An Analysis of the Ribosomal Ribonucleic Acids of *Escherichia coli* by Hybridization Techniques

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From analyses of the hybridization of *Escherichia coli* rRNA (ribosomal RNA) to homologous denatured DNA, the following conclusions were drawn. (1) When a fixed amount of DNA was hybridized with increasing amounts of RNA, only $0.35 \pm 0.02\%$ of *E. coli* DNA was capable of binding (16s + 23s) rRNA. Although preparations of 16s and 23s rRNA were virtually free from cross-contamination, the hybridization curves for purified 16s or 23s rRNA were almost identical with that of the parent specimen containing 1 weight unit of 16s rRNA mixed with 2 weight units of 23s rRNA. The 16s and 23s rRNA also competed effectively for the same specific DNA sites. It appears that these RNA species each possess all hybridizing species typical of the parent (16s + 23s) rRNA specimen, though probably in different relative amounts. (2) By using hybridization-efficiency analysis of DNA-RNA hybridization curves (Avery & Midgley, 1969) it was found that (a) 0.45% of the DNA would hybridize total rRNA and (b) when so little RNA was added to unit weight of DNA that the DNA sites were not saturated, only 70–75% of the input RNA would form hybrids. The reasons for the discrepancy between the results obtained by the two alternative analytical approaches were discussed. (3) For either 16s or 23s rRNA, hybridization analysis indicated that two principal weight fractions of rRNA may exist, hybridizing to two distinct groups of DNA sites. However, these groups seem to be incompletely divided between the 16s and 23s fractions. Analysis suggested that (a) 85% of the 16s rRNA was hybridized to about half the DNA that specifically binds rRNA (0.23% of the total DNA). (b) 70% of the 23s rRNA hybridized to a further 0.23% of the DNA and (c) the minor fraction (15%) of 16s rRNA may be competitive with the major fraction (70%) of 23s rRNA. Conversely, the minor fraction (30%) of the 23s rRNA may compete with the major fraction (85%) of 16s rRNA. Models were proposed to explain the apparent lack of segregation of distinct RNA species in the two subfractions of rRNA. (4) If protein synthesis and ribosome maturation were inhibited in cells of an R^{Cre1} mutant, *E. coli* W 1665, by depriving them of an amino acid (methionine) essential for growth, the inhibition had no discernible effect on the relative rates of synthesis of rRNA species. The rRNA that accumulates in R^{Cre1} strains of *E. coli* after amino acid deprivation is apparently identical in its content of RNA species with that of the pre-existing mature RNA in the ribosomes. On the other hand, the messenger RNA is stabilized, and accumulates as about 15% of the RNA formed after withdrawal of the amino acid.

The 16s and 23s rRNA* of species of the genus *Bacillus* hybridize without mutual competition to distinct, though closely linked, regions of denatured DNA (Yankofsky & Spiegelman, 1963; Oishi & Sueoka, 1965; Mangiarotti, Apirion, Schlessinger & Silengo, 1968; Smith, Dubnau, Morell & Marmur, 1968; Avery & Midgley, 1969).

* Abbreviations: rRNA, ribosomal RNA; mRNA, messenger RNA.

With the corresponding *E. coli* system, however, there appears to be considerable cross-competition between the 16s and 23s rRNA species for the same specific DNA sites (Attardi, Huang & Kabat, 1965; Avery & Midgley, 1968; Mangiarotti *et al.* 1968).

Analysis of the 3'-hydroxyl-terminal nucleotide sequences of *E. coli* rRNA has indicated some degree of heterogeneity in the 16s and 23s fractions (Midgley & McIlreavy, 1966; McIlreavy & Midgley,

1967; Midgley & McIlreavy, 1967; Nichols & Lane, 1967). Molecular heterogeneity has also been demonstrated by other methods (Aronson & Holowczyk, 1965; Young, 1968; Schaup, Best & Goodman, 1969).

End-group analysis of periodate-oxidized rRNA with [^{14}C]isonicotinic acid hydrazide has shown that the 16s rRNA of *E. coli* K 12 terminates chiefly with -pPypA and 23s rRNA chiefly with -pPypU (Midgley & McIlreavy, 1966, 1967). However, significant amounts of the terminal nucleotide sequences predominating in one fraction were found in the other as a minor component. In agreement with these studies, the alkaline digestion of 16s and 23s rRNA species showed that the 3'-hydroxyl-terminal nucleotide adenylic acid predominated in digests of 16s rRNA (as the derived nucleoside adenosine) and that uridylic acid (as uridine) predominated in 23s rRNA digests (Lane, 1962; Nichols & Lane, 1967).

We have designed our present experiments to define the hybridization characteristics of *E. coli* rRNA fractions and to observe any species heterogeneity, by using two analytical approaches developed for hybridization curves (Pigott, 1967; Avery & Midgley, 1968; Midgley, 1968; Avery & Midgley, 1969). The detailed analysis of the hybridization of 16s and 23s rRNA species has thus been used to determine, in a different way, the extent of heterogeneity and the distribution of specifically hybridizing groups of RNA species within the 16s and 23s fractions. From the results, we have proposed models of rRNA structure in *E. coli* to account for our observations.

MATERIALS AND METHODS

Organisms. The following strains of *Escherichia coli* were used: K 12, laboratory strain; B3005, an auxotroph derived from strain B, which requires adenine and thymine; W1665, an RC^{rel} mutant of K 12 requiring methionine. In the RC^{rel} mutant of *E. coli* used, there are at least two mutations present. One of these gives rise to a specific requirement for an amino acid (methionine) for bacterial growth; the other permits the organisms to synthesize nucleic acids, but not protein, in the absence of the required amino acid.

Growth of organisms. *E. coli* K 12 was maintained on 1% Davis agar slopes containing 8g. of tryptone (Oxo Ltd., London E.C.4)/l. and 5g. of NaCl/l. *E. coli* B3005 was maintained on slopes containing, in addition to the above, 0.02g. adenine/l. and 0.1g. of thymine/l. *E. coli* W1665 was maintained on slopes as described for strain, K 12, except that 0.05g. of DL-methionine/l. was also added.

In liquid culture, *E. coli* K 12 was grown as described by Pigott & Midgley (1968). *E. coli* B3005 was grown in the same medium, with the additional supplements of 0.02g. of adenine/l. and 0.005g. of thymine/l. *E. coli* W1665 was also grown in the same medium as strain K 12, supplemented with 0.05g. of DL-methionine/l.

Cultures were grown, harvested and disintegrated, and

DNA, rRNA and rapidly labelled RNA were prepared as described by Pigott & Midgley (1968).

Incorporation of radioactive substances into cultures. [^{14}C]Uracil or [^3H]uracil was used to label *E. coli* rRNA as described by Pigott & Midgley (1968). [^3H]Thymidine or [^{14}C]thymidine was used to label DNA, by using the thymine-requiring auxotroph. Cultures were grown over several hours in the presence of labelled thymidine.

Hybridization of RNA to DNA. The method of Gillespie & Spiegelman (1965) was used. Denatured *E. coli* DNA was immobilized on cellulose nitrate membrane filters (Sartorius Membranfilter MF 50, 2.5 cm. diam., V. A. Howe & Co. Ltd., London W.11) and the RNA was hybridized after incubation for 16 hr. in a suitable buffer (Gillespie & Spiegelman, 1965). The concentrations of RNA and DNA used were as described by Avery & Midgley (1969).

When labelled RNA was hybridized with unlabelled DNA, the RNA and DNA were assayed as described by Pigott & Midgley (1968). When both the RNA and DNA were labelled, the assay method of Avery & Midgley (1969) was used.

Separation of 16s and 23s rRNA. The chromatographic method of Mandell & Hershey (1960) was used. Purified rRNA, labelled with [^{14}C]uracil, was eluted from a kieselguhr column coated with bovine serum albumin (British Drug Houses, Ltd., Poole, Dorset) methylated in methanol-HCl as described by Mandell & Hershey (1960). The NaCl-sodium phosphate gradient described by Avery & Midgley (1969) was used as eluent. The ^{14}C -labelled 16s and 23s rRNA species were eluted as shown in Fig. 1. Fractions (3 ml.) round the peaks corresponding to 16s and 23s rRNA were pooled and dialysed into $6 \times \text{SSC}$ buffer, pH 7.2, ($\text{SSC} = 0.15 \text{ M-NaCl} - 0.015 \text{ M-sodium citrate}$) at 4° .

Sucrose-density-gradient sedimentation was performed as described by Avery & Midgley (1969).

Radiochemicals. [^{14}C]Uracil, sp. radioactivity 40-60 mc/m-mole, [^3H]uracil, sp. radioactivity 1000 mc/m-mole,

Fig. 1. Column chromatography of ^{14}C -labelled rRNA from *E. coli* K 12 on methylated-serum-albumin-coated kieselguhr (MAK), with a non-linear elution gradient of NaCl-sodium phosphate buffer (see the text). The figure shows only that part of the chromatogram where the rRNA fractions were eluted. Fraction size, 3 ml.

[6-³H]thymidine, sp. radioactivity 18100 mc/m-mole and [2-¹⁴C]thymidine, 54.5 mc/m-mole were obtained from The Radiochemical Centre, Amersham, Bucks.

RESULTS

Sucrose-density-gradient analysis of rRNA

³H-labelled *E. coli* rRNA was extracted from cultures of strain K 12 grown over several cell divisions in the continuous presence of [5-³H]uracil. It was then fractionated by the chromatographic column method of Mandell & Hershey (1960) with the elution gradient of Avery & Midgley (1969). Fractions of the peaks corresponding to 16s and 23s rRNA were then analysed by sucrose-density-gradient sedimentation to check that the column was fractionating the 16s and 23s rRNA species completely. Fig. 2 shows that this was the case. The column separated 1 weight unit of 16s rRNA from 2 weight units of 23s rRNA. 16s rRNA is derived from 30s ribosomal subunits and 23s rRNA from 50s subunits (Kurland, 1960; Iwabuchi, Kono, Oumi & Osawa, 1965; Midgley, 1965a) and molecular weights of the RNA species have a 1:2 relationship (Kurland, 1960; Stanley & Bock, 1965). As there are equal numbers of 50s and 30s subunits in the cells, this indicates that the rRNA was fractionated by the column into RNA species, each derived wholly from the appropriate ribosomal subunits.

Hybridization analysis

Analysis of hybridization of RNA and denatured DNA has been developed at length by Avery & Midgley (1969), for *Bacillus subtilis* rRNA and DNA.

The fact that the two rRNA fractions of *B. subtilis* hybridize independently to different DNA regions simplifies the analyses considerably. However, the arguments presented can also be used for the analysis of specimens that do not conform to this pattern, so long as (a) the materials are not extremely complex and (b) the equilibrium constant for the equilibrium between hybridized and non-hybridized RNA is small (Avery & Midgley, 1969). The following experiments were designed to define the essential characteristics of rRNA hybridization in *E. coli*, so that the species content of 16s and 23s fractions could be defined in these terms.

Hybridization competition between 16s and 23s rRNA species. ¹⁴C-labelled rRNA was prepared from cultures of *E. coli* B3005 as described by Pigott & Midgley (1968). It was then fractionated into 16s and 23s rRNA by using the protein-coated kieselguhr column (Mandell & Hershey, 1960). Unlabelled 16s and 23s rRNA samples were similarly prepared. Hybridization of ¹⁴C-labelled RNA and ³H-labelled DNA was carried out in the presence of unlabelled competitors, at a DNA labelled RNA ratio 300:1 (w/w). This ratio was chosen because it is the minimum ratio at which 16s and 23s rRNA first hybridize maximally with the DNA. Figs. 3(a) and 3(b) show the results of competition between 16s and 23s rRNA in the hybridization experiments.

In agreement with Attardi *et al.* (1965) and Mangiarotti *et al.* (1968), 16s and 23s rRNA species of *E. coli* compete efficiently for each other's binding sites on the DNA. However, the competition for hybridization sites between dissimilar RNA fractions is in neither case as effective as the

Fig. 2. Sucrose-density-gradient sedimentation of ³H-labelled *E. coli* rRNA fractions from the MAK column (Fig. 1) + unlabelled *E. coli* total rRNA. Linear density gradients (20 ml.) contained 5–20% (w/v) sucrose in 0.04 M-NaCl–0.01 M-EDTA–0.01 M-sodium acetate, pH 6.0. Centrifugation was for 18 hr. at 25000 rev./min. and 4° in the 3 × 20 ml. swing-out rotor of the MSE Superspeed 50 ultracentrifuge. Each fraction contained 13 drops. (a) ³H-labelled *E. coli* RNA [Peak I on MAK columns (Fig. 1)] + unlabelled *E. coli* total rRNA; (b) ³H-labelled *E. coli* RNA [Peak II on MAK columns (Fig. 1)] + unlabelled *E. coli* total rRNA. ●, *E*₂₆₀; ○, ³H radioactivity in RNA.

Fig. 3. Competition of ^{14}C -labelled *E. coli* 16S or 23S rRNA with unlabelled 16S or 23S rRNA samples for hybridization sites on the DNA. (a) Experiments with ^{14}C -labelled 16S rRNA. The DNA ^{14}C -labelled 16S rRNA ratio was 350:1 (w/w) throughout. O, ^{14}C -Labelled 16S rRNA against unlabelled 16S rRNA; ●, ^{14}C -labelled 16S rRNA against unlabelled 23S rRNA. (b) Experiments with ^{14}C -labelled 23S rRNA. The DNA ^{14}C -labelled 23S rRNA ratio was 300:1 (w/w) throughout. O, ^{14}C -Labelled 23S rRNA against unlabelled 23S rRNA; ●, ^{14}C -labelled 23S rRNA against unlabelled 16S rRNA. Values for percentages of RNA hybridized are normalized to 100% maximum.

competition between identical labelled and unlabelled fractions. If there are very similar species present in both RNA types, their weight ratios must differ in each fraction.

Hybridization of DNA with excess of rRNA. To a fixed amount of ^{14}C -labelled DNA, increasing amounts of total, 16S or 23S ^3H -labelled rRNA were added. After hybridization, the filters, with hybridized RNA, were washed and incubated with pancreatic ribonuclease (freed from deoxyribonuclease) as described by Gillespie & Spiegelman (1965). The results are shown in Figs. 4(a), 4(b) and 4(c). A 'saturation plateau' was attained when the specific DNA sites on the filter-bound material were saturated with rRNA, in each of the three cases. The amount of RNA finally bound/unit weight of DNA was the same for 16S, 23S or total rRNA. However, the RNA/DNA ratio at which the 'saturation plateau' was first attained was greater with 16S and 23S rRNA separately than with the total rRNA. This indicates that, even when the

Fig. 4. Hybridization of total, 16S or 23S rRNA to DNA. (a) Total (16S+23S) rRNA; (b) 16S rRNA; (c) 23S rRNA.

rRNA is fractionated into 16S and 23S components, these contain all RNA species capable of hybridizing to all rRNA-specific DNA regions, though in different relative amounts. This confirms the competition analysis, which also indicated considerable similarities in hybridization behaviour between the 16S and 23S rRNA species of this organism.

The 'saturation plateau' was flat in all three cases, giving an average value of 0.35 ± 0.02 weight units of rRNA bound/100 weight units of DNA. This value may be compared with those of McConkey & Dubin (1966) and Kennell (1968).

Analysis of the efficiency of hybridization of RNA and DNA. A fixed amount of ^{14}C -labelled rRNA was hybridized with increasing amounts of either unlabelled or ^3H -labelled DNA. Unlabelled DNA was obtained from *E. coli* K12 and ^3H -labelled DNA from *E. coli* B3005.

Since a high percentage of the input RNA was hybridized when an excess of binding sites on DNA was available, it was unnecessary to use the ribonuclease-digestion step to remove spurious hybrids. Tests on the effect of adding ribonuclease to remove non-specifically bound RNA showed that only 1–2% of the input RNA was both bound to the filters and sensitive to the action of the enzyme, in the conditions described by Gillespie & Spiegelman (1965).

Samples of total, 16s and 23s ^{14}C -labelled rRNA were hybridized to DNA at DNA/RNA ratios that allowed a high proportion of the RNA to hybridize. Figs. 5(a), 5(b) and 5(c) show the results obtained. The results for *E. coli* K12 and B3005 were indistinguishable.

In Fig. 5(a), the curve for total rRNA is almost linear and cuts the ordinate at a value of about 2% hybridization. This is ascribed to the presence of mRNA, which is hybridized efficiently to DNA at low DNA/RNA ratios and is co-chromatographed with the rRNA species on the fractionating column. In support of this explanation, the hybridization curves for rRNA freed from mRNA extrapolate to the origin (J. E. M. Midgley, unpublished work). There is a rapid change of slope at DNA/RNA ratios 220:1 (± 10 :1) and thereafter about 70–75% of the input RNA will hybridize. The DNA/RNA ratio at the point of rapid change from positive to zero slope is almost identical with that found for *B. subtilis* (16s + 23s) rRNA. However, the efficiency of RNA hybridization in the presence of excess of specific binding sites on DNA appears to be greater with the RNA of this organism than with the RNA of *E. coli* (Avery & Midgley, 1969). If we take the DNA/RNA ratio 220:1 as a measure of the percentage of DNA responsible for the hybridization of rRNA, it is obvious that from the equation describing the hybridization curve (Avery & Midgley, 1969) this DNA/RNA ratio is either correct or an overestimate of the true value. However, the linearity of the hybridization curve at DNA/RNA ratios below 210:1 suggests that the measurement gives a correct value (Avery & Midgley, 1969). Thus, the analysis of rRNA hybridization by this method gives the percentage of DNA capable of binding rRNA as $1/220 \times 100\%$, or 0.45%. This figure is considerably higher than that obtained from analyses by saturation of specific DNA sites with RNA (Fig. 4). Possible reasons for this discrepancy will be discussed later. It is, however, unlikely to be due to distortions caused by mass-

action effects. If a filter containing DNA-RNA hybrids was thoroughly washed free from non-hybridized RNA and incubated in a hybridization medium for a further 30 hr. at 65°, only 3–5% of the hybridized RNA was lost. It was expected that mass-action effects would have established a new equilibrium by this time between hybridized and non-hybridized RNA, thus indicating that the true equilibrium lies far on the side of hybrid formation.

Figs. 5(b) and 5(c) show the hybridization curves for the efficiency of binding 16s and 23s rRNA respectively, over a range of DNA/RNA ratios from 5:1 to 400:1 (w/w). In both figures, an extrapolation of the earlier part of the hybridization curve can be made, cutting the line of maximum hybridization at a DNA/RNA ratio close to 200:1. With 16s rRNA (Fig. 5b) the accuracy of the extrapolation is very low, if based on the few experimental points at low DNA/RNA ratios. This is due to the presence of mRNA, which is efficiently bound to DNA at low DNA/RNA ratios, and also to the fact that a change in slope of the hybridization curve occurs so soon after the origin. The placing of this extrapolation can be justified from the results

Fig. 5. Hybridization of total, 16s or 23s rRNA to DNA (a) Total (16s + 23s) rRNA; (b) 16s RNA; (c) 23s RNA.

shown in Figs. 4(a), 4(b) and 4(c), demonstrating that at low DNA/RNA ratios, all specific DNA sites capable of binding total (16s + 23s) rRNA also hybridize with either of the purified RNA fractions. Thus, the extrapolation in Fig. 5(b) for 16s rRNA may be based on the hybridization curve obtained in Fig. 5(a) for total rRNA, independently of the experimental points for 16s rRNA actually obtained.

If the hybridization curves of 16s and 23s rRNA are compared with that for total rRNA, it can be seen that, whereas the hybridization curve for total RNA is linear until DNA/RNA ratios become greater than 200:1 (Fig. 5a), breaks in the early slope of the curve occur with purified 16s or 23s fractions. Thus, the practical maximum of 75% of input RNA hybridized is not reached until DNA/RNA ratios are much greater than 200:1. From analysis of the curve (Avery & Midgley, 1969), this indicates that there may be at least two classes of RNA or RNA fragments capable of hybridizing independently to different regions of the specific DNA. Further, since the purified 16s and 23s rRNA species will hybridize with all the specific DNA at low DNA/RNA ratios, this indicates that both fractions contain both classes of hybridizing RNA, though probably in different relative amounts. Thus, since the hybridization curves for 16s and 23s rRNA have two distinct slopes before maximum hybridization of input RNA is achieved at high DNA/RNA ratios, we may estimate approximately the contribution to either 16s or 23s rRNA fractions of each of the two types of RNA.

If a line is drawn from the origin, parallel to the second part of the hybridization curve, where RNA is still in excess of at least some of the available DNA sites, this cuts the line of maximum hybridization at a DNA/RNA ratio roughly corresponding to the amount of DNA hybridizing with the major RNA fraction in the specimen (Avery & Midgley, 1969). For both 16s and 23s rRNA (Figs. 5b and 5c), this construction gives the percentage of the DNA hybridizing with the major RNA fraction of both species as about 0.23%. Thus, in both cases, a further 0.23% of the DNA hybridizes to the minor RNA fractions, as the percentage of the DNA hybridizing with total rRNA is 0.45% (Fig. 5a). The simplest interpretation of these results is that, since the 16s and 23s rRNA compete for the same bindingsites on DNA and contain all the hybridizing RNA species typical of the total material (Figs. 4a, b and c) each of the two groups of RNA species distinguishable by hybridization in, for example, 16s rRNA, must have its complement in 23s rRNA.

If the second (intermediate) slope of the hybridization curves for 16s and 23s rRNA (Figs. 5b and 5c) is extrapolated to the ordinate, the construction cuts the ordinate between the origin and the value for maximum percentage of hybridization of the

RNA (75% of input) approximately in the weight ratio of the two RNA species present (Avery & Midgley, 1969). Thus, for 16s rRNA (Fig. 5b), about 15% of the RNA makes up the minor fraction and about 85% the major fraction of the two species. The added complication of the mRNA has been ignored as it contributes only some 2% extra hybridization to the curve near the origin (Fig. 5b). However, the minor fraction of the 16s rRNA is likely to be overestimated slightly owing to the effects of mRNA hybridization at low DNA/RNA ratios. For 23s rRNA, a similar analysis indicates that about 30% of the RNA forms a minor fraction, and about 70% a major fraction, of this species.

Thus, analysis of the curves in Figs. 5(b) and 5(c) suggests that about 85% of the 16s rRNA will hybridize with 0.23% of *E. coli* DNA and is competitive with either 30% or 70% of the 23s rRNA species for the same DNA regions. The remaining 15% of the 16s rRNA also competes with a different fraction of 23s rRNA. We conclude that 16s rRNA hybridizes to DNA as a more homogenous species than 23s RNA, as its minor components contribute a much smaller proportion of the total 16s component than is the case with 23s rRNA.

Hybridization of rRNA from an RC^{rel} mutant of E. coli. *E. coli* W1665, an auxotroph requiring methionine, was grown in the presence of DL-methionine and glucose-salts medium at 37° (see the Materials and Methods section). When the culture (11.) had reached a turbidity of 0.60 at 650nm., it was harvested by centrifuging for 10 min. at 10000g. The bacterial pellet was then washed once with methionine-free growth medium and was then resuspended in 11. of the same medium. Incubation at 37° was continued, and after 10 min., when the residual methionine had been exhausted, 25 μ C of [2-¹⁴C]uracil was added, suitably diluted with unlabelled carrier, to allow the culture to grow for at least 1 hr. in the continuing presence of exogenous uracil. The cells were then harvested and the RNA was purified as described in the Materials and Methods section. Transfer RNA was removed by gel filtration on Sephadex G-200 columns (Midgley, 1965b). During the period of methionine deprivation, the *E. coli* W1665 cultures continued to synthesize nucleic acids, but protein synthesis was severely inhibited (Borek, Ryan & Rockenbach, 1955; Stent & Brenner, 1961; Fleissner & Borek, 1962; Dagley, Turnock & Wild, 1963; Turnock & Wild, 1964, 1965). This behaviour is ascribed to the effects of a mutation in the RC locus of the genome, which relaxes the normally stringent relationship between nucleic acid and protein synthesis. It was thus of interest to see if the apparent co-ordination of transcription of the DNA sites responsible for rRNA synthesis was maintained when translation of RNA was stopped and

Fig. 6. Hybridization of ^{14}C -labelled RNA obtained from the RC^{rel} mutant, *E. coli* W1665, after the cultures had been suspended in amino acid-free growth medium with exogenous $[2\text{-}^{14}\text{C}]\text{uracil}$ (see the text).

subribosomal RNA-containing particles accumulated in the cells (Dagley *et al.*, 1963).

Fig. 6 shows the hybridization curve for the ^{14}C -labelled RNA (free from transfer RNA) produced after methionine deprivation of the cultures. Three features of the curve are immediately apparent. First, the hybridization curve, though linear for most of its length at DNA/RNA ratios less than 220:1, does not pass through the origin. Extrapolation of the curve to the ordinate cuts it at a value of about 10% hybridization. This is a feature of specimens containing mRNA, which hybridizes efficiently with DNA at DNA/RNA ratios less than 5:1. The curve indicates that, since 70% of the labelled RNA would hybridize at DNA/RNA ratios greater than 220:1, then $10/70 \times 10 = 14\%$ of the labelled RNA formed is mRNA (assuming equal efficiencies of hybridization of mRNA and rRNA). Recent evidence has shown that mRNA accumulates in the cells of amino acid-requiring RC^{rel} strains of *E. coli* when they are deprived of the amino acid, probably due to stabilization of the mRNA fraction (Turnock & Wild, 1965; Lavallé & de Hauwer, 1968; Stubbs & Hall, 1968).

Secondly, the hybridization curve is linear between DNA/RNA ratios of 10:1 and 220:1. This shows that the mode of synthesis of the various species of rRNA that may be distinguished by hybridization is unchanged after protein synthesis is halted. Thirdly, the DNA/RNA ratio at which the slope of the hybridization curve becomes zero is 220:1, in close agreement with the hybridization curve for (16s + 23s) rRNA in Fig. 5(a). Thus, it appears that the prevention of translation in a bacterial cell has no effect on the number of rRNA-specific DNA sites transcribed.

DISCUSSION

In the present paper we have analysed the hybridization of *E. coli* rRNA and DNA by the method originally devised for the simpler case of *B. subtilis* RNA and DNA (Avery & Midgley, 1969).

The results have essentially confirmed the earlier work of Attardi *et al.* (1965) and Mangiarotti *et al.* (1968), in that the rRNA of *E. coli* hybridizes with a small specific fraction of the DNA and that the 16s and 23s species compete for the same binding sites. Our results suggest that 0.35–0.45% of the DNA will hybridize with rRNA, in good agreement with the results of others (McConkey & Dubin, 1966; Moore & McCarthy, 1967; Kennell, 1968; Mangiarotti *et al.* 1968). Taking the molecular weight of *E. coli* DNA to be 3×10^9 (Kit, 1963) this indicates that there are 19–25 cistrons of mol.wt. 5.5×10^5 (i.e. of the size of 16s rRNA) specific for rRNA.

Our two methods of hybridization analysis have given more discrepant results than was the case with the corresponding RNA and DNA from *B. subtilis* (Avery & Midgley, 1969). This we ascribe to the observed competition of *E. coli* 16s and 23s rRNA in hybridization experiments. If, during the course of saturation of the specific DNA sites with excess of rRNA (saturation analysis), some fractions of the RNAs were bound to very similar but not sequentially identical DNA sites, then the treatment of the hybrids with ribonuclease to remove spuriously bound material might very well remove also a fraction of the RNA bound with the specific regions of DNA. Thus, we would expect that, in this case, saturation-curve analysis of hybridization of rRNA would underestimate the amount of DNA taking part in the process, compared with the estimate from hybridization-efficiency analyses. As there is no reason to suspect that the hybridization-efficiency analysis overestimates the amount of specific DNA capable of binding rRNA, we believe that the larger value obtained by this method is probably more correct than the smaller value obtained by saturation analysis. In the case of *B. subtilis*, the 16s and 23s rRNA species hybridize independently to different DNA cistrons (Yankofsky & Spiegelman, 1963; Oishi & Sueoka, 1965; Mangiarotti *et al.* 1968; Avery & Midgley, 1969), so that this situation probably does not arise. Indeed, for *B. subtilis* rRNA, both methods of analysis give concordant results (Avery & Midgley, 1969). It is notable that the higher value is identical with that found for *B. subtilis* RNA and DNA, indicating that there are the same numbers of specific cistrons per genome in each case.

The observations of mutual competition of 16s and 23s rRNA species for the same hybridization sites on DNA and the analyses of the hybridization curves for purified 16s and 23s species indicate considerable internal heterogeneity in both fractions. At least two groups of RNA species were discerned in sedimentationally homogeneous 16s and 23s fractions. Also, it is probable that these two groups hybridize to different regions of the specific DNA. In support of this interpretation, analyses of

the 3'-hydroxyl-terminal nucleotide sequences of *E. coli* rRNA, by using periodate oxidation and addition of [¹⁴C]isonicotinic acid hydrazide to terminally oxidized RNA molecules (Midgley, 1965*b*; Midgley & McIlreavy, 1966, 1967; McIlreavy & Midgley, 1967), also indicate a similar heterogeneity in both RNA species.

It is impossible to make a firm decision as to whether the two types of RNA in intact 16s and 23s species may be defined as two distinct molecular types with different nucleotide sequences throughout, or whether, in some of the RNA molecules, in either or both fractions, there are long stretches of sequences homologous with both specific regions of the DNA. As the RNA samples are subject to considerable chain-scission at the elevated temperatures of the hybridization process (Attardi *et al.* 1965), we cannot draw a distinction between these alternatives. However, the results show that the 16s RNA hybridizes as a more homogenous species than does 23s RNA.

These observations add further relevance to earlier speculations about the mode of synthesis of the 16s and 23s rRNA species in *E. coli*. A considerable body of evidence has accumulated that suggests that the 23s rRNA is synthesized by dimerization of a 16s precursor. For example, estimates of the length of the 16s and 23s polynucleotide chains by the periodate-[¹⁴C]isonicotinic acid hydrazide method (Midgley, 1965*b*; McIlreavy & Midgley, 1967; Midgley & McIlreavy, 1967; Riley, 1969) indicate that 16s rRNA has one reactive terminal region per molecule (5.5×10^5 mol.wt.) and 23s rRNA has two such regions per molecule (1.1×10^6 mol.wt.). Riley (1969), in confirming these findings, has extended them with corroborative studies by using digestion of polynucleotide chains with exonuclease as a measure of chain length.

Mangiarotti *et al.* (1968) have also studied the kinetics of synthesis of 16s and 23s rRNA species in *E. coli*. Using osmotically fragile cultures, they studied the flow of exogenous labelled nucleic acid precursors through uncompleted, though growing, chains of rRNA to the finished 16s and 23s RNA species in the ribosomes. An important conclusion reached was that the kinetics of synthesis of the 16s and 23s rRNA was very similar, in that the same delay time was noted for the entry of labelled precursors into 16s and 23s fractions. The two RNAs are presumably synthesized by the stepwise assimilation of nucleoside 5'-triphosphates into the growing RNA chains (Maitra & Hurwitz, 1965). Thus, if 16s rRNA is half the molecular size of 23s rRNA (Stanley & Bock, 1965) and the DNA-dependent RNA polymerase located at the specific DNA sites forms each RNA at the same rate, then it should take 23s RNA twice as long to be completed as 16s RNA. We have studied the incorporation of

[5-³H]uracil into the 16s and 23s RNA and the kinetics of rRNA synthesis in *E. coli* B3005, and have obtained results that are identical with those of Mangiarotti *et al.* (1968) (R. J. Avery, unpublished work). Similar conclusions were reached by the earlier findings of McCarthy, Britten & Roberts (1962), in that the kinetics of synthesis of the RNA of 50s ribosomal subunits in *E. coli* (i.e. 23s rRNA) showed a two-stage entry of labelled precursors into the polynucleotide fraction.

Some support for the idea that the 23s rRNA may be a covalently linked dimer of 16s precursors has also come from the findings of Fellner & Sanger (1968). They discovered that the nucleotide sequences near methylated nucleotides in the chains of 23s rRNA have a different pattern from those in 16s rRNA. More importantly, there were two weight units of all nucleotide sequences containing methylated nucleotides per molecule of 23s rRNA, and only one weight unit of all such sequences per molecule of 16s rRNA. The interpretation of this work was that 23s rRNA had arisen either by a 'gene doubling' of a 16s cistron during the process of evolution of DNA cistrons specific for 23s rRNA, or that dimerization of 16s rRNA precursors had occurred in the synthesis of the larger RNA species.

This model of rRNA synthesis is fully supported by the results reported here. If a pool of 16s material is built up by stepwise elongation of growing RNA chains, then precursors could be withdrawn from this pool, with appropriate selection of species, and dimerization to the 23s RNA. The observed lack of species segregation between 16s and 23s fractions would be explained as being partly due to the need to produce equal numbers of 30s and 50s ribosomal subunits and partly to the fact that the two hybridizationally distinct specific regions of the DNA are about equal in size.

Finally, our results show that the mode of transcription of the multiple DNA cistrons specific for rRNA synthesis is not affected by the presence or absence of simultaneous translation processes. Inhibition of translation appears to have no effect on the relative amounts of rRNA species produced, so that in the RC^{rel} mutant of *E. coli* used, RNA-rich particles accumulate and contain all species detectable in the RNA of normal ribosomal particles. Thus, if the above model of rRNA synthesis is correct, the hypothetical dimerization of 16s rRNA must occur at a relatively early stage in ribosome synthesis.

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