract, resist dissociation up to a level of 1 mM Mg^{++} (Fig. 2b, 2c, 4). The extra stability of these ribosomes is probably due to the peptidyl-tRNA associated with them (19).

The concentrations of Mg^{++} just discussed are comparable to those found in *E. coli* (15, 16, 18).

Below 1 mM Mg^{++}, nascent protein remains attached to the 50*S* subunit, as in *E. coli* (8). However, contrary to earlier work with *E. coli*

(18), this nascent protein does not dissociate from the 50*S* subunit at 0.04 mM Mg^{++} when 0.35 to 0.5 M NH_4^+ is present. Release requires a suitably low level of both Mg^{++} and NH_4^+ ions and, since it is reversible, it presumably involves the dissociation of peptidyl-tRNA from the 50*S* subunit. Thus, the association of peptidyl-tRNA with ribosomes must be specifically dependent on both Mg^{++} and NH_4^+ ions. This result is consistent with the work of Spyrides (21) on *E. coli* and may not be inconsistent with the results of Schlessinger and Gros (18) which were obtained in the absence of monovalent cations.

The labeled peptidyl-tRNA that is released at 0.04 mM Mg^{++} in the absence of NH_4Cl sediments at about 15*S* to 20*S* (Fig. 5b, 6a, 6b). The reason for this is not known. Since the UV pattern is unaltered by the omission of NH_4^+, it seems unlikely that the peptidyl-tRNA has remained attached to the 50*S* subunit and that this whole complex has changed. It seems more likely that the peptidyl-tRNA molecules have aggregated under the conditions in the gradient.

In the present system, where there is an abundance of other cations, it is clear that Mg^{++} and NH_4^+ serve specific functions in the binding of mRNA and peptidyl-tRNA to ribosomes. This result, and the concentrations of Mg^{++} and NH_4^+ found to be necessary for polyribosomal stability, are consistent with observations on the amino acid-incorporating system (1). Furthermore, the present results with NH_4^+ ions help to explain the need for these ions in the transfer of amino acids but not in their activation (Griffiths and Bayley, *in preparation*).

The actual nature of the functions Mg^{++} and NH_4^+ ions serve in binding is still not clear. These ions, and Mg^{++} in particular, may form ionic linkages between phosphate groups on the RNA molecules involved. Much of the specificity of these ions may be due to their hydrated radii, which enable them to form linkages or even merely to neutralize charge in such a way that the polyanions can assume the secondary and tertiary structures necessary for activity. Whatever function they serve, however, these ions must clearly form an integral part of the macromolecular complex, since they cannot be in dynamic equilibrium with the excess of other cations (1).

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