The Role of Ribonuclease II in the Maturation of Precursor 16S Ribosomal Ribonucleic Acid in *Escherichia coli*

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(Received 24 January 1974)

The suggested involvement of ribonuclease II in the maturation of rRNA has been examined directly by determining the activity of the enzyme and the amount of p16S rRNA in cell-free extracts from *Escherichia coli* A19 and its temperature-sensitive derivative N464 grown under experimental conditions designed to vary the amounts of enzyme and precursor independently. In strain A19 the enzyme showed maximum activity in circumstances where the amount of p16S rRNA was normal (e.g. exponential-phase cells) or raised eight times (e.g. during inhibition of growth by methionine starvation of the relaxed auxotroph or by chloramphenicol or puromycin treatment). In strain N464 at the non-permissive temperature the ribonuclease II activity may be decreased by 50% without effect upon the amount of p16S rRNA, whereas in methionine starvation of this strain the enzyme activity is at a maximum and the p16S rRNA is eight times that in exponential-phase cells. These observations are discussed in relation to the previously implied role of ribonuclease II in the maturation of rRNA within ribosome precursors.

The ribosomal RNA (rRNA) species in bacteria are all subject to considerable modification between transcription and their final incorporation into the mature ribosomes. Further, in *Escherichia coli* the cistrons for these rRNA species (5S, 16S and 23S) are closely grouped and there is some evidence to suggest that the immediate transcription product for 16S and 23S species may be a single high-molecular-weight species analogous to the 45S rRNA precursor in eukaryotes (Pettijohn *et al.*, 1970). Undoubtedly the mature ribosomal RNA species (m5S, m16S and m23S) have individual precursors of higher molecular weight (p5S, p16S and p23S) which, after transcription and during their incorporation into mature 30S and 50S ribosomes, are subject to limited specific nucleolytic attack and enzyme-catalysed methylation of certain of their bases and ribose residues. Most experimental evidence now indicates that these modifications take place at late stages in ribosome assembly, since submethylated high-molecular-weight precursor rRNA species have been detected in the penultimate precursors of 30S and 50S ribosomes (Nierhaus *et al.*, 1973; Hayes & Hayes, 1971; Lindahl, 1973). It is therefore apparent that ribosome assembly *in vivo* involves a number of essential enzymic steps not shown in the reconstruction of ribosomes *in vitro* (Nomura, 1970). *E. coli* p16S rRNA is known to have additional nucleotides at the 3' and 5' termini compared with m16S rRNA. The nucleotides at the 5' end are removed as a single unit of approximately 150–200 nucleotides in an endonucleolytic scission (Adesnik & Levinthal, 1969; Dahlberg & Peacock, 1971). The evidence for a simple endonucleolytic scission is indirect and rests upon the demonstration that the

conversion of p16S into m16S rRNA is discrete and a spectrum of intermediates is not observed. Feunteun *et al.* (1972) have also observed that maturation of p5S rRNA in *E. coli* proceeds via an endonucleolytic cleavage of a small number of nucleotides from the 5' terminus and in *Bacillus subtilis* a similar process removes rather more nucleotides from the 5' terminus (Pace *et al.*, 1973). The conversion of p23S into m23S rRNA is unclear since the presence of p23S rRNA is not readily detected by the techniques usually adopted although extra nucleotides have been reported in p23S rRNA (Sogin *et al.*, 1973).

The nuclease enzymes involved in these important transformations are largely unknown. In the specific case of the 5S tyrosine transfer RNA precursor Robertson *et al.* (1972) have demonstrated the presence of a specific ribonuclease (RNAase P) associated with ribosomes, which cleaves a single phosphodiester bond to release a 41 nucleotide fragment from the 5' end of the precursor. The reported specificities and modes of action *in vitro* of the other known ribonucleases (i.e. ribonucleases I–IV) together with the properties of strains of *E. coli* carrying mutations of some of these enzymes would indicate that these enzymes are unlikely to be involved in the distinctive conversion of precursor into mature rRNA. However, there are reports implicating ribonuclease III in the specific cleavage of RNA to generate early messenger RNA species of T7 bacteriophage (Robertson *et al.*, 1968; Dunn & Studier, 1973). Further, precursor rRNA species accumulate in temperature-sensitive ribonuclease II mutants of *E. coli* grown at non-permissive temperatures and the conversion of isolated p16S rRNA (Corte *et al.*, 1971; Yuki, 1971) and

p23S rRNA (Venkov, 1972) into their corresponding mature forms *in vitro* has been claimed by using purified ribonuclease II. On the basis of this type of evidence without direct assays for activity *in vivo* and despite its known exonucleolytic function and cation requirements (Singer & Tolbert, 1965), ribonuclease II was claimed to be involved in the maturation of p16S and p23S rRNA *in vivo*.

Accordingly in the present paper the suggested role of ribonuclease II in the maturation of rRNA is examined by direct determinations of the activity of the enzyme *in vivo* together with the amounts of precursor rRNA species, in experimental circumstances designed to vary their respective amounts. The amounts of precursors are altered by starvation of a relaxed amino acid auxotroph for the required amino acid or by treatment of growing *E. coli* cultures with the inhibitors puromycin and chloramphenicol. The activity of enzyme is varied by growing a derived temperature-sensitive ribonuclease II strain of *E. coli* at the non-permissive temperature.

Materials and Methods

Bacterial strains and growth media

E. coli A19 (*rel*⁻, *met*⁻, *rns*⁻)* was obtained from Dr. G. Turnock, University of Leicester, Leicester, U.K. *E. coli* N464 (*rel*⁻, *met*⁻, *tyr*⁻, *rns*⁻, *rne*^{ts}, *pnp*^{ts}), a strain derived by *N*-methyl-*N*-nitro-*N*-nitrosoguanidine treatment of strain A19, was obtained from Professor D. Schlessinger, University of Washington, St. Louis, Mo., U.S.A. Both organisms were grown on a minimal medium containing, per litre, 9g of KH₂PO₄, 1g of (NH₄)₂SO₄, 1.6g of MgSO₄·7H₂O, 4g of glucose, adjusted to pH7.5 with NaOH and supplemented with 100mg of L-tyrosine/l and 50mg of DL-methionine/l as appropriate. In certain experiments a peptone-enriched medium was used, containing per litre 10g of bacteriological peptone (Oxoid, Ltd., London S.E.1, U.K.), 9g of KH₂PO₄, 1g of (NH₄)₂SO₄, 0.8g of MgSO₄·7H₂O, 5g of glucose, adjusted to pH7.5 with NaOH. All cultures were grown in 250ml volumes in 21 conical flasks in a gyrotary shaker at 30°C (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.). When required an increase in temperature was effected by transferring a culture to a pre-set gyrotary shaker or water-bath shaker.

Bacteria were starved for methionine by first centrifuging the cells from a growing culture, washing once with minimal medium minus methionine and finally resuspending in a pre-warmed quantity of the same

* Abbreviations used for mutations as follows: *rel*⁻, relaxed at the RC locus; *met*⁻, *tyr*⁻, requirements for L-methionine or L-tyrosine respectively; *rns*⁻, ribonuclease I negative; *rne*^{ts}, *pnp*^{ts}, temperature-sensitive ribonuclease II and polynucleotide phosphorylase respectively; RNAase, ribonuclease.

medium. Bacteria were treated with chloramphenicol (Parke, Davis and Co., Hounslow, Middx., U.K.) or puromycin dihydrochloride (Calbiochem Ltd., London W.1, U.K.) by harvesting the cells from a culture in minimal medium and resuspending the bacteria in fresh pre-warmed medium containing 50µg of chloramphenicol/ml or 100µg of puromycin/ml. In view of the known antagonistic effect of Mg²⁺ on puromycin inhibition (Sells, 1964), before cells were treated with puromycin they were washed twice in minimal medium containing 0.5mM-MgSO₄·7H₂O and finally resuspended in this medium containing the puromycin.

Cell-free extracts of bacteria were prepared by harvesting the cells from a culture by centrifugation at 4°C (MSE 18 centrifuge, 6 × 250ml rotor, 12000rev./min, 10min) and crushing the bacterial paste in a press at -20°C (Hughes, 1951). The crushed frozen cell paste was dispersed in 3.5vol. of 10mM-Tris-HCl buffer, pH7.8, containing 30mM-NH₄Cl, 0.3mM-MgCl₂ and 6mM-β-mercaptoethanol, with the aid of an homogenizer. The resulting homogenate was centrifuged in the Spinco 30 rotor (Beckman Instruments, Glenrothes, Fife, U.K.) at 54500g_{av} for 45min at 5°C. The optically clear supernatant from this step was the crude cell-free extract used in the analytical-ultracentrifugation experiments. For the RNAase II assay the crude cell-free extracts were prepared exactly as above but in 10mM-Tris-HCl (pH7.5)-1.5mM-MgCl₂-1mM-β-mercaptoethanol.

Extraction of RNA

RNA was extracted from the cell-free extracts by shaking the latter twice with the phenol-*m*-cresol-8-hydroxyquinoline mixture described by Kirby (1956). The final aqueous phase from these extractions was treated with 2vol. of 2% (w/v) potassium acetate in absolute ethanol and left at -20°C to allow complete precipitation of the nucleic acids. The precipitate was collected by centrifugation and washed several times with 75% (v/v) ethanol before dissolving in 0.01M-sodium acetate buffer, pH4.6, containing 0.1M-NaCl. If necessary this solution was clarified by 20min centrifugation at 24148g_{av} and the RNA concentration of the clear supernatant estimated by absorbance at 260nm (Kurland, 1960).

The RNA was also directly extracted from the crushed cell pastes by suspension in 3.5vol. of 0.01M-Tris-HCl-1mM-magnesium acetate, pH7.8, and adding a final concentration of 0.2% (w/v) sodium dodecyl sulphate (specially pure grade, British Drug Houses, Poole, Dorset, U.K.) before shaking with the phenol-*m*-cresol-8-hydroxyquinoline mixture.

Polyacrylamide-gel electrophoresis

The RNA preparations were analysed by electrophoresis in 3% (w/v) polyacrylamide gels prepared as

described by Loening (1967). The acrylamide and *NN'*-methylenebisacrylamide were recrystallized before use and the gels pre-run for 1 h. The electrophoresis buffer was 36mM-Tris–30mM- NaH_2PO_4 –1mM-EDTA (disodium salt), pH7.6, and contained 0.2% (w/v) sodium dodecyl sulphate to minimize nuclease action (Loening, 1969). The cylindrical gels were 6cm long $\times$ 0.7cm diam. and were prepared in glass or silica tubes. Electrophoresis was for 4½ or 5 h at room temperature (approx. 20°C) at a constant 5mA/gel and 6V/cm. Gels run in glass tubes were carefully removed and scanned at 260nm (Robinson & Sykes, 1971). Gels in silica tubes were scanned *in situ* at 260nm in a Unicam SP.500 spectrophotometer fitted with a Gilford linear-transport accessory. The areas under the peaks on the electrophoretograms were measured with a planimeter.

Ribonuclease II assay

Potassium-activated phosphodiesterase activity (ribonuclease II) was measured by using the assay described by Singer & Tolbert (1965) with non-radioactive polyadenylic acid as substrate (K^+ salt, mol.wt. 100000, Koch–Light Ltd., Colnbrook, Bucks., U.K.). The standard assay mixture contained (in 0.1ml) 100mM-KCl, 100mM-Tris–HCl, pH7.5, 1.5mM- MgCl_2 , 2mM-polyadenylic acid and enzyme. The optimum amount of enzyme to give a linear rate of hydrolysis with enzyme concentration was in the range up to 30µg of crude cell-free extract of protein (see below). After incubation at 37°C for 20min, 0.4ml of ice-cold 0.156% (w/v) uranyl acetate in 2.5% (v/v) HClO_4 was added. The small centrifuge tubes containing the mixture were kept on ice for 10min and then centrifuged at 4°C for 15min at 2000g_{av.}. The absorbance at 260nm of the final supernatant was recorded together with the absorbance of zero time and appropriate substrate and reagent blanks. One unit of enzyme activity is equivalent to the production of 1µmol of 5'-AMP/h. Polyadenylic acid concentrations were measured spectrophotometrically by assuming a molar extinction coefficient of 10500 litre·mol⁻¹·cm⁻¹ at 257nm (Singer *et al.*, 1962). 5'-AMP concentrations were also measured spectrophotometrically by assuming a molar extinction coefficient of 15000 litre·mol⁻¹·cm⁻¹ at 260nm and acid pH.

Protein was determined by the biuret (Gornall *et al.*, 1949) and Folin–Ciocalteu reactions (Lowry *et al.*, 1951) with crystallized bovine serum albumin as standard.

Analytical ultracentrifugation

Crude cell-free extracts prepared as described above were examined in the Spinco model E analytical ultracentrifuge (Beckman Instruments) with schlieren

optics and the R.T.I.C. (rotor temperature indicator and control) unit.

Results

Assay of ribonuclease II activity in cell-free extracts

A reliable estimate of the maximum realizable ribonuclease II activity in the cell-free extracts obtained from the bacteria grown under a variety of circumstances is required. A number of factors likely to bear upon this were therefore examined, namely (i) other nuclease activities giving rise to cold acid-soluble u.v.-absorbing products, (ii) possible 'latency' of this predominantly ribosome-bound enzyme, cf. ribonuclease I (Spahr, 1964; Datta & Burma, 1972), and (iii) the presence of inhibitors in the extract, e.g. oligodeoxyribonucleotides inhibiting the enzyme (Gorelic & Apirion, 1971) or ribosomes binding and protecting the substrate (Castles & Singer, 1969).

The possibility of other ribonuclease activities contributing to the measured activity was eliminated by incorporating appropriate blank determinations in all assays, by the assay conditions, by the nature of the substrate and by using ribonuclease I-negative strains. Even under optimal conditions for ribonuclease I the activity of this enzyme in negative mutants, e.g. *E. coli* A19 and M.R.E. 600, is less than 0.5% of the wild-type activity (Gesteland, 1966; Robinson & Sykes, 1973). This activity is further decreased under the optimal conditions of ribonuclease II assays (J. Sykes & E. Metcalf, unpublished observations). Further, in all the assays a variety of control determinations were included to check for the production of acid-soluble u.v.-absorbing materials before and during the incubation from the cell-free extract alone in the buffer and the substrate alone in the buffer. All the activities recorded are corrected for these control determinations. The other ribonuclease activities potentially contributing to the observed activity are also eliminated by the assay conditions since they either require double-stranded RNA as a substrate (for ribonuclease III; Robertson *et al.*, 1968) or the presence of phosphate (for polynucleotide phosphorylase).

Ribonuclease II activity is predominantly associated with the ribosomes in *E. coli* extracts. The extracts used in the assays contain intact ribosomes and these may act to partially inhibit ribonuclease II activity by binding the enzyme or the substrate. To examine these possibilities and also to show the dependence of the measured activity on K^+ and Mg^{2+} the experiment detailed in Table 1 was performed. The results in Table 1 show that the measured activity depends on the presence of K^+ and Mg^{2+} . Further, pretreatment with EDTA to bring about degradation of the ribosomes in the extract is without significant

Table 1. Dependence of ribonuclease II activity in cell-free extracts of *E. coli* A19 on ribosomes, Mg^{2+} and K^+

Cell-free extracts of *E. coli* A19 for ribonuclease II assay were prepared as described in the Materials and Methods section. The extract was divided and (i) assayed without further treatment, (ii) treated for 3 h at 4°C with an equal volume of extracting buffer containing 0.1M-EDTA and 0.2M-KCl. The extract was then dialysed against the extracting buffer overnight (approx. 16h) at 4°C before enzyme assays.

RNAase II assay conditions	Activity (units/mg of protein)	
	Untreated extract	Treated extract
Complete	16.20	15.28
- K^+	2.32	1.32
- Mg^{2+} and in the presence of 50mM-EDTA	1.47	1.05

Fig. 1. Effect of dialysis and deoxyribonuclease (EC 3.1.4.5) on the ribonuclease II activity in cell-free extracts from *E. coli* A19

For details see the text. ●, Undialysed extract; △, dialysed extract; □, extract treated with deoxyribonuclease and then dialysed.

effect on the activity. The ribosomes in the crude cell-free extracts are therefore unlikely to influence the activities measured.

The reported inhibition of ribonuclease II by oligodeoxyribonucleotides present in extracts (Gorelic & Apirion, 1971) was not found in the extracts used in these experiments. Fig. 1 shows that dialysis of the cell-free extract with or without previous deoxyribonuclease (EC 3.1.4.5) treatment has no effect on the measured activity. Other workers, e.g. Holmes & Singer (1971), have also failed to observe the inhibition by oligodeoxyribonucleotides. The data in Fig. 1 show that the hydrolysis of polyadenylic acid in this assay is linearly dependent on enzyme concentration except at high enzyme concentrations when 80% of

Fig. 2. Thermal inactivation of ribonuclease II in cell-free extracts from *E. coli* strains A19 and N464

Samples (1 ml) of cell-free extracts (containing between 4 and 7 mg of protein/ml) in 10mM-Tris-HCl buffer, pH 7.5, containing 1.5mM- $MgCl_2$ and 1mM- β -mercaptoethanol were kept at the temperatures shown for 5 min and then immediately chilled in ice before assay for ribonuclease II activity as described in the Materials and Methods section. Certain preparations were also dialysed overnight at 4°C against the buffer before the above treatment. ●, Extract from *E. coli* A19 grown in minimal medium, undialysed; ○, extract from *E. coli* A19 grown in minimal medium, dialysed; ▲, extract from *E. coli* N464 grown in minimal medium, undialysed; △, extract from *E. coli* N464 grown in minimal medium, dialysed; ■, extract from *E. coli* N464 grown in peptone medium, undialysed.

the added substrate has been hydrolysed (Singer, 1966). Accordingly, in these experiments all the assays were carried out in the optimum range of crude extract of protein in the assay, i.e. up to 30 μg of extract of protein/assay. It may be concluded from the observations in this section that the assay procedure adopted specifically measures the maximum ribonuclease II activity in the cell-free extracts.

The relative temperature sensitivity of the ribonuclease II activity in *E. coli* N464 compared with strain A19 is shown in Fig. 2. These results essentially confirm the observations of Weatherford *et al.* (1972). Dialysis of the cell-free extract makes no significant difference to the temperature versus activity profile.

Fig. 3. Analytical-ultracentrifuge schlieren diagrams for cell-free extracts from *E. coli* A19

Schlieren photographs were taken after 12 min at 50740 rev./min and with bar angle 45° with crude cell-free extracts from *E. coli* A19 prepared as described in the Materials and Methods section from: (a) exponential-phase cells in minimal medium at 30°C. Temperature of run 22.5°C, protein concentration 10.0 mg/ml; (b) cells after 2h starvation for methionine in minimal medium at 30°C. Temperature of run 20.5°C, protein concentration 10.0 mg/ml; (c) cells after 2h treatment with chloramphenicol in minimal medium. Temperature of run 24.2°C, protein concentration 10.0 mg/ml; (d) cells after 2h treatment with puromycin in minimal medium at 30°C. Temperature of run 20°C, protein concentration 10.0 mg/ml.

Table 2. Precursor 16S rRNA amounts and ribonuclease II activities in cell-free extracts from *E. coli* A19 grown under various conditions

For details see the text. p16S/m16S rRNA ratios were obtained by planimetry of areas on densitometric records of the RNA separations on polyacrylamide gels (see Fig. 4). The values in parentheses are enzyme activities for extracts from cells grown in media containing a lowered amount of Mg^{2+} (see the Materials and Methods section).

Culture conditions	Ratio of p16S/m16S rRNA	Ribonuclease II activity (units/mg of protein)
Exponential-phase cells, complete minimal medium	0.112	20.5 (16.7)
Exponential-phase cells transferred to fresh minimal medium minus methionine for 2h	0.92	18.0
Exponential-phase cells transferred to fresh complete minimal medium + 50 µg of chloramphenicol/ml for 2h	0.88	21.2
Exponential-phase cells transferred to fresh complete minimal medium + 100 µg of puromycin dihydrochloride/ml for 2h	0.47	(17.0)

p16S rRNA amounts and ribonuclease II activity in E. coli A19 under various growth conditions

Figs. 3(a), 4(a) and Table 2 show the analytical-ultracentrifuge schlieren diagram for a crude cell-free extract from exponential-phase *E. coli* A19 grown in minimal medium, the electrophoretogram of the total RNA fraction from this extract and the ribonuclease II activity respectively. The corresponding data for a culture starved for methionine in minimal medium for 2h are shown in Figs. 3(b) and 4(b) and Table 2. The response to methionine starvation is typical for a relaxed strain, i.e. growth is inhibited, RNA accumulates in the cells to two times prestarvation amounts and ribonucleoprotein particles sedimenting between 12 and 22S appear in the ultracentrifuge diagram (compare Figs. 3a and 3b). The electrophoretograms show that p16S rRNA also accumulates in these circumstances (compare Figs.

4a and 4b) so that the p16S/m16S rRNA ratio rises from 0.112 in exponential-phase cells to 0.92 after 2h methionine starvation (Adesnik & Levinthal, 1969). p23S rRNA also accumulates during starvation but is not resolved from m23S rRNA in these gels.

The addition of 50 µg of chloramphenicol/ml to a growing culture of *E. coli* A19 in minimal medium also strongly inhibits growth, brings about the accumulation of ribonucleoprotein particles in the cell-free extract (compare Figs. 3a and 3c) and RNA within the cells. The p16S/m16S rRNA ratio rises from 0.112 to 0.88 during the 2h inhibition (compare Figs. 4a and 4c). A similar pattern occurs with inhibition by puromycin (compare Figs. 3a and 3d and 4a and 4d) and here the p16S/m16S rRNA ratio rises from 0.112 to 0.47.

The data in Table 2 summarize the corresponding ribonuclease II activities in the cell-free extracts from these inhibited cells and the p16S/m16S rRNA ratios

Fig. 4. Accumulation of p16S rRNA in *E. coli* A19 in various media

Densitometer records for the electrophoretic separation of rRNA preparations in polyacrylamide gels are shown. The rRNA preparations were extracted from cells grown as described below, electrophoresed in 3% gels and the latter scanned at 260 nm as described in the Materials and Methods section. (a) Exponential-phase A19 cells in minimal medium. RNA (50 μ g) was loaded and electrophoresis was for 5 h. (b) Strain A19 grown in minimal medium and transferred to fresh medium minus methionine for 2 h. RNA (50 μ g) was loaded and electrophoresis was for 4 $\frac{1}{2}$ h. (c) Strain A19 grown in minimal medium and transferred to fresh medium plus 50 μ g of chloramphenicol/ml. RNA (50 μ g) was loaded and electrophoresis was for 4 $\frac{1}{2}$ h. (d) Strain A19 grown in minimal medium and transferred to fresh medium plus 100 μ g of puromycin dihydrochloride/ml. RNA (40 μ g) was loaded and electrophoresis was for 5 h.

on the electrophoretograms. Clearly in circumstances that lead to an inhibition of growth and mature ribosome synthesis but continued RNA synthesis, precursor 16S rRNA accumulates while the ribonuclease II activities in the cells remain the same, or slightly increase, compared with uninhibited exponential-phase cultures.

Precursor 16S rRNA amounts and ribonuclease II activity in cell-free extracts of E. coli N464 grown at normal and non-permissive temperatures

In agreement with Corte *et al.* (1971), *E. coli* N464 displayed exponential growth in media at 30°C, and at

43°C growth was temporarily accelerated (see Table 3.) *E. coli* N464 grew on agar plates at 37°C but not at 43°C. In view of the relative temperature sensitivity of the ribonuclease in this strain (Fig. 2) and the preceding results with *E. coli* A19 similar experiments were made at 30°C and 43°C with strain N464 grown in minimal and peptone media. The p16S/m16S rRNA ratios found in the electrophoretograms of the rRNA and the ribonuclease II activities for the crude cell-free extracts in each experiment are summarized in Table 3. p16S rRNA does not accumulate in the cells in either growth medium at the non-permissive temperature. Raising the temperature to 45°C and harvesting the cells by pouring on crushed ice con-

Table 3. Amounts of precursor 16S rRNA and ribonuclease II activity in cell-free extracts from *E. coli* N464 grown under various conditions

For details see the text. N.D., not determined.

Growth conditions	Doubling time (h)	Ratio of p16S/m16S rRNA	Ribonuclease II activity (units/mg of protein)
Exponential-phase cells, minimal medium, 30°C	2.72	0.045	6.40
Exponential-phase cells, peptone medium, 30°C	1.73	0.076	12.50
Minimal medium after 2h at 43°C	1.80	0.047	2.85
Peptone medium after 2h at 43°C	1.17	0.087	6.00
Exponential-phase cells in minimal medium at 30°C transferred to minimal medium minus methionine for 2h at 30°C	—	0.358	N.D.

taining KCN gave the same results. In the analytical ultracentrifuge the cell-free extracts from *E. coli* N464 grown at 43°C for 2h in mineral-salts or peptone media showed a small new component sedimenting at approximately 23.7S.

E. coli N464 carries the *rel*⁻ and *met*⁻ mutations and withdrawal of methionine by resuspending exponential-phase cells in fresh minimal medium at 30°C strongly inhibits growth (measured by absorbance) and after 2h there is a 50% increase in the total RNA and no detectable increase in the protein content of the cells.

Additional ribonucleoprotein particles were found in the cell-free extract sedimentation diagram (cf. Fig. 3b). In these circumstances the p16S/m16S rRNA ratio rises to approximately eight times that in exponential-phase cultures in complete minimal medium (Table 3).

The results in Table 3 show that although the activity of ribonuclease II falls at 43°C it is not totally abolished in this strain and the amount of precursor 16S rRNA does not rise in these circumstances. On the other hand, during methionine starvation at 30°C the amount of precursor RNA increased markedly.

Discussion

The specific enzymic processing of precursor RNA species into their mature forms is not doubted. An enzyme responsible for the maturation of tyrosine tRNA (RNAase P) has been characterized by Robertson *et al.* (1972) but the enzyme(s) involved in rRNA maturation are unknown. Recent studies (Corte *et al.*, 1971; Yuki, 1971), mainly based on indirect evidence obtained from ribonuclease II temperature-sensitive mutants (e.g. strain N464) at the non-permissive temperature, have strongly implicated this enzyme in rRNA maturation. However, the experiments involving direct enzyme assays reported in the present paper clearly show that a significant proportion of the enzyme is still active

after 2h at 43°C and normal activities persist in circumstances where p16S rRNA accumulates in this strain. Further, although the direct conversion *in vitro* of p16S rRNA, both free and combined in late ribosome precursor particles, into an m16S rRNA-like form has been claimed by using purified ribonuclease II (Corte *et al.*, 1971; Yuki, 1971), this has also been questioned by Hayes & Vasseur (1973). Hayes & Vasseur (1973) found that such conversions *in vitro* with cell extracts as a source of enzyme would take place in the absence of monovalent cations which are clearly essential for ribonuclease II activity (e.g. see Table 1 and Spahr, 1964; Singer & Tolbert, 1965).

It is clear from the results reported in the present paper that p16S rRNA may accumulate in the presence of normal (or slightly enhanced) ribonuclease II activities and conversely that lowered ribonuclease II activities do not give rise to substantial accumulation of the precursor RNA. The amounts of enzyme also do not appear to fluctuate in direct relation to the growth rate. Therefore if this enzyme is involved in the maturation of p16S rRNA it would appear that the activity of the enzyme is not the limiting factor in maturation. Maturation of rRNA is known to take place at a very late stage in 30S ribosome biogenesis and at this time the RNA is associated with 9 ribosomal proteins and 12 more remain to be added (Nierhaus *et al.*, 1973). The accessibility and/or conformation of the p16S rRNA may therefore be a determining factor in the maturation step. Such a view could account for the raised amounts of precursor 16S rRNA co-existing with high ribonuclease II activity observed here in methionine starvation and drug-inhibited growth. In all of these circumstances rRNA synthesis continues but ribosome biogenesis is blocked at a stage when few (if any) ribosomal proteins are associated with the precursor RNA species. In preliminary experiments it has been found that purified ribonuclease II (Singer & Tolbert, 1965) does not degrade the p16S rRNA present in the ribonucleoprotein particles accumulated in these inhibited states

(J. R. Dean & J. Sykes, unpublished observations). On the other hand both Corte *et al.* (1971) and Yuki (1971) found that p16S rRNA in the late ribosome precursors and isolated p16S rRNA were susceptible to purified ribonuclease II.

At least two enzymes are now implicated in the maturation of transfer RNA species (Schedl & Primakoff, 1973; Robertson *et al.*, 1972) and the maturation of rRNA species may also involve more than one enzyme acting in concert or at different stages in the complex process of ribosome biogenesis.

In view of the results reported here, and those of Hayes & Vasseur (1973) and Weatherford *et al.* (1972), the correlation previously claimed for assumed ribonuclease II activities and observed p16S rRNA amounts may be fortuitous. Another monovalent cation-independent ribonuclease activity may also be involved in maturation. However, although ribonuclease II crypticity is excluded in the extracts used in the present experiments (see Table 1) there remains the possibility in all these investigations that the enzyme(s) involved could exhibit variable degrees of masking *in vivo* which is not detectable *in vitro*. If ribonuclease II is involved then the present evidence shows that it must act at a later ribosome precursor stage with the RNA in a susceptible conformation. Chang & Irr (1973) have also argued for a model of rRNA maturation which involves the ribosomal proteins to protect the precursor rRNA. Finally, it must be noted that although it has been suggested that the failure to detect intermediates between p16 and m16S rRNA implies that maturation is endonucleolytic rather than exonucleolytic (Adesnik & Levinthal, 1969; Pace *et al.*, 1970) it is also possible that the combined activity of exonucleases and endonucleases may be so rapid that the intermediates may escape detection by the methods used. No intermediates between p16S and m16S rRNA were detected in the present experiments.

We are indebted to the Science Research Council for a studentship (to J.R.D.) and to Mrs. E. Metcalf for her skilful technical assistance throughout this work.

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