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Studies of Newly Synthesized Ribosomal Ribonucleic Acid of *Escherichia coli*

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1. RNA synthesized by *Escherichia coli* during one-hundredth of the generation time contains two fractions distinguishable by hybridization with homologous DNA. One fraction, approximately 30% of the newly synthesized RNA, did not compete with ribosomal RNA, being apparently messenger RNA. The other fraction, approximately 70% of the newly made RNA, hybridized as ribosomal RNA. These values are comparable with previous estimates (McCarthy & Bolton, 1964; Pigott & Midgley, 1968). 2. Hybridization-competition experiments showed that the newly made RNA associated with 70S ribosomes and larger ribosome aggregates was a mixture of ribosomal RNA and messenger RNA, whereas that associated with nascent ribosomal subunits consisted exclusively of ribosomal RNA. This observation provides means by which newly synthesized ribosomal RNA can be isolated free from messenger RNA. 3. Newly made ribosomal RNA in nascent ribosomal subunits was sensitive to shear under conditions where ribosomal RNA in mature ribosomes was shear-resistant. Thus, when RNA was extracted from cells of *E. coli* disrupted by mechanical means, newly made ribosomal RNA appeared heterogeneous in size, sedimenting as a broad peak extending from 8S to 16S. 4. Newly synthesized ribosomal RNA in nascent ribosomal subunits was rapidly degraded in the presence of actinomycin D and during glucose starvation. 5. Newly synthesized ribosomal RNA stimulated amino acid incorporation in a system synthesizing protein *in vitro* to the same extent as the RNA which contained the messenger RNA fraction.

Various criteria, based on the properties predicted for mRNA* (Jacob & Monod, 1961) have been used in identifying an RNA fraction as mRNA: (1) heterogeneity of size (Gros *et al.* 1961); (2) temporary association with ribosomes (Gros *et al.* 1961); (3) homology with DNA (Spiegelman & Hayashi, 1963); (4) metabolic instability (Levinthal, Keynan & Higa, 1962); (5) stimulation of amino acid incorporation (Willson & Gros, 1964; Forchhammer & Kjeldgaard, 1967). In these and numerous other studies (reviewed by Singer & Leder, 1966) it has been assumed that each of these properties is exclusively that of mRNA.

We were interested in the question of whether these properties are unique for mRNA. The present investigation indicates that most of the properties attributed to mRNA are those of newly synthesized rRNA, which together with the former constitutes the pulse-labelled RNA fraction.

* Abbreviations: mRNA, messenger RNA; rRNA, ribosomal RNA; tRNA, transfer RNA; SSC, 0·15M-NaCl–0·015M-trisodium citrate, pH 7·0–7·2.

MATERIALS AND METHODS

Cells. Escherichia coli B and a strain K12 were used. Stock cultures were maintained on nutrient Difco agar slopes and were transferred every 3 weeks. For experiments, the cells were grown in liquid media with agitation at 37°. The following growth media were used. (1) The glucose–salts medium of Davis & Mingioli (1950), which contained (per l.): 7g. of K_2HPO_4, 3g. of KH_2PO_4, 1g. of $(NH_4)_2SO_4$, 0·1g. of $MgSO_4,7H_2O$, 5g. of trisodium citrate dihydrate, and 0·2g. of D-glucose. (2) The same medium supplemented with 0·04% of vitamin-free casamino acids (casein hydrolysate) (Difco, Detroit, Mich., U.S.A.). (3) Bacto-Tryptone broth, which contained (per l.): 10g. of Bacto-Tryptone, 1g. of yeast extract (Difco), 1g. of D-glucose and 5g. of NaCl.

Labelling experiments. Exponentially growing cultures at a density of 2×10^8 cells/ml. were exposed to [^{3}H]uracil (Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A.) for one-hundredth of the generation time and quickly chilled by pouring them on to crushed frozen buffer of the desired composition. Such cells are referred to as pulse-labelled cells. For hybridization-competition experiments, the cells were pulse-labelled with 2·5μc of [^{3}H]uracil (sp.

radioactivity 23c/m-mole)/ml. of medium. Otherwise the cells were exposed to 0·2 μc of [^{3}H]uracil (sp. radioactivity 60mc/m-mole)/ml. of medium. To obtain cells with the isotope present in stable RNA only, the culture was exposed to [^{3}H]uracil for 45sec. and allowed to grow exponentially for another two to three generations with a 100-fold excess of non-radioactive uridine. The cells were collected as described above.

Preparation of bacterial extracts by mechanical means. Extracts were obtained by either sonication or alumina grinding. (i) Sonication. Exponentially growing cells were harvested, washed once in 10mM-tris–HCl buffer, pH 7·4, containing 10mM-magnesium acetate, resuspended in the same buffer at a density of 10^{10} cells/ml. of buffer and disrupted for 2min. in the 20kHz MSE ultrasonic disintegrator. Unbroken cells and cell debris were removed by centrifugation at 15000*g* for 15min. at 4°. The supernatant fraction was used immediately. (ii) Alumina grinding. Exponentially growing cells were harvested as described above and the pellet was frozen and ground with twice their wet weight of alumina. The ground bacterial paste was extracted with 10mM-tris–HCl buffer, pH 7·4, containing 10mM-magnesium acetate and 60mM-KCl. Alumina, bacterial debris and unbroken cells were removed by centrifugation at 15000*g* for 15min. RNA from the extracts was prepared as described below. The preparation of S-30 extracts used in the experiments on amino acid incorporation is described in detail under a separate heading.

Preparation of spheroplasts, polyribosomes and non-fragmented pulse-labelled RNA. Spheroplasts were made by a modification of the method used by Kohn(1960). A 100ml. culture was grown in glucose–salts medium, supplemented with casamino acids, to a density of 2×10^8 cells/ml., pulse-labelled, poured on to crushed frozen 10mM-tris–HCl buffer, pH 7·75, containing 30% (w/v) sucrose (ribonuclease-free; Mann Research Laboratories Inc., New York, N.Y., U.S.A.) and 15mM-magnesium acetate, centrifuged, washed once with 10ml. of the same buffer and resuspended in 0·5ml. of the above buffer to which 0·6mg. of egg-white lysozyme (Sigma Chemical Co., St Louis, Mo., U.S.A.) was added. The cells were twice frozen in anacetone–solid CO_2 bath and thawed in an ice bath. For the preparation of polyribosomes, the spheroplasts were lysed by incubation with 0·015ml. of 10% (w/v) sodium deoxycholate for 3min. at 0° (Ron, Kohler & Davis, 1966). The released DNA and cell debris were sedimented by centrifugation at 15000*g* for 15min. in the cold and the supernatant fraction containing polyribosomes was analysed by sucrose-density-gradient centrifugation.

For the preparation of non-fragmented pulse-labelled RNA the spheroplasts were collected by centrifugation in the cold and gently suspended, with a wide-bore pipette, in 2·7ml. of 10mM-tris–HCl buffer, pH 7·4, containing 10mM-magnesium acetate, and lysed by adding 0·3ml. of 15% (w/v) sodium dodecyl sulphate and incubating the mixture at 0° for 1–3min. (Fry & Artman, 1968).

Preparation of RNA from various types of ribosome. Spheroplasts and extracts from pulse-labelled cells disrupted by sonication or alumina grinding were prepared as described above and fractionated by sucrose-density-gradient centrifugation. Fractions containing the various types of ribosome were pooled and the RNA was extracted.

Preparation of rRNA free of mRNA for hybridization-competition experiments. A 300ml. culture of *E. coli* was grown overnight in the glucose–salts medium as described above, disrupted by sonication and centrifuged at 15000*g* for 15min. The supernatant fraction was centrifuged in the Spinco model L ultracentrifuge at 120000*g* for 70min. The ribosomal pellet was resuspended in 10mM-tris–HCl buffer, pH 7·4, containing 10mM-magnesium acetate, to give a concentration of 1·5mg. of ribosomes/ml. mRNA was selectively degraded to acid-soluble material by incubating the ribosomal suspension at 37° for 3hr. (Artman, Silman & Engelberg, 1967). After this incubation the ribosomal suspension was dialysed against 500vol. of 10mM-tris–HCl buffer, pH 7·4, containing 0·05mM-magnesium acetate, to dissociate the 70s ribosomes into the 50s and 30s subunits.

The derived subunits were sedimented by centrifugation at 120000*g* for 3·5hr. at 4° and the RNA was extracted. rRNA free of mRNA could also be obtained by extracting RNA from cells starved of glucose (see below).

Extraction of RNA. The different preparations were poured on to an equal volume of freshly distilled 90% (v/v) phenol together with enough sodium dodecyl sulphate to give a final concentration in the aqueous phase of 1·5%. The mixture in stoppered glass centrifuge tubes was gently agitated by turning the tubes upside down for 5min. in the cold and was then centrifuged at 10000*g* for 10min. The upper aqueous layer was carefully collected and freed from phenol by repeated extractions with ether. The dissolved ether was removed by blowing air, the solution was incubated with 10 μg. of ribonuclease-free deoxyribonuclease/ml. (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) and the RNA precipitated with 2 vol. of ice-cold ethanol. The product was stored under ethanol at −20°.

Breakdown of pulse-labelled RNA during glucose starvation and in the presence of actinomycin D. (i) Glucose starvation. *E. coli* B cells were grown in glucose–salts medium as described above. Pulse-labelled cells and cells with the isotope present in stable RNA were used. The radioactively labelled cells were quickly chilled by pouring them on to crushed frozen starvation medium of the following composition (per l.): 7g. of K_2HPO_4, 3g. of KH_2PO_4 and 0·1g. of $MgSO_4,7H_2O$ (pH 7·0) and collected by centrifugation in the cold. The cells were resuspended in one-tenth of their original volume of cold starvation medium of the above composition. At zero time the cells were diluted tenfold with the same starvation medium, prewarmed to 37°, and incubated at this temperature with agitation. At zero time and after different times of incubation, 2ml. samples were added to 2ml. of ice-cold 10% (w/v) trichloroacetic acid and left for 20min. The samples were then filtered through Millipore filters, the filters were rinsed with several portions of cold 5% (w/v) trichloroacetic acid, dried and counted. For the analysis of RNA broken down during glucose starvation, cells at zero time and after 30min. of incubation in starvation medium were disrupted by alumina grinding, the extracts were centrifuged in sucrose-density gradients and the fractions collected and analysed as described below and in the figure legends.

(ii) Actinomycin D. *E. coli* strain K12 was grown in glucose–salts medium supplemented with casamino acids as described above. Exponentially growing cells were harvested at a density of 2×10^8 cells/ml., centrifuged, washed once at room temperature with 10mM-tris–HCl buffer, pH 8, and resuspended in the same buffer at a density of 2×10^9 cells/ml. EDTA (0·2mM final concn.) was added and 2min. later the cells were diluted tenfold with prewarmed

complete medium (Leive & Kollin, 1967). After 10min. of growth at 37°, the cells were pulse-labelled and actinomycin D (a gift of Merck, Sharp and Dohme, Rahway, N.J., U.S.A.) was added to give a final concentration of the antibiotic of 10μg./ml. of medium. For the experiments on the effect of actinomycin D on stable RNA, the cells were exposed to [^{3}H]uracil and 'chased' with non-radioactive uridine before sensitization with EDTA. The loss of acid-insoluble radioactivity and the analysis of RNA which became degraded in the presence of actinomycin D were determined as described above for glucose-starved cells.

Sedimentation of extracts (ribosomes) and RNA on linear sucrose gradients. Linear sucrose gradients, 5–20% or 15–30% (w/v), in suitable buffers and ionic conditions were used throughout this work (the buffers and salts present in gradients are listed in the figure legends). For analytical purposes gradients of volume 4·4ml. were run in the Spinco model L ultracentrifuge (SW39 rotor). Extracts obtained by alumina grinding, or spheroplast lysates, in suitable buffer and ionic composition in a volume of 0·2–0·4ml. were layered on top of sucrose gradients and centrifuged at 4° for different times, as listed in the figure legends. RNA samples in 10mM-tris–HCl buffer, pH7·4, containing 0·1M-NaCl and 1mM-EDTA, were centrifuged in sucrose gradients at 4° for 4hr. From each gradient 24–30 fractions (0·15ml.) were collected. Each fraction was made up to 1ml. with water, 0·1M-NaCl or an appropriate buffer and the E_{260} value was determined. To each fraction 2 drops of 1% albumin solution and 1ml. of ice-cold 10% (w/v) trichloroacetic acid were added. The trichloroacetic acid-insoluble material was collected on Millipore filters, washed with several portions of cold 5% (w/v) trichloroacetic acid and after drying placed in vials for the determination of radioactivity. Sucrose-gradient centrifugations were also used for the isolation of the different types of ribosome. Fractions from the gradients containing the various classes of ribosome were pooled and the RNA was extracted as described above.

Hybridization-competition experiments. E. coli DNA was isolated by a method mainly derived from those of Marmur (1961) and Thomas, Berns & Kelly (1966). DNA was denatured, immobilized and adsorbed on Millipore cellulose nitrate membrane filters essentially as described by Gillespie & Spiegelman (1965). All these procedures were described in detail by Fry & Artman (1969). Pulse-labelled [^{3}H]RNA from lysed spheroplasts, polyribosomes, 70s ribosomes and native ribosomal subunits and various amounts of unlabelled rRNA free of mRNA were annealed with a constant amount of immobilized heat-denatured DNA bound on cellulose nitrate membrane filters (60μg. of DNA/filter) at 66° for 20hr. in 6×SSC in scintillation vials (total volume 0·5ml.). After annealing, each side of the filter was washed with 10ml. of 6×SSC and incubated at 37° for 1hr. in 1ml. of 6×SSC containing 25μg. of pancreatic ribonuclease (Worthington) freed of deoxyribonuclease activity by being heated at 80° for 10min. The filters were then washed each side with 60ml. of 6×SSC, dried and analysed for the retention of hybridized RNA on the filters. Background corrections were done for each point of the curve from samples containing pulse-labelled RNA, the appropriate amounts of unlabelled rRNA and filters without DNA.

Subcellular protein-synthesizing system. S-30 extracts from *E. coli* were prepared by the method of Nirenberg & Matthaei (1961) as used by Capecchi (1966). A 500ml. culture of *E. coli* B was grown overnight in the Bacto-Tryptone medium. This culture was used to inoculate 12l. of a similar medium and the diluted culture was grown exponentially at 37° with rapid agitation to E_{550} 0·8. The cells were harvested and washed twice with 250ml. of 10mM-tris–HCl buffer, pH7·5, containing 10mM-magnesium acetate. The pellet (12g. wet wt. of cells) was stored at −20°. The S-30 extracts were prepared by grinding the frozen cells with twice their weight of alumina and extracting them with 18ml. of the standard buffer (10mM-tris–HCl–60mM-KCl–14mM-magnesium acetate–0·1mM-dithiothreitol, pH7·8). Ribonuclease-free deoxyribonuclease (5μg./ml.) was added and the extract was clarified by centrifugation at 15000rev./min. for 15min. in the Servall RC-2 refrigerated centrifuge. The resulting supernatant fraction was centrifuged in the Spinco model L ultracentrifuge at 30000*g* for 30min. The pellet was discarded and the supernatant fraction again centrifuged at 30000*g* for 30min. The supernatant fraction (S-30) was then dialysed for 8hr. at 4° against 500 vol. of the standard buffer. After dialysis, the extracts were stored for 1 week at −20°. The extracts contained 25mg. of protein and approximately 12mg. of ribosomes/ml. The system for amino acid incorporation *in vitro* directed by the various RNA species consisted of the S-30 fraction preincubated for 15min. at 37° in the presence of an ATP-generating system. The preincubation mixture contained (per ml.): 0·5ml. of S-30 fraction, 3μmoles of ATP, 0·3μmole of GTP, 5μmoles of phosphoenolpyruvate (potassium salt), 20μg. of pyruvate kinase, 0·14μmole of each of the protein amino acids, 75μmoles of KCl and 1μmole of the dithiothreitol. After this preincubation, the mixture was chilled and immediately used for amino acid-incorporation experiments. The reaction mixture for the incorporation experiments contained (per ml.): 0·5ml. of preincubated S-30 fraction, the various RNA species as indicated in the Results section, 3μmoles of ATP, 0·3μmole of GTP, 5μmoles of phosphoenolpyruvate (potassium salt), 20μg. of pyruvate kinase, 0·14μmole of each of the 19 protein amino acids other than leucine, 0·14μmole of [^{14}C]leucine (15mc/m-mole), 75μmoles of KCl, 11μmoles of magnesium acetate, 50μmoles of tris, pH7·8, and 1μmole of dithiothreitol. The incubation was at 36° for 15min. After the incubation, 0·2ml. samples were pipetted on to small Whatman no. 3 filter-paper discs and placed into a 500ml. beaker containing 10% (w/v) trichloroacetic acid. Batches were washed twice with 5% (w/v) trichloroacetic acid, heated at 95° for 15min. in 5% (w/v) trichloroacetic acid, washed again in 5% trichloroacetic acid, washed twice with ethanol–ether (1:1, v/v), washed twice with diethyl ether, dried in air and their radioactivity was counted.

Scintillation counting. All samples were counted in a Packard Tri-Carb scintillation counter. Samples on filter-paper discs or Millipore filters were counted in 10ml. of a scintillation fluid containing 0·4% of 2,5-diphenyloxazole and 0·1% of 1,4-bis-(5-phenyloxazol-2-yl)benzene intoluene.

RESULTS

Sedimentation behaviour and hybridization of pulse-labelled RNA from E. coli. We have shown that RNA, like other high-molecular-weight polymers, is highly sensitive to mechanical shear

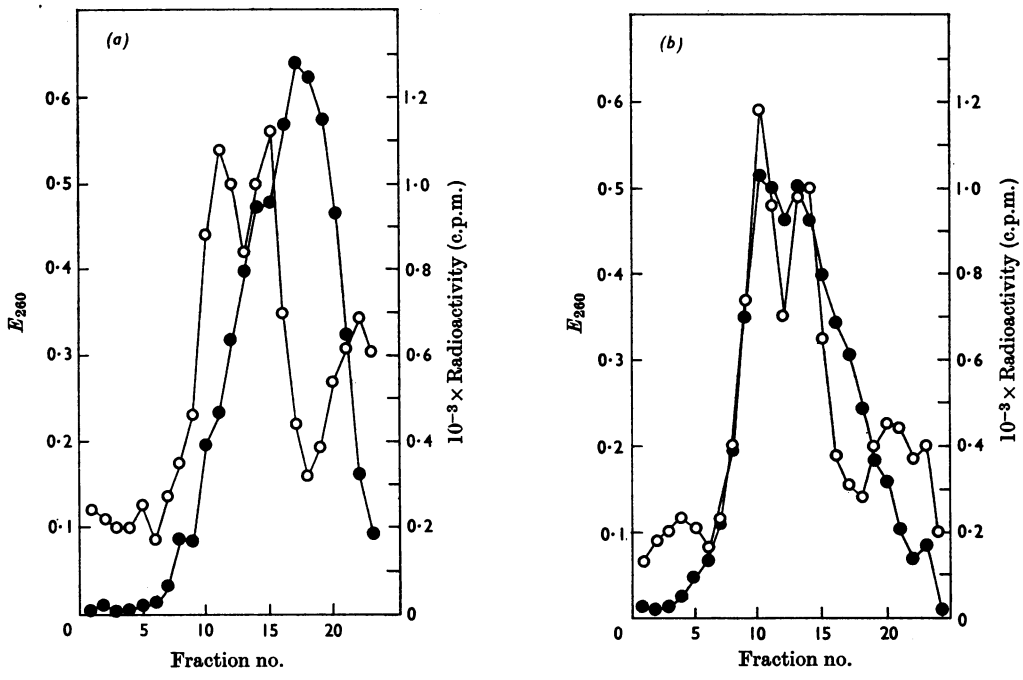

Fig. 1. Sedimentation pattern of pulse-labelled RNA and rRNA from *E. coli* B. A 100ml. culture of *E. coli* B was grown in glucose–salts medium supplemented with casamino acids and pulse-labelled with 0·2 μc of [^{3}H]uracil (sp. radioactivity 60mc/m-mole)/ml. of medium for one-hundreth of the generation time. One portion of the pulse-labelled cells was converted into spheroplasts and lysed with sodium dodecyl sulphate, the other portion was disrupted by sonication and the RNA extracted as described in the Materials and Methods section. The RNA was dissolved in 10mM-tris–HCl buffer, pH 7·4, containing 0·1M-NaCl and 1mM-EDTA. Sucrose solutions (5% and 20%, w/v) used to prepare the gradients were made in the same buffer. The RNA samples were centrifuged in sucrose gradients in the SW 39 rotor at 37000rev./min. at 4° for 4hr. and the gradients fractionated. (*a*) RNA obtained from sonicated cells; (*b*) RNA obtained from lysed spheroplasts. ○, E_{260}; ●, trichloroacetic acid-precipitable radioactivity.

(Fry & Artman, 1968). The two rRNA species, which sediment at 23s and 16s, become sheared by short sonication to chains sedimenting as a broad peak of 10–12s. Sonication of ribosomes before extraction of RNA did not lead to fragmentation of rRNA. Hence the critical shear for rRNA in mature ribosomes is greatly increased, presumably because the interaction of ribosomal proteins and RNA in mature ribosomes gives protection to the RNA moiety against shear. Correspondingly, high-molecular-weight RNA not protected in this way should be fragmented during the preparation of bacterial extracts by mechanical means. Fig. 1(*a*) shows the sedimentation pattern of rRNA and pulse-labelled RNA prepared from *E. coli* cells disrupted by short sonication. Here, the u.v.-absorbing material comprising the two rRNA species and tRNA is well resolved into three fractions sedimenting at 23s, 16s and 4s. The pulse-labelled RNA consists of short chains with a mean sedimentation coefficient of 10–12s, and a second rather ill-defined peak at about 16s. Similar results were obtained with *E. coli* cells broken by alumina grinding. That the slow-sedimenting chains of pulse-labelled RNA were produced by shear could be shown in experiments in which excessive shear was avoided at every step of the preparation of bacterial extracts and during all subsequent manipulations. Fig. 1(*b*) shows the sedimentation pattern of a similar RNA preparation obtained from spheroplasts lysed by sodium dodecyl sulphate. Here, the distribution of radioactivity coincides with the distribution of material absorbing at 260nm., both sedimenting at 23s and 16s. From the results presented in Fig. 1(*a*), it follows that practically all the pulse-labelled RNA was fragmented by short sonication, suggesting that both components of pulse-labelled RNA, mRNA and the newly synthesized rRNA, are susceptible to shear under these conditions. To obtain direct evidence for shear-sensitivity of newly synthesized rRNA we attempted to isolate the latter free from mRNA. To

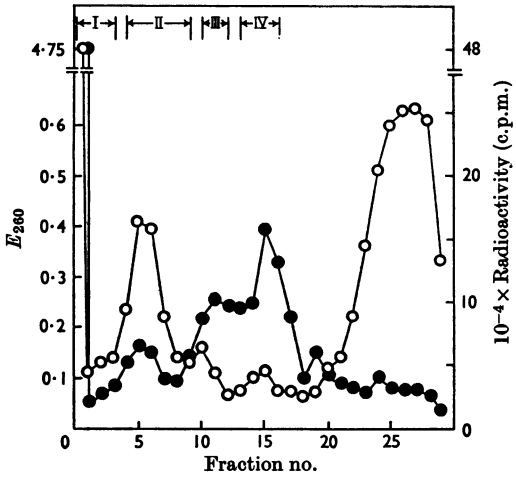

Fig. 2. Sedimentation pattern of a lysate of *E. coli* B centrifuged for long enough to separate native ribosomal subunits. The total cell lysate from 40ml. of a pulse-labelled culture grown in glucose–salts medium supplemented with casamino acids was layered on a 4·4ml. sucrose density gradient (15–30%, w/v) in 10mM-tris–HCl buffer, pH 7·4, containing 10mM-magnesium acetate and 60mM-KCl, and centrifuged in the SW 39 rotor at 37000rev./min. at 4° for 3·5hr. The polyribosomes sedimented to the bottom of the gradient tube. The profile was divided into four regions: I, polyribosomes; II, 70s ribosomes; III and IV, 50s and 30s ribosomal subunits respectively. The fractions from each region were pooled; the RNA was extracted and used for hybridization-competition experiments as described in the Materials and Methods section. ○, E_{260}; ●, trichloroacetic acid-precipitable radioactivity.

this end we have studied the distribution of pulse-labelled RNA among the various types of ribosome and analysed it by hybridization-competition experiments. Cells growing exponentially in glucose–salts medium supplemented with casamino acids were pulse-labelled, converted into spheroplasts, lysed with sodium deoxycholate, centrifuged in sucrose gradients for 70min. and for 3·5hr. and analysed for the distribution of pulse-labelled RNA among the different classes of ribosomes. After centrifugation for 70min., polyribosomes and 70s ribosomes were clearly resolved, whereas the ribosomal subunits were not separated from the 70s ribosomes. After centrifugation for 3·5hr. (Fig. 2) the entire polyribosomal fraction (64% of the total ribosomal population) had pelleted and the 70s ribosomes, which constituted 24% of the total ribosomal population, sedimented near the bottom of the gradient tube. The ribosomal subunits, which under these growth conditions constitute 12% of the total ribosomal population, sedimented as two peaks at 50s and 30s. It should be pointed out that the lower the rate of growth of the cells, the greater the proportion of ribosomal subunits. When the cells were grown in the glucose–salts medium without casamino acids, ribosomal subunits constituted 40% of the total ribosome population (Fig. 6*a*). These results are in agreement with those of Godson & Sinsheimer (1967), who reported on the variation in the relative amount of ribosomal subunits present in the cell under different growth conditions. The percentage distribution of pulse-labelled RNA among the various classes of ribosome, however, remained unchanged by growth conditions. In all our experiments approximately 55% of pulse-labelled RNA was associated with nascent ribosomal subunits and the remaining 45% with 70s ribosomes and larger ribosome aggregates (Figs. 2 and 6*a*). In order to distinguish between mRNA and the newly synthesized rRNA, the two RNA species comprising the pulse-labelled RNA, fractions containing polyribosomes, 70s ribosomes and ribosomal subunits were isolated from sucrose gradients and pooled; the RNA was extracted and used in hybridization-competition experiments. In these experiments, increasing quantities of unlabelled rRNA, free from mRNA, were mixed with a constant amount of pulse-labelled RNA extracted from unfractionated cell lysates and from each of the pooled sucrose gradient fractions, and annealed with immobilized heat-denatured DNA bound to cellulose nitrate membrane filters at a DNA/pulse-labelled RNA ratio of 400:1. Efficiency of annealing without competitor RNA was 45–55%. The amount of radioactivity hybridized without competitor RNA was taken as 100% hybridization.

(i) Competition between pulse-labelled RNA from whole-cell lysates and unlabelled rRNA. A series of filters were annealed with pulse-labelled RNA obtained from unfractionated cell lysates. Addition of increasing quantities of unlabelled rRNA to the annealing mixture resulted in a steady lowering of the amount of pulse-labelled RNA binding to the DNA. At an excess of rRNA added (rRNA/pulse-labelled RNA ratio of 100:1) a value of 30% hybridization of pulse-labelled RNA was attained. Further addition of unlabelled rRNA did not decrease this value (Fig. 3*a*). (ii) Competition between pulse-labelled RNA samples from different regions of the sucrose gradient and unlabelled rRNA. With an excess of unlabelled rRNA added the amount of hybridization of pulse-labelled RNA from the polyribosome region was 65% and that of pulse-labelled RNA extracted from the region of 70s ribosomes was 33% (Figs. 3*b* and 3*c*). Pulse-labelled RNA extracted from the ribosomal subunits hybridized as rRNA (Figs. 3*d* and 3*e*). Its annealing with the DNA decreased steadily with increasing quantities of unlabelled rRNA until its complete

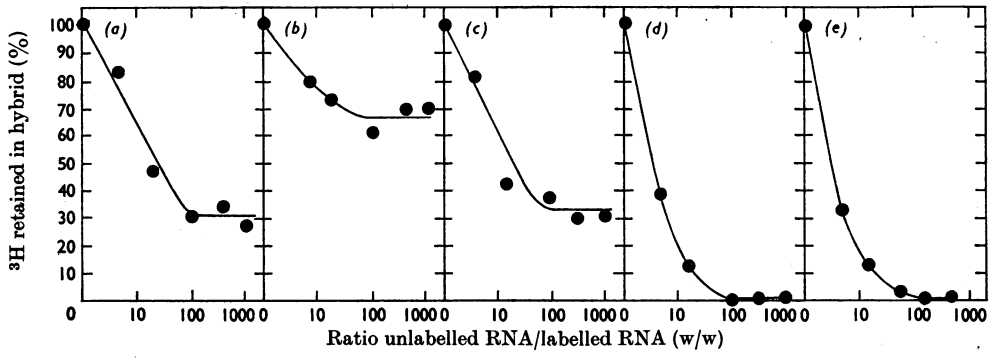

Fig. 3. Competition of *E. coli* unlabelled rRNA with *E. coli* pulse-labelled RNA for hybridization sites on homologous DNA. Pulse-labelled RNA (0·15 μg.) from (*a*) unfractionated lysates, (*b*) polyribosomes, (*c*) 70s ribosomes, (*d*) 50s ribosomal subunits, (*e*) 30s ribosomal subunits, and various quantities of unlabelled rRNA free from mRNA were annealed with immobilized heat-denatured DNA bound to cellulose nitrate membrane filters (60 μg. of DNA per filter) as described in the Materials and Methods section. Efficiency of hybridization without competitor RNA was 45–55%. The amount of radioactivity hybridized without competitor RNA represents 100% hybridization. Background corrections were done for each point of the curve from control samples containing pulse-labelled RNA, the appropriate quantities of unlabelled competitor RNA and cellulose nitrate filters without DNA.

exclusion from the hybrid at a 100-fold excess of unlabelled rRNA. Similar results were obtained in hybridization-competition experiments with pulse-labelled RNA from extracts obtained by alumina grinding. In this case too, the pulse-labelled RNA extracted from ribosomal subunits consisted of rRNA exclusively. The pulse-labelled RNA associated with the 70s ribosomes and the few polyribosomes remaining unbroken consisted of a mixture of newly synthesized rRNA and mRNA. Thus newly synthesized rRNA free from mRNA can be readily isolated from sucrose-gradient regions where ribosomal subunits sediment, regardless of the method used for preparation of the extract.

Shear-sensitivity of newly synthesized rRNA in nascent ribosomal subunits. An exponentially growing culture of *E. coli* B in glucose–salts medium was pulse-labelled, disrupted by alumina grinding, and fractionated by centrifugation in sucrose gradients. The ribosomal subunits were pooled and the RNA was extracted. Fig. 4 shows the sedimentation analysis of rRNA extracted from ribosomal subunits. The u.v.-absorbing material that represents the two rRNA components of mature ribosomal subunits is resolved into two fractions sedimenting at 23s and 16s. The radioactivity representing newly synthesized rRNA from nascent ribosomal subunits sediments as a broad peak extending from 8s to 16s, indicating extensive shear brought about by alumina grinding of the cells. It can thus be concluded that newly synthesized rRNA in nascent ribosomal subunits is sensitive to shear in conditions in which rRNA in mature ribosomes is shear-resistant.

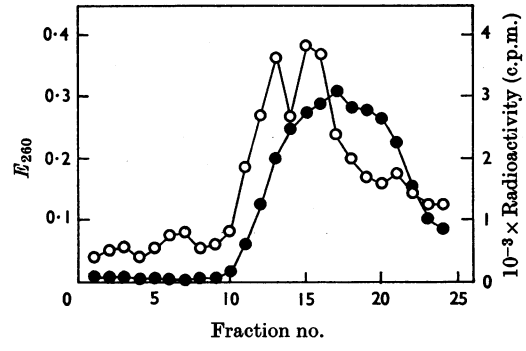

Fig. 4. Sedimentation pattern of RNA from mature and nascent ribosomal subunits. Cells were grown in glucose–salts medium without casamino acids, pulse-labelled and disrupted by alumina grinding. The extracts were layered on 4·4ml. sucrose gradients (5–20%, w/v) in 10mM-tris-HCl buffer, pH 7·4, containing 10mM-magnesium acetate and 60mM-KCl, centrifuged in a SW 39 rotor at 37000rev./min. at 4° for 80min. and the fractions collected. Fractions from the region of native ribosomal subunits were pooled and the RNA was extracted as described in the Materials and Methods section. The RNA was dissolved, centrifuged in sucrose gradients and analysed as described in the legend to Fig. 1. ○, E_{260} of RNA from mature ribosomal subunits; ●, trichloroacetic acid-precipitable radioactivity of newly made rRNA from nascent ribosomal subunits.

Breakdown of newly synthesized rRNA during glucose starvation and in the presence of actinomycin D. Having shown that the heterogeneity of size displayed by pulse-labelled RNA is not a reflection

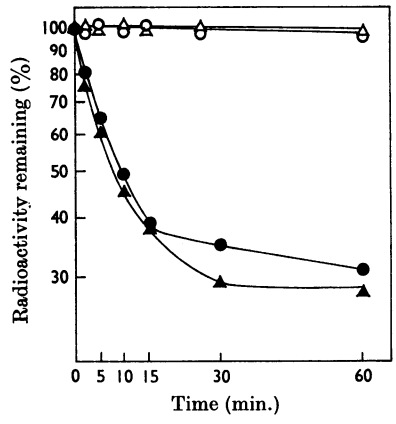

Fig. 5. Degradation of pulse-labelled RNA of *E. coli* during glucose starvation and in the presence of actinomycin D. Cells of *E. coli* B grown exponentially in glucose–salts medium were either pulse-labelled with [^{3}H]uracil or exposed to radioactive uracil for long enough to label stable RNA. The cells were then incubated in starvation medium devoid of glucose. At zero time and after different times of starvation, 2ml. samples were taken into an equal volume of cold 10% (w/v) trichloroacetic acid. *E. coli* K12 were grown exponentially in glucose–salts medium supplemented with casamino acids, sensitized with EDTA and pulse-labelled. Another culture with the ^{3}H in stable RNA was similarly sensitized with EDTA. To the sensitized cells in flasks wrapped in aluminium foil enough actinomycin D (2mg./ml.) was added to bring the final concentration of the antibiotic to 10μg./ml. of medium. Samples were taken at zero time and after different times of exposure to actinomycin and added to cold 10% (w/v) trichloroacetic acid. All samples in trichloroacetic acid were filtered through Millipore filters. The filters were rinsed with several portions of cold 5% (w/v) trichloroacetic acid, dried and their radioactivity was counted. The ^{3}H radioactivity insoluble in 5% trichloroacetic acid is plotted against time of starvation and time of exposure to actinomycin D. The results are plotted on a logarithmic scale as a percentage of the value at zero time. ●, Pulse-labelled cells starved for glucose; ▲, pulse-labelled cells exposed to actinomycin D; ○, cells with the ^{3}H in stable RNA, starved of glucose; △, cells with the isotope in stable RNA exposed to actinomycin D.

of the true size of mRNA, but an artifact produced by shear of newly synthesized rRNA (and probably of mRNA too), we next attempted to show whether the decay of pulse-labelled RNA *in vivo* in the presence of actinomycin D (Levinthal *et al.* 1962; Artman & Engelberg, 1965), the breakdown of polyribosomes during glucose starvation (Dresden & Hoagland, 1967) and the breakdown of pulse-labelled RNA *in vitro* (Artman & Engelberg, 1964) can be interpreted to mean that mRNA is metabolically unstable and turns over rapidly. Since under these conditions more that 70% of pulse-labelled RNA became degraded, we suspected that in these earlier experiments newly synthesized rRNA was also degraded. Had only mRNA been involved, not more than 30% of the pulse-labelled RNA would appear unstable. To test this, two series of experiments were performed. (i) Pulse-labelled cells and cells with radioactively labelled stable RNA were starved in media devoid of an energy source or were exposed to actinomycin D. At zero time and after various times of incubation the loss of acid-insoluble radioactive material derived from [^{3}H]uracil was measured. (ii) Pulse-labelled cells were either starved of glucose or exposed to actinomycin D. At zero time and after 30min. of incubation in starvation media or in the presence of actinomycin D, the cells were disrupted by alumina grinding and the extracts analysed by sucrose-density-gradient centrifugation. Fig. 5 shows that the tRNA and rRNA components in mature ribosomes remained stable both in the presence of actinomycin D and during glucose starvation. On the other hand approximately 71% of the pulse-labelled RNA became degraded to acid-soluble material under these conditions. The degradation was exponential. It should be recalled that from the rate of decay of pulse-labelled RNA the half-life of mRNA was computed to be 4·8min. (Zimmermann & Levinthal, 1967). The apparent half-life of the RNA that became degraded in our experiments was 4–5min. The sedimentation analysis of RNA that became degraded during glucose starvation is presented in Fig. 6. Fig. 6(*a*) shows a typical pattern of sedimentation of various types of ribosome and of pulse-labelled RNA in *E. coli* cells before glucose starvation, and Fig. 6(*b*) shows a similar profile after glucose starvation. For the estimation of the extent of breakdown of pulse-labelled RNA after glucose starvation, the 'specific radioactivity' of the pulse-labelled RNA before and after glucose starvation was computed by calculating the $^3H/E_{260}$ ratio, where the ^{3}H-labelled material was synthesized during the pulse and E_{260} represents stable RNA. The total amount of 260nm.-absorbing material/cell remained unaltered during glucose starvation. It can be estimated from Fig. 6 that out of 38249c.p.m. representing pulse-labelled RNA, 38% (14636c.p.m.) was associated with 70s monomers and larger ribosome aggregates, 47% (17653c.p.m.) with nascent subunits and 15% 6005c.p.m.) with tRNA. After 30min. of incubation in media devoid of an energy source, breakdown of 60% of the pulse-labelled RNA occurred. There was a loss of 66% of the pulse-labelled RNA originally associated with 70s and larger ribosome aggregates (4770c.p.m. remained) and a loss of 73% of newly synthesized rRNA originally associated with nascent subunits (4720c.p.m. remained). tRNA labelled during the

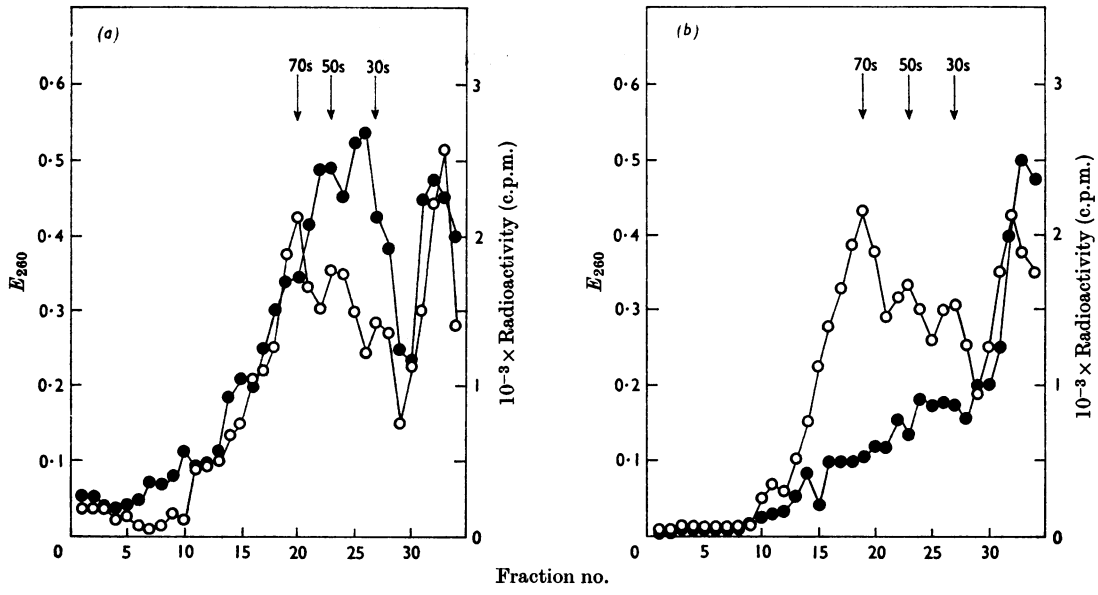

Fig. 6. Sedimentation pattern of extracts from exponentially growing cultures and after glucose starvation. A culture of *E. coli* B was grown exponentially in glucose–salts medium, pulse-labelled (as described in the legend to Fig. 1) and divided into two portions. One was immediately poured on to crushed frozen 10mM-tris–HCl buffer, pH7·4, containing 10mM-magnesium acetate and the other was starved of glucose for 30min. The cells were then disrupted by alumina grinding and eluted with 10mM-tris–HCl buffer, pH7·4, containing 10mM-magnesium acetate and 60mM-KCl. The extracts were layered on a 4·4ml. sucrose gradient (5–20%, w/v) in the same buffer and centrifuged in a SW39 rotor at 37000 rev./min. at 4° for 80min. The gradient was fractionated and E_{260} values and acid-insoluble radioactivity were determined as described in the Materials and Methods section. (*a*) Sedimentation pattern of extracts from exponentially growing cells. (*b*) Sedimentation patterns of extracts from cells after glucose starvation. ○, E_{260}; ●, trichloroacetic acid-insoluble radioactivity.

pulse remained intact. Similar results in all respects were obtained with pulse-labelled cells exposed to actinomycin D. The results of these experiments show that the rate of breakdown of pulse-labelled RNA does not reflect the half-life of any RNA species, since under these conditions an otherwise stable rRNA is degraded.

Stimulation of amino acid incorporation by newly synthesized rRNA. We have examined the response of an amino acid-incorporating system to various concentrations of rRNA from glucose-starved cells and from exponentially growing cultures. RNA extracted from glucose-starved cells was practically free from newly synthesized RNA, the latter having been broken down during starvation of the cells. RNA obtained from exponentially growing cultures contained newly synthesized rRNA when extracted from ribosomal subunits, or a mixture of mRNA and newly made rRNA when extracted from whole cell lysates or from polyribosomes and 70s ribosomes. The results are illustrated in Fig. 7. They show that rRNA from mature ribosomes is devoid of all stimulatory activity. With the various RNA preparations obtained from exponentially growing cells, linear response curves were obtained. Although the stimulatory activity of newly synthesized rRNA appeared to be twice that of the RNA that contained mRNA, the relative efficiency of the two fractions of newly made RNA was the same. This was borne out by the finding (see Fig. 6*a*) that, per unit weight, ribosomal subunits contained twice the amount of radioactivity and hence twice the amount of newly made RNA (Bremer & Yuan, 1968) that the 70s ribosomes and polyribosomes combined.

DISCUSSION

The term newly synthesized (or newly made) rRNA as used throughout this work refers to RNA synthesized by *E. coli* during one-hundredth of the generation time, which hybridizes as rRNA. The results of hybridization-competition experiments presented in this work confirmed the findings of McCarthy & Bolton (1964) and of Pigott & Midgley (1968) that approximately 70% of pulse-labelled RNA consists of newly made rRNA. Newly synthesized rRNA was found in polyribosomes, in

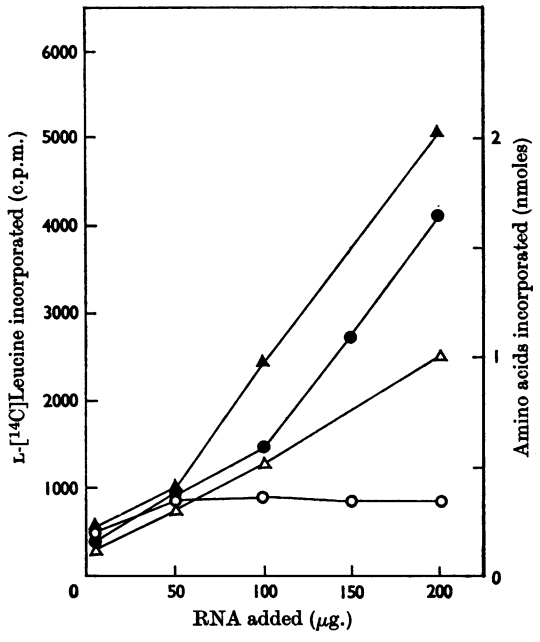

Fig. 7. Stimulation of amino acid incorporation by newly synthesized RNA. The extracts were prepared from *E. coli* B grown in Bacto-Tryptone broth. Conditions were as described in the Materials and Methods section. The extracts were stimulated with RNA from; ○, *E. coli* B cells grown in glucose–salts medium and starved of glucose for 90min.; △, cells grown exponentially in glucose–salts medium; ●, polyribosomes and 70s ribosomes from cells grown exponentially in the above medium; ▲, native and nascent subunits of the above cells. The left-hand ordinate shows the amount of L-[14C]leucine incorporated into hot-trichloroacetic acid-precipitable material. The right-hand ordinate shows the number of nmoles of amino acids incorporated, calculated on the basis of a counting efficiency of 83% and a molar fraction of leucine of 9·2% (Goldstein, Goldstein & Lowney, 1964).

70s ribosomes and in nascent ribosomal subunits. Pulse-labelled RNA associated with 70s ribosomes and larger ribosome aggregates was a mixture of mRNA and rRNA. This finding is at variance with that of Mangiarotti & Schlessinger (1967), who reported that pulse-labelled RNA associated with polyribosomes of *E. coli* growing in high-salts medium with a generation time of 2hr. consisted entirely of mRNA. In our experiments, with cells growing in normal physiological conditions with a generation time of 55min., newly made rRNA was found in polyribosomes even after the shortest pulses given. The newly synthesized RNA in nascent ribosomal subunits, on the other hand, consisted exclusively of rRNA. Thus the isolation of labelled mRNA free from newly made rRNA is not feasible, but newly made rRNA free from mRNA can be readily isolated from nascent ribosomal subunits.

Sedimentation behaviour of newly made rRNA. Since the greater proportion of pulse-labelled RNA consists of rRNA, the sedimentation behaviour of the former should resemble that of rRNA from mature ribosomes. It has been shown, however, in the present and in previous work (Fry & Artman, 1968) that the sedimentation behaviour of pulse-labelled RNA depends on the method of preparation of bacterial extracts. When bacteria were disrupted by sonication or alumina grinding the pulse-labelled RNA obtained from whole extracts or from nascent ribosomal subunits had a mean sedimentation coefficient of 8–16s. Sonicated deproteinized rRNA from mature ribosomes also sedimented as a broad peak at 8–10s. On the other hand, when the pulse-labelled RNA was extracted from gently lysed spheroplasts, its sedimentation behaviour was similar to that of rRNA and both sedimented as two peaks at 23s and 16s.

From these results it is concluded that (i) the low sedimentation coefficient and the apparent heterogeneity of size displayed by newly synthesized rRNA, and indeed of the pulse-labelled RNA as a whole, obtained from mechanically disrupted cells resulted from shear of pulse-labelled RNA during preparation of the extract, and cannot be regarded as reflecting the size of mRNA. (ii) Deproteinized rRNA from mature ribosomes is as shear-sensitive as is pulse-labelled RNA. (iii) In mature ribosomes the association of RNA and ribosomal proteins gives protection to the RNA moiety against mechanical shear. (iv) The difference in shear-sensitivity of rRNA in mature and in nascent ribosomes may be explained by the fact that the latter lack certain specific ribosomal proteins (Osawa, Otaka, Itoh & Fukui, 1964), which through their association with the RNA render the latter resistant to shear.

Breakdown of newly made rRNA during glucose starvation and in the presence of actinomycin D. The metabolic instability and rapid turnover of mRNA were inferred mainly from studies on the decay of pulse-labelled RNA in the presence of actinomycin D (Levinthal *et al.* 1962) and on breakdown of polyribosomes during glucose starvation (Dresden & Hoagland, 1967). The results reported in this work show conclusively that during glucose starvation most of the newly synthesized rRNA associated with nascent ribosomal subunits is rapidly degraded to acid-soluble compounds. The experiments on the breakdown of rRNA associated with nascent ribosomes during starvation of their energy source draw attention to the capacity of bacterial cells to degrade rRNA molecules which cannot be properly made into ribosomes. Similar degradation of rRNA in animal cells under conditions

of amino acid deprivation has been reported (Darnell, 1968). The results of our experiments, which show that the bulk of newly made rRNA is broken down in the presence of actinomycin D, support the assumption of Acs, Reich & Valanju (1963) that this antibiotic may induce the breakdown of otherwise stable rRNA molecules before they had acquired their protective protein component. This action of actinomycin D is difficult to explain on the assumption that the sole function of this antibiotic is to block transcription of RNA from a DNA template through the formation of an actinomycin–DNA complex based on specificity for deoxyguanosine residues. Semmel & Huppert (1965) have shown that actinomycin D can also form complexes with rRNA and the work of Gomatos, Tamm, Dales & Franklin (1963) and of Gomatos, Krug & Tamm (1964) has shown that actinomycin D interferes with the multiplication of reovirus and inhibits the priming ability of reovirus RNA *in vitro*, probably through binding to critical areas of the RNA template. Müller & Crothers (1968) have proposed that the actinomycin chromophore is actually intercalated into the DNA adjacent to G·C base-pairs. It is known that an acknowledged intercalating agent such as proflavine brings about a degradation of pulse-labelled RNA and even a slow destruction of mature ribosomes (Soffer & Gros, 1964). Proflavine has the ability to become intercalated between adjacent base-pairs of a double helix in both DNA and RNA (Waring, 1968) and to bind to RNA in ribosomes (Miall & Walker, 1967). The number of binding sites in RNA in mature ribosomes is very small but is greatly increased when the tertiary structure is altered by unfolding the ribosome in EDTA. The selective destruction of newly made rRNA in the presence of intercalating agents can thus be explained by assuming that RNA in nascent ribosomes, which lacks certain specific protein components, has a large number of sites available for intercalation. Nascent ribosomes with intercalated RNA cannot be properly made into ribosomes and their RNA becomes degraded, as during glucose starvation. It remains still to be proved that actinomycin D can become intercalated between adjacent base-pairs of RNA in nascent ribosomes.

Stimulation of amino acid incorporation by newly made rRNA. The template activity of rRNA from chloramphenicol-treated cells of *E. coli* and from RC-particles from methionine-requiring relaxed-control mutants of *E. coli* was reported by Otaka, Osawa & Sibatani (1964), Muto (1968) and Nakada (1965). In the present work we have presented evidence to show that rRNA isolated from native ribosomal subunits of cells growing exponentially under physiological conditions stimulated amino acid incorporation in a system synthesizing protein *in vitro*. The stimulatory activity of RNA from ribosomal subunits was lost when the cells had been previously starved of glucose. Since during glucose starvation mature ribosomal subunits remained intact and only newly made rRNA became degraded, the conclusion reached was that the stimulatory activity was that of newly synthesized rRNA. The extent to which newly synthesized rRNA stimulated amino acid incorporation was similar to that of the RNA fraction that contained mRNA. The template activity of newly synthesized rRNA and of RNA from chloramphenicol- or RC-particles may be related to their lack or low content of methyl groups compared with that of RNA from mature ribosomes (Srinivasan & Borek, 1964; Gordon & Boman, 1964; Gordon, Boman & Isaksson, 1964). This assumption is supported by the finding of Takanami & Yan (1965) that methylation of polyribonucleotides may abolish their template activity. Whether newly synthesized rRNA associated with nascent ribosomal subunits ever serves as a template in the bacterial cell is a matter of speculation. Although Nakada (1965) and Muto (1968) interpreted the results of their experiments on the template activity of rRNA accumulated during the inhibition of protein synthesis to mean that it serves as messenger for the synthesis of its own ribosomal proteins, conclusive evidence for this is still lacking.

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