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Stability and Homogeneity of Preparations of Ribosomal Particles from *Escherichia coli*†

Jennifer Littlechild and Michael Spencer*

ABSTRACT: Techniques have been developed for preparing ribosomal particles from *Escherichia coli* (MRE 600) so that the particles are unusually stable against autodegradation when kept unfrozen for long periods. Criteria for stability were constancy of RNA and RNP migration on polyacrylamide gels, and preservation of activity in poly(U)-directed incorporation of amino acid. Stability could be maintained for 1 week at 25° or 4 weeks at 4°. Gel migration was also used to assess the homogeneity of both monosome and subunit preparations. Evidence for multiple conformations was obtained, and some progress was made toward improving homogeneity.

Our aim in starting this work was to stabilize ribosomal particles from *Escherichia coli* for *in vitro* crystallization trials. For the same purpose we also set out to assess any lack of homogeneity in our preparations, not just for cross-contamination between different particles but for the existence of more than one conformation of each particle type. We believe that improved stability and homogeneity are prerequisites for any serious attempt to crystallize ribosomes; the work is also expected to be of some interest to other users of cell-free systems.

The part of a ribosome most sensitive to damage is its rRNA. Extracts of ribosomes always contain some endonucleases which attack the exposed regions of rRNA; such enzymes are more dangerous than exonucleases, since the latter can only attack one end of the molecule which may in any case be protected. Nucleases may in some cases form part of the ribosomal "split" protein fraction (Spahr, 1964; Szer, 1969). *E. coli* MRE 600, used in this work, is a mutant which contains only low levels of the endonuclease RNase I, but RNase II (an exonuclease) is present in usual amounts (Singer and Tolbert, 1965) and endonuclease IV has been found (Spahr and Gesteland, 1968). We have endeavored to reduce the activity of endogenous nucleases, and to distinguish their effects from those of nucleases introduced accidentally from outside during the preparations.

The stability requirements for crystallization are probably

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more stringent than those for retention of biological activity. Ribosomes containing degraded rRNA can still retain activity in a protein synthesizing system (Szer, 1969; Cahn *et al.*, 1970; Kuechler *et al.*, 1972). For crystallization, however, it is quite likely that the rRNA exposed on the surface must remain intact, because it is by surface interactions that particles arrange themselves in a crystalline lattice. Since ribosomes containing degraded rRNA can still appear intact, we have used polyacrylamide gel electrophoresis to examine extracted rRNA. We have also used this technique on ribosomal particles to assess their homogeneity.

Materials and Methods

E. coli MRE 600 cells were obtained as a frozen paste from the Microbiological Research Establishment, Porton, and were stored at −20° until required. They contained very few polyribosomes, since the harvesting technique led to the accumulation of runoff 70S particles (J. Littlechild, unpublished work).

Preparation of Ribosomes. Cells were disrupted by one of two methods, both using a ribosome buffer containing 0.1 M KCl–0.02 M $MgCl_2$–0.01 M Tris (pH 7.8). All operations were carried out at 4° and buffer solutions were autoclaved before use. The cells were washed in buffer before the preparation.

For *alumina extraction* cells were ground by hand for 10 min, after which buffer containing purified bentonite (Fraenkel-Conrat *et al.*, 1961) at 1 mg/g of cells was added, and the monosomes were separated by differential centrifugation. Mechanical grinding was tried, but found to produce damaged ribosomes. The yield was 0.2–1.0 g of rRNA from 100 g wet weight of cells.

For *lysozyme extraction* the method of Ron *et al.* (1966) was used, with the addition of purified bentonite, as above, after the second freeze–thaw cycle. Ribosomes were separated from the supernatant by differential centrifugation incorporating steps to remove free subunits and polysomes. The yield obtained was about 3 g of rRNA from 100 g wet weight of cells. The ribosomes were stored as pellets at −20°.

Ribosomal Subunits. Fractionation was by centrifugation in a 10–25% w/v sucrose gradient using a Beckman SW 25.2 rotor (17 hr at 21,000 rpm) or the Ti14 zonal rotor (2 hr at 48,000 rpm). In later experiments a hyperbolic gradient (Eikenberry *et al.*, 1970) was adapted for the Ti14 zonal rotor. Ribosomes containing 100 mg of RNA were treated in each run. Sucrose solutions were made up in autoclaved buffer and treated with bentonite before use. *Low Mg^{2+} subunits* were isolated in 0.0005 M $MgCl_2$–0.01 M Tris (pH 7.8); *high salt subunits* were isolated in 0.8 M KCl–0.01 M $MgCl_2$–0.01 M Tris (pH 7.8). Subunit fractions were later concentrated by centrifugation and stored at −20°.

rRNA Extraction. rRNA was extracted from ribosomal particles with phenol in the presence of bentonite, or from whole cells by the method of Robinson and Wade (1968) with the addition of further deproteinization steps with phenol and bentonite to eliminate RNase contamination. The material was stored as a dry powder at −20°.

Gel Electrophoresis. Cylindrical gels of 0.7-cm diameter were made with a composition of 2.25% acrylamide and 0.17% *N,N'*-methylenebisacrylamide, using *N,N,N',N'*-tetramethylenediamine as initiator and ammonium persulfate to complete polymerization. Overheating and pH changes during electrophoresis were eliminated by enclosing gel tubes in buffer and arranging continuous circulation from upper to lower reserviors. The load per gel was equivalent to 0.32 A_{260} unit (about 14 μg dry wt of rRNA). *For rRNA gels*, the buffer 0.04 M Tris (pH 8.0) was used and electrophoresis was carried out for 1 hr, 45 min at 5 mA/tube. *For RNP gels*, buffers in which the particles were isolated were used for electrophoresis except for high-salt subunits, when we used 0.6 M KCl–0.01 M $MgCl_2$–0.01 M Tris (pH 7.8). Monosomes and polysomes were run for 4 hr at 15 mA/tube, low-salt subunits for 2 hr at 5 mA/tube and high-salt subunits for 5.5 hr at 40 mA/tube. Gels were stained in 1% Pyronin Y–15% acetic acid and destained electrophoretically.

Poly(U)-Directed Incorporation of [^{14}C]Phenylalanine. The reaction mixture of 1 ml contained 100 μl of β-mercaptoethanol (60 mM), 50 μl of pyruvate kinase (10 mg/ml), 100 μl of energy source (1.5 mg/ml of GTP, 6.05 mg/ml of ATP, and 10.35 mg/ml of P-enolpyruvate), 250 μl of ribosome suspension (6.0 mg/ml of RNA in ribosome buffer), 100 μl of enzyme extract (see below), and a stock salt solution to give final concentrations of 0.1 M KCl–0.02 M $MgCl_2$–0.01 M Tris (pH 7.8). After incubation at 30° for 10 min, 100 μl of energy source, 20 μl of poly(U) (5 mg/ml), and 20 μl of [^{14}C]phenylalanine (high specific activity 513 mCi/mmol, 50 μCi/ml) were added. The total mixture was incubated at 30°, 50-μl aliquots being removed at various time intervals and pipetted onto filter paper disks (Whatman 3MM) previously stapled onto rubber mats. The washing procedure was as described by Mans and Novelli (1961). The disks were analyzed using Permablend I scintillant (Packard Ltd.) in a scintillation counter working at 60–80% efficiency. All tests were performed in duplicate with controls containing no energy, no ribosomes, and no poly(U). Ribosomes tested after stability trials in the presence of ethanol were precipitated by addition of an equal volume of ethanol and centrifuged, and the pellet was resuspended in ribosome buffer. The enzyme extract used was the supernatant obtained after the first high-speed centrifugation of a ribosome preparation using alumina or other mechanical disruption. The extract was in some cases purified by DEAE-cellulose chromatography and stored at −20° in 50% glycerol, unfractionated tRNA being added before the assay.

Sedimentation Analysis. A Beckman Model E ultracentrifuge was used to study ribosomal particles using the schlieren optical system at 42,040 rpm.

Chemicals. Alumina was obtained from Norton Abrasives and acid washed before use. Lysozyme was purchased from Worthington Biochemicals or Sigma; only batches free of nuclease activity (J. Littlechild, paper in preparation) were used. Acrylamide, *N,N'*-methylenebisacrylamide, and *N,N,N',N'*-tetramethylenediamine were supplied by Eastman Kodak. Pyruvate kinase and ATP were obtained from Sigma, GTP and P-enolpyruvate from Boehringer, poly(U) from Miles, and [^{14}C]phenylalanine from the Radiochemical Centre, Amersham.

Results

Preliminary Trials. Crystallization involves slowly bringing a solute out of solution. Salting-out would be undesirable for ribosomes because of the risk of dissociating proteins from rRNA. We therefore decided first to investigate the effect of various organic solvents on ribosome structure. Monosomes prepared with alumina as described above were used in trials where the solvent was added in small aliquots to the ribosome suspension until precipitation occurred. The precipitate, once spun down, was resuspended in ribosome buffer and examined in the analytical ultracentrifuge. We found the solvents ethanol, methanol, acetone, and trimethyl phosphate to give best

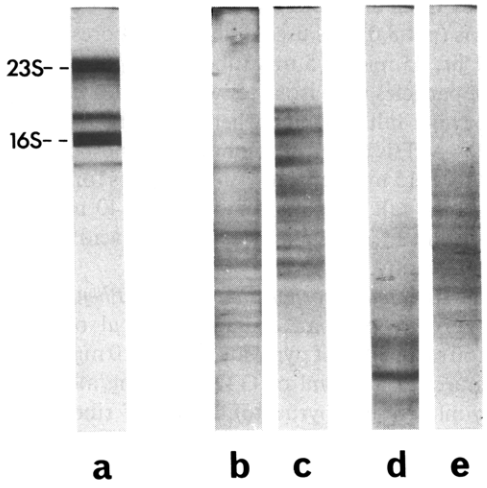

FIGURE 1: Incubations for 1 week at 25° without the use of sterile techniques. Polyacrylamide gels were used as described under Materials and Methods. Migration is from top to bottom: (a) unincubated rRNA control; (b) incubated rRNA; (c) as in b but with ethanol; (d) extracted rRNA from incubated ribosomes; (e) as in d but with ethanol.

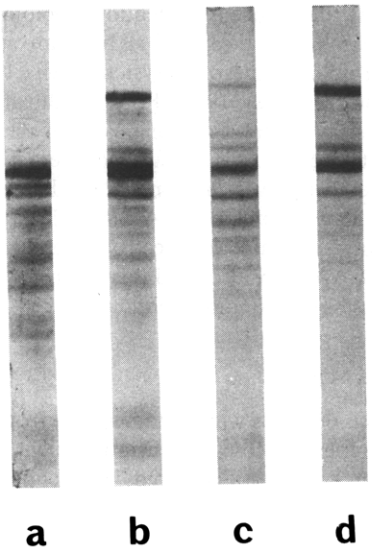

FIGURE 3: Ribosome stability trials using sterile techniques, rRNA gels: (a) incubated for 1 week at 25°; (b) as in a but with ethanol; (c) incubated for 4 weeks at 4°; (d) as in c but with ethanol.

recovery of ribosomal particles which appeared unaltered by the precipitation process on the basis of sedimentation behavior. Some solvents (tetrahydrofuran, polyethylene glycol 200, 2-methylpentane-2,4-diol, dimethyl sulfoxide, formamide, and glycerol) caused dissociation or aggregation of the particles, or failed to precipitate them. Ethanol was used in the stability trials described at a concentration just below that needed to precipitate the rRNA or ribosomal particles. However, when ribosomes prepared without the use of sterile techniques were kept for 1 week at 25°, conditions under which we intended to attempt crystallization, the rRNA was shown by polyacrylamide gels to be extensively degraded (Figure 1a,d,e).

rRNA Stability Trials. We decided next to investigate the stability of isolated rRNA, where degradation due to nucleases present as part of the ribosomal protein would be eliminated. The stability of rRNA extracted from ribosomes and from whole cells was tested by incubation for 1 week at 25° in the presence and absence of ethanol at a final concentration of 23%. In all cases examination of the rRNA on acrylamide gels showed that a large amount of degradation had occurred, though this was less for the sample containing ethanol (Figure 1b,c). We excluded the possibility of nuclease contamination from glassware or fingertips by carefully cleaning and heating all glassware before use and wearing disposable gloves when setting up stability trials.

Bacteria, visible under the microscope, were found in trials where extensive rRNA degradation had occurred. We therefore took precautions to eliminate bacteria by using autoclaved buffers, passing samples through Millipore filters and setting up the trials in a sterile air cabinet (Microflow Ltd., Fleet, Hants, England); these procedures greatly enhanced rRNA

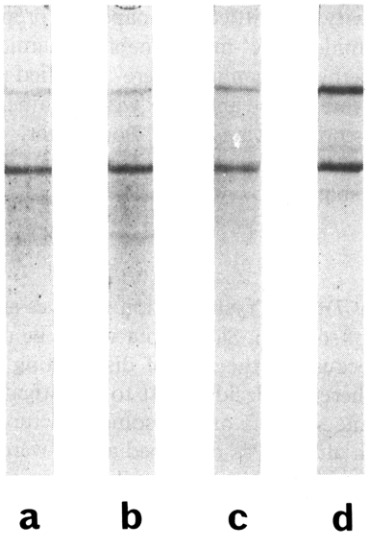

FIGURE 2: rRNA stability trials using sterile techniques: (a) incubated for 1 week at 25°; (b) as in a but with ethanol; (c) incubated for 4 weeks at 4°; (d) as in c but with ethanol.

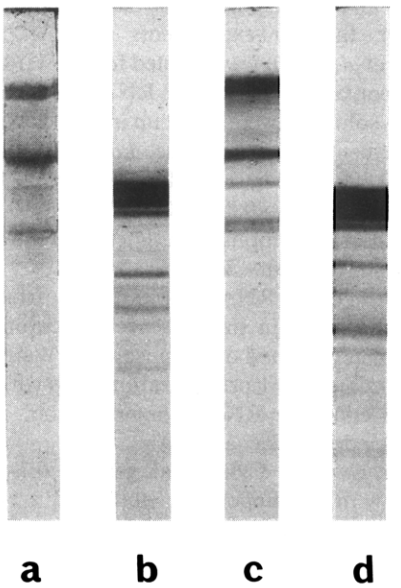

FIGURE 4: Ribosome subunit stability trials for 1 week at 25° using sterile techniques, gels of extracted rRNA: (a) low-Mg^{2+} large subunit; (b) low-Mg^{2+} small subunit; (c) high-salt large subunit; (d) high-salt small subunit.

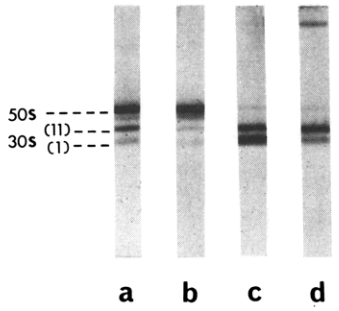

FIGURE 5: Low-Mg^{2+} ribosome subunits, RNP gels: (a) unfractionated subunits; (b) low-Mg^{2+} large subunit; (c) low-Mg^{2+} small subunit, fast side of gradient peak; (d) low-Mg^{2+} small subunit, slow side of gradient peak.

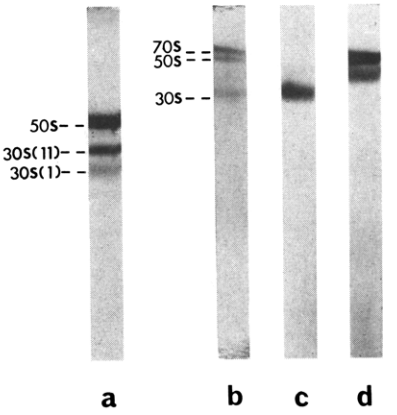

FIGURE 6: Further dissociation conditions, RNP gels: (a) dissociation in 0.1 M KCl–0.005 M $MgCl_2$–0.01 M Tris (pH 7.8) (Eikenberry *et al.*, 1970); (b) unfractionated material in high-salt buffer; (c) high-salt small subunit; (d) high-salt large subunit.

stability. It was then possible to incubate rRNA solutions for 1 week at 25° or 4 weeks at 4° with little degradation. The inclusion of ethanol at a final concentration of 23% further enhanced stability (Figure 2).

Ribosome Stability Trials. Stability trials with purified monosomes, using extracted rRNA from the same ribosomes as a control, showed after acrylamide gel electrophoresis of rRNA that more degradation had occurred in the ribosome samples than in the rRNA controls. In particular, most of the 23S RNA from incubations without ethanol had suffered at least one break (Figure 3a,c). This suggested that nuclease was present with the ribosomal protein. Ribosomes could however be incubated in the presence of ethanol for 1 week at 25° or 4 weeks at 4°, with little degradation of rRNA (Figure 3b,d). The bulk of the rRNA appeared entirely intact.

Subunits obtained by dissociation of monosomes in high-salt and low Mg^{2+} buffers appeared stable after incubation at 25° for 1 week or 4° for 4 weeks, both in the presence and absence of ethanol. The extracted rRNA from all subunits (Figure 4) showed somewhat more degradation than for monosomes, but some of each species had remained entirely intact.

Homogeneity of Ribosomes. Purified monosomes as described in the Experimental Section ran as a single band on acrylamide gels. However, several of the procedures commonly used for purifying monosomes produced more complicated patterns. We found that monosomes washed with 0.5 or 1.0 M NH_4Cl ran as two closely separated bands on gels. The method using $(NH_4)_2SO_4$ fractionation for purifying ribosomes (Kurland, 1966) was avoided due to the known loss of proteins after such a procedure (Voynow and Kurland, 1971). This increases the heterogeneity of the ribosomes and removes proteins required for polypeptide synthetic activity (Kurland *et al.*, 1969; Randall-Hazelbauer and Kurland, 1972). The passage of 70S ribosomes through a sucrose gradient produced an increase in the proportion of subunits, due to the dissociating effect of hydrostatic pressure (Infante and Baierlein, 1971; Infante and Krauss, 1971; Spirin *et al.*, 1972).

Low Mg^{2+} subunits when examined in low Mg^{2+} buffer on acrylamide gels showed the 30S subunit to be composed of two well-separated bands, though the same material examined in the analytical ultracentrifuge showed only one 30S peak. Examination of fractions obtained after sucrose gradient centrifugation showed that the two 30S bands on gels, which we call 30S (i) and (ii), had migrated at different rates in the gradient (Figure 5). The slower moving band on the acrylamide gel moved slower in the sucrose gradient, indicating that 30S (ii) might be unfolded or changed in conformation from 30S (i) during the process of dissociation. The 50S subunit isolated after low Mg^{2+} dissociation migrated on gels mainly as a single band, but traces of a faster component were sometimes observed. Ribosomes purified by $(NH_4)_2SO_4$ fractionation (Kurland, 1966) when dissociated and run on gels in low Mg^{2+} also showed this minor 50S band, but only 30S (i) was seen. Monosomes washed in NH_4Cl (0.5 and 1.0 M) and examined on low Mg^{2+} gels showed two 50S bands and both 30S (i) and (ii) bands. The amount of 30S (i) band had increased when compared with unwashed material. High-salt washing procedures or passage through a sucrose gradient increased the amount of 30S (i) component.

Observation of two 30S bands was not confined to low Mg^{2+} gels, but also occurred with ribosomes dissociated and run in other buffers. Two bands were seen in a buffer similar to our ribosome buffer, but containing 5 mM $MgCl_2$ (Eikenberry *et al.*, 1970) (Figure 6a), in a buffer used by Traub and Nomura (1968) (10^{-2} M Tris (pH 7.8)–10^{-4} M $MgCl_2$–5×10^{-2} M NH_4Cl), and in several other K^+–Mg^{2+} mixtures. On the other hand, *high-salt subunits*, run on gels in the same medium, showed only one 30S band. The faster sedimenting peak from the high-salt gradient showed two bands on the gel. The faster migrating band could be due to 30S contamination from an intermediate 30S:50S $\rightleftharpoons$ 30S + 50S sedimenting zone since the ionic conditions used did not cause complete 70S dissociation (Figure 6b–d). We used this medium to prepare 30S particles for crystallization trials.

The possibility that 30S (i) and (ii) contain different proteins (Van Duin and Kurland, 1970; D'Alessio *et al.*, 1972) is still under investigation; our gel electrophoresis of ribosomal proteins shows variations in the protein content of subunits prepared by different methods. Preliminary results suggest that under conditions where there is only one 30S band on the gel, the small subunits lack high molecular weight proteins. A large ribosomal protein such as S1 (mol wt 65,000), which is known to be in limited supply could, if only bound to half of the 30S subunits, result in the appearance of the two 30S components.

Biological activity of ribosomes was assayed in a poly(U) system as described in the Materials and Methods section. Ribosomes not purified beyond the first high-speed centrifugation showed high activity with added poly(U) when assayed in the presence of the enzyme extract described in the Materials

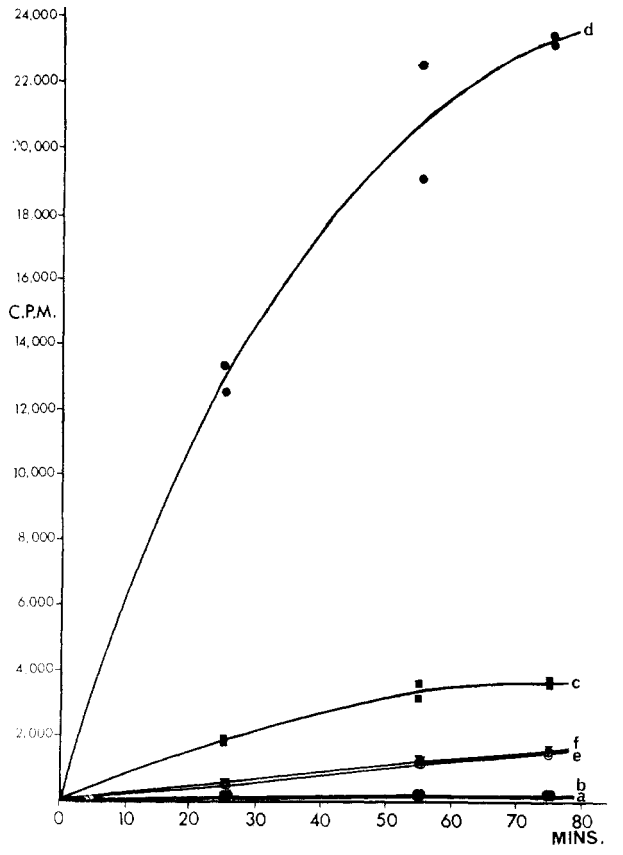

FIGURE 7: Ribosome biological activity, [^{14}C]phenylalanine incorporation with unpurified ribosomes from first high-speed pellet. Assays using the enzyme extract described in the Materials and Methods section: (a) control without ribosomes, (b) control wtihout "energy;" (c) control without poly(U); (d) all components. Assays using the enzyme extract obtained after freeze–thaw lysozyme ribosome extraction: (e) control without poly(U); (f) all components.

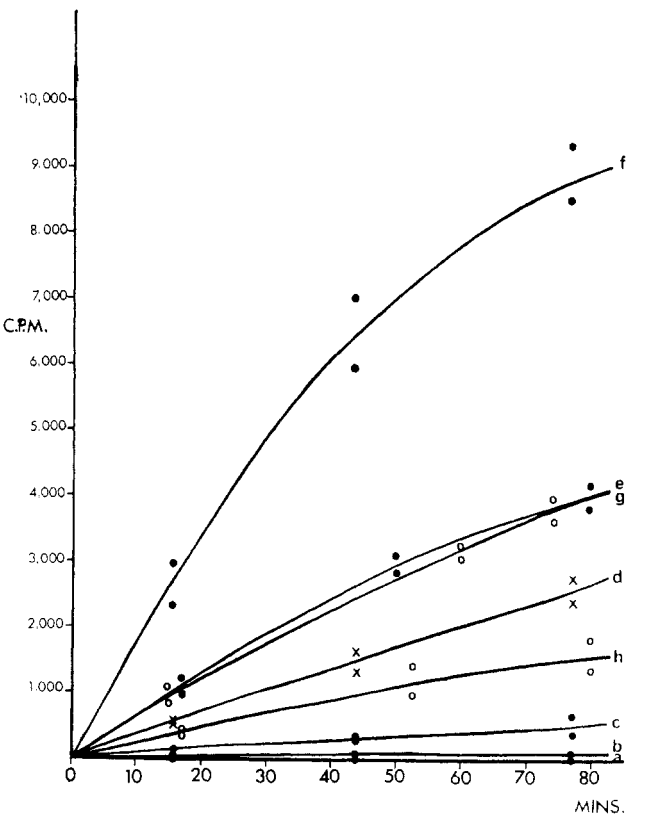

FIGURE 8: Ribosome biological activity, [^{14}C]phenylalanine incorporation with ribosomes purified by differential centrifugation. Activity of ribosomes from stability trails using sterile techniques, all components present. All assays used the enzyme extract described in the Materials and Methods section. "Control" points represent experiments with ribosomes incubated as in (f) below. Controls were also carried out after the other incubation experiments and gave similar results; these are not plotted: (a) control without ribosomes; (b) control without "energy;" (c) control without poly(U); (d) unincubated ribosomes; (e) incubated for 1 week at 25° with ethanol; (f) incubated for 1 week at 25°; (g) incubated for 4 weeks at 4° with ethanol; (h) incubated for 4 weeks at 4°.

and Methods section (Figure 7d). Some endogenous activity was always observed (Figure 7c). The enzyme extract obtained after freeze–thaw lysozyme ribosome extraction was found to be inactive (Figure 7e,f) but ribosomes prepared by this method, if assayed with active extract, appeared undamaged. Purified 70S ribosomes showed a decrease in activity in all samples studied. However this loss was reversible, since purified 70S ribosomes incubated under sterile conditions showed an increase in activity after 1 week at 25° with or without the addition of ethanol (Figure 8e,f) or after 4 weeks at 4° with the addition of ethanol (Figure 8g). Incubation for 4 weeks at 4° without ethanol resulted in no increase in activity and in some cases a slight decrease (Figure 8h). The purified 70S ribosomes incubated for 1 week at 25° without ethanol showed up to $\times 10$ increase in activity, making the results comparable to those with unpurified material. Ribosomes before and after stability trials under sterile and unsterile conditions showed activities which could be directly correlated with the appearance of the RNP on acrylamide gels. Samples in which no 70S material remained gave high incorporation of [^{14}C]phenylalanine without the addition of energy to the system, indicating that the ribosomes had become contaminated with bacteria. After monosome incubations at elevated temperatures in the absence of ethanol an additional slower migrating band was seen on acrylamide gels, and the biological activity of the samples had increased from that of the unincubated material.

Ribosomes and ribosome subunits were kept at $-20°$ before stability trials. Freezing did not appear to affect biological activity or appearance on acrylamide gels. After several months at $-20°$ slight degradation of the rRNA was observed.

Discussion

We find that suitably prepared ribosomes can be incubated in suspension at 25° for 1 week or 4° for 4 weeks with little change according to the criteria defined. Hence crystallization *in vitro* can be attempted under these conditions, provided the precautions described are taken; there is clearly a risk of nucleases arising from airborne bacteria.

The attainment of homogeneity is much more difficult than that of stability, and we claim little more than to have defined the problem. Our purified 70S particles were mainly runoff ribosomes, as observed by others (Kohler *et al.*, 1968; Flessel, 1968; MacDonald and Yeater, 1968; Algranati *et al.*, 1969; Tai and Davis, 1972; Chliamovitch and Anderson, 1972). However, some endogenous activity was observed in the poly(U) system described; the uptake of ^{14}C increased linearly with incubation time, indicating that existing mRNA was not being degraded. Runoff 70S ribosomes should by definition be free of mRNA. This activity could be due to a

small contamination by polysomal ribosomes in the preparation, or to the possible presence of fragmented mRNA in the enzyme extract. For absolute homogeneity of ribosomes one would need to remove this mRNA, without damaging the ribosomes. However, it is clear from our results that washing procedures such as the use of NH_4Cl may introduce physical heterogeneity, as shown by the appearance of the two bands on gels.

An explanation of the increased activity of purified monosomes after high-temperature incubation could be that during the earlier purification procedure the ribosomes have undergone a change in conformation which can be reversed on incubation. Bodley (1969), when investigating the irreversible thermal denaturation of *E. coli* ribosomes, found that the first effect of heating on ribosome activity was an increase in activity, followed at higher temperatures by inactivation. No explanation was provided for the initial activation, but it was suggested that it was due to an increase in the number of active ribosomes. Kikuchi and Monier (1971) report that ribosome suspensions were preincubated for 20 min at 50° to bring them to maximum activity. The two bands observed in the 70S range on acrylamide gels could represent different monosome conformations. These results need further investigation.

The problem of heterogeneity in subunit preparation is also complicated, and no set of dissociating conditions tried by us has given both subunits in a completely homogeneous form. Many recent reports suggest that conformational changes may easily be induced in ribosomes. In the absence of monovalent cations, such as NH_4^+, K^+, Rb^+, and Cs^+ (Zamir *et al.*, 1971; Miskin *et al.*, 1968, 1970; Ravel *et al.*, 1970; Vogel *et al.*, 1969, 1971), various catalytic properties of the ribosome in protein synthesis are lost, probably due to conformational changes of the ribosomal subunits. Kikuchi and Monier (1970) found that ribosomes dissociated in low concentrations of monovalent (NH_4^+, K^+) or divalent (Mg^{2+}) cations lost their ability to reassociate to 70S ribosomes. Incubation at elevated temperature in the presence of higher concentrations of these cations restored this function. The loss of activity was mainly attributed to a conformational change occurring in the 30S subunit. Kagawa *et al.* (1971) found that for low-salt subunits to reassociate to 70S particles preincubation of the 30S subunit only, with magnesium and monovalent ions, was sufficient for the recovery but incubation of the 50S subunit only was ineffective.

The two 30S components observed by us could be the native and altered conformations or both could be of biological significance. Dahlberg *et al.* (1969) using agarose–acrylamide gels observed two 30S subunit bands in the presence of a Tris-EDTA buffer. The two 30S subunits had 16S rRNA bands of very similar, if not identical mobility. rRNA extracted by us from a mixture of the two 30S subunits yields only one 16S band. This, together with our other evidence, leads us not to support the idea that one of the particles is a precursor for the other. We observed on acrylamide gels of NH_4Cl-washed ribosomes two monosome bands of nearly similar migration rate. The monosome bands we obtained after incubation of ribosomes (preparation as in the Materials and Methods section) have a larger difference in migration rate and are produced by incubation without any additional factors. Dissociation of NH_4Cl-washed ribosomes by dissociation factor (Talens *et al.*, 1970) resulted in the appearance of at least two classes of 50S subunits and two classes of 30S subunits. These results are similar to those we obtain using NH_4Cl-washed ribosomes on low Mg^{2+} gels.

In summary, we believe that our procedures enable one to make preparations of ribosomes and their subunits that are sufficiently stable for crystallization trials to be attempted. We have carried out preliminary trials ourselves (unpublished work) but have not so far observed crystallization. The problem of heterogeneity is, however, a much more complex one which requires further study. All separation procedures seem to carry the risk of increasing the heterogeneity. This is particularly true of subunit preparation. It may even apply to monosome purification where we observe a temporary loss of biological activity. This seems due to a change in conformation rather than loss of proteins. The use of acrylamide gel electrophoresis for characterizing ribosomes and ribosome subunits yields information which cannot be obtained by other methods and should also prove useful in the investigation of changes in ribosome structure occurring naturally during the process of protein synthesis.

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Cross-Linking of Elongation Factor EF-G to the 50S Ribosomal Subunit of *Escherichia coli*†

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ABSTRACT: A wide variety of bifunctional protein reagents will covalently cross-link the elongation factor EF-G to the 50S ribosomal subunit of *Escherichia coli*. About 120,000 daltons of ribosomal proteins become cross-linked to EF-G upon extensive reaction. About 30,000 daltons of this material is made up of proteins L7 and L12 (mol wt 13,000 daltons). Thus, these two components, whose presence on the ribosome is required for the EF-G interaction, must be located at the EF-G binding site.

There is reason to believe that elongation factors EF-G[1] and EF-Tu interact with the 50S subunit of the procaryotic ribosome at the same site or at very closely related sites. Antibiotics such as siomycin, thiostrepton, and thiopeptin which inhibit the interaction of EF-G with the ribosome also block the binding of aminoacyl-tRNA to ribosomes catalyzed by EF-Tu and its attendant hydrolysis of GTP (Bodley and Lin, 1970; Bodley *et al.*, 1970; Tanaka *et al.*, 1970; Weisblum and Demohn, 1970a,b; Pestka, 1970; Highland *et al.*, 1971; Kinoshita *et al.*, 1971; Modolell *et al.*, 1971a,b). Moreover, when EF-G is bound to the ribosome, EF-Tu will no longer interact with it (Richter, 1972; Miller, 1972; Cabrer *et al.*, 1972; Richman and Bodley, 1972). These results all seem to point to the existence of a critical region on the 50S subunit involved in both tRNA binding (the "A site") and translocation. Clearly it would be of greatest interest to know what the structure of the ribosome is in this region.

The one fact we do know about this binding center of the 50S subunit is that two 50S proteins, L7 and/or L12, must be present in order for its activities to be expressed. When these proteins are removed from the 50S subunit, the particle which remains is inactive with respect to both EF-G- and EF-Tu-catalyzed interactions (Hamel and Nakamoto, 1971, 1972; Hamel *et al.*, 1972; Sander *et al.*, 1972; Brot *et al.*, 1972). Proteins L7 and L12 (mol wt 13,000 each) have identical primary structures except that the N terminus of L7 is acetylated whereas that of L12 is free (Möller *et al.*, 1972; Terhorst *et al.*, 1972). The activity of particles lacking L7 and L12 can be restored by adding these components back. Recently Highland *et al.* (1973) have tested a large number of antibodies specific for 50S proteins for their ability to block the EF-G interaction. Only the antibodies raised against L7 or L12 have this effect. Accordingly, it has been suggested that L7 and L12 are in the EF-G binding site on the ribosome.

A direct method for finding out which ribosomal proteins are in the factor binding sites would be to identify the ribosomal components which can be cross-linked to factors with bifunctional reagents when they are bound to ribosomes. In this article we report the results of a study of the EF-G binding site using bifunctional reagents. The cross-linking of EF-G to the ribosome is demonstrated. Extensive cross-linking attaches about 120,000 daltons of 50S protein (which is about 20–25% of the total) to EF-G. Included in this material are about 2 equivalents of protein (L7–L12). This result proves that L7 and L12 are indeed at or very near the EF-G binding site.

Materials and Methods

Materials. Adiponitrile, suberonitrile, sebaconitrile, glutaraldehyde, and difluorodinitrobenzene were obtained from

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[1] Abbreviations used are: GMPPCP, β,γ-methyleneguanosine triphosphate; EF-G, peptide chain elongation factor G; EF-Tu, peptide chain elongation factor Tu.