A New Approach to the Analysis of Hybridization of Bacterial Nucleic Acids

ANALYSIS OF THE RIBOSOMAL RIBONUCLEIC ACIDS OF *BACILLUS SUBTILIS*

By R. J. AVERY AND J. E. M. MIDGLEY

Department of Biochemistry, University of Newcastle upon Tyne

(Received 7 May 1969)

A new graphical analytical technique is described for the hybridization of bacterial RNA with denatured homologous DNA immobilized on cellulose nitrate membrane filters. To a constant amount of DNA, various amounts of bacterial RNA were added and the percentage of input RNA bound was plotted against the DNA/RNA weight ratio in a given experiment. When RNA samples were used that hybridize to denatured DNA as a single species, the resulting curves (RNA-hybridization-efficiency curves) could be analysed to show the percentage of the DNA capable of specifically binding the RNA and could also be used to detect the presence of minor RNA contaminants in a purified specimen. The method could also estimate the relative amounts of two species of RNA in a mixture when these were hybridized independently to different DNA cistrons or cistron groups. As an example of RNA that can be studied in this way, the 16s and 23s ribosomal RNA species of *Bacillus subtilis* were chosen. These each behave in DNA-RNA hybridization as a single species and bind independently to different groups of DNA cistrons. The results obtained from hybridization-efficiency curves were compared with those obtained by the more usual method of saturating the specific DNA regions with excess of ribosomal RNA (hybridization-saturation curves). It was confirmed by both approaches that 0.15 (± 0.02)% of *B. subtilis* DNA would hybridize with 16s ribosomal RNA, 0.30 (± 0.02)% would hybridize with 23s ribosomal RNA, and 0.46 (± 0.02)% would hybridize with (16s + 23s) ribosomal RNA. This agreement suggested that mass-action equilibria between hybridized and free RNA had a negligible effect on the hybridization curves over the range of DNA and RNA concentrations employed.

Microbial rRNA* is an excellent example of an RNA species produced by transcription of a very limited region of DNA and as such can be used as a model system in the study of quantitative aspects of DNA-RNA hybridization. This fraction, comprising the bulk of the RNA in the cell, has been shown to hybridize specifically to as little as 0.5% of the homologous denatured DNA (Yankofsky & Spiegelman, 1963; Attardi, Huang & Kabat, 1965; Oishi & Sueoka, 1965; McConkey & Dubin, 1966; Cutler & Evans, 1967; Takahashi, Saito & Ikeda, 1967; Kennell, 1968; Kennell & Kotoulas, 1968; Smith, Dubnau, Morell & Marmur, 1968). A considerable species specificity has been demonstrated (Dubnau, Smith, Morell & Marmur, 1965; Doi & Igarashi, 1965, 1966; Takahashi *et al.* 1967; Pigott & Midgley, 1968). Thus, although the rRNA species of closely related species of the genus

Bacillus will bind with efficiency to each other's DNA sites (Dubnau *et al.* 1965; Doi & Igarashi, 1965, 1966; Takahashi *et al.* 1967), there is little detectable hybridization between the heterologous nucleic acids of *Pseudomonas aeruginosa* and *Escherichia coli* (Pigott & Midgley, 1966, 1968).

Earlier quantitative studies on DNA-RNA hybridization, with rRNA, have generally employed the technique of challenging a fixed amount of denatured microbial DNA with increasing quantities of RNA. When no more rRNA will bind with unit weight of DNA, the sites responsible for its transcription are presumed to be saturated with stable hybrids. The value of the 'saturation plateau', in terms of weight of RNA bound/100 weight units of DNA, is taken to represent the percentage of DNA responsible for the formation of the RNA. However, this value must always be considered as a minimum, since with DNA immobilized on membrane filters, as the method most commonly used,

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

we are not dealing with the hybridization of DNA and RNA in free solution. Further, the possibility of shearing of RNA and DNA during the hybridization process, the presence of impurities such as protein on the support and the physical interference of hybrid formation between RNA and DNA strands immobilized intimately on the surface of the support may seriously affect any quantitative assessments. We also have to be certain that ribonuclease, which is used to remove unstable hybrids from the filters, has no effect on the stability of correctly formed hybrids (Gillespie & Spiegelman, 1965).

In the present paper, we have developed the analysis of hybridization curves for the 16s and 23s rRNA species of *Bacillus subtilis* by using a different graphical approach (hybridization-efficiency analysis). This method has the considerable advantage that the test with ribonuclease for RNA-DNA-hybrid stability is no longer necessary, since most of the experimental points are obtained at concentrations of the RNA not greatly in excess of the concentration of the specific DNA sites to which it will bind.

We have chosen *B. subtilis* rRNA to develop this analytical technique for two reasons. First, the 16s and 23s rRNA species do not compete for each other's hybridization sites on *B. subtilis* DNA (Yankofsky & Spiegelman, 1963; Oishi & Sueoka, 1965; Smith *et al.* 1968). As there is a possibility that within either the 16s or the 23s fraction the RNA molecules are sequentially so similar as to hybridize at any DNA site specific for 16s or 23s rRNA, we can regard each of these two RNA fractions as essentially single species. Secondly, the weight ratio of the DNA regions specific for 16s and 23s rRNA is 1:2 (Oishi & Sueoka, 1965; Smith *et al.* 1968), and the weight ratio of the 16s and 23s rRNA species produced is also 1:2 (Kurland, 1960; Spirin, 1961). Thus, for hybridization purposes, we can also treat the total (16s + 23s) rRNA as a single species, as it appears that, within this fraction, the RNA species are present in the same weight ratio as the DNA cistrons capable of binding them. These considerations make the rRNA species of *B. subtilis* ideal for a quantitative assessment of DNA-RNA hybridization processes.

Thus we have compared critically the estimations of the percentages of DNA specifically hybridizing with rRNA in analyses with either RNA-saturation or hybridization-efficiency curves. A preliminary account of this work has been given (Midgley, 1968).

MATERIALS AND METHODS

Organism. *Bacillus subtilis* 168 ind⁻ thy⁻, an auxotroph requiring tryptophan and thymine, was used.

Growth of bacterial cultures. The organism was grown, under forced aeration, at 37° in a medium containing

(per l.): 3g. of KH₂PO₄, 6g. of Na₂HPO₄, 3g. of NaCl, 0.12g. of Na₂SO₄, 0.05g. of MgCl₂, 8g. of casein hydrolysate ('casamino acids'), 0.1g. of yeast extract, 2.5g. of D-glucose, 0.1g. of DL-tryptophan and 0.25g. of thymine. The cultures were grown to the late-exponential phase of growth, and were harvested at a turbidity of 1.0 at 650 nm., as measured in the Unicam SP.500 spectrophotometer in glass cuvettes of 1 cm. light-path. Stock cultures of the organism were maintained on 1% Davis agar slopes containing the same growth medium.

Incorporation of radioactive substances into cultures. This was carried out as described by Pigott and Midgley (1968) for *Escherichia coli*. ³H- or ³²P-labelled rRNA samples were produced by adding either [5-³H]uracil or [³²P]phosphate to exponentially growing cultures. In the latter case, the growth medium was decreased in phosphate content. The cultures were permitted to incorporate the radioactive supplements for several generations. ¹⁴C-labelled DNA was produced by adding [2-¹⁴C]thymidine to growing cultures. Harvesting of cultures and preparation of bacterial cell extracts were as described by Pigott & Midgley (1968).

Preparation of bacteria DNA. This was isolated by the method (Pigott & Midgley, 1968) derived from those of Marmur (1961) and Kirby (1964). Some alterations in procedure were necessary to lyse the bacterial cells. Wet packed cells of *B. subtilis* were suspended in 0.1 M-EDTA-0.4 M-NaCl buffer, pH 8.0, at a concentration of about 100 mg. wet wt. of cells/ml. Lysozyme (500 µg./ml.; Koch-Light Ltd., Colnbrook, Bucks.) (EC 3.2.1.17) was added and the mixture was incubated for 1 hr. at 37°. The procedure of Pigott & Midgley (1968) was then followed to the first deproteinization. After this treatment, the DNA solution was very opaque owing to the presence of suspended polysaccharide. It was centrifuged at 80000g in the MSE Superspeed 50 centrifuge for 30 min. at 5°, giving a clear supernatant and a pellet of polysaccharide. The purification was continued as described by Pigott & Midgley (1968). Taking the extinction coefficient as $E_{1\text{cm}}^{1\%} = 200$, the assay of DNA by its E_{260} agreed with an assay by the diphenylamine method of Burton (1956).

Preparation of bacterial rRNA. This was described by Pigott & Midgley (1968).

Sucrose-density-gradient sedimentation of RNA. rRNA was centrifuged in a 3 × 20 ml. swing-out rotor in the MSE high-speed 50 centrifuge. A linear 5–20% (w/w) sucrose gradient was made up in 20 ml. of 0.01 M-sodium acetate-0.01 M-EDTA-0.04 M-NaCl buffer, pH 6.0, in a polypropylene tube. The RNA solution, containing 1–2 mg. of RNA in 0.5 ml. of 0.01 M-sodium acetate, pH 4.6, was layered on top of the gradient. To ensure an even distribution of the RNA solution on the gradient, the tubes were gently twirled during loading. The sucrose gradients were then centrifuged for 18 hr. at 25000 rev./min. at 4°. After the rotor had come to rest without braking, the tubes were pierced at the bottom with a hypodermic needle and 13-drop fractions were collected (about 50). The E_{260} of the solutions in each fraction was measured in quartz micro-cells of 1 cm. light-path in the Unicam SP.500 spectrophotometer.

Determinations of nucleotide base compositions. These were carried out as described by Midgley (1962).

Hybridization procedure. The hybridization of RNA to denatured DNA, immobilized on cellulose nitrate membrane filters (Sartorius Membranfilter MF50, 2.5 cm. diam.,

Fig. 1. Time-course of hybridization of *B. subtilis* (16s + 23s) RNA with DNA at 66° and a DNA/RNA ratio 400:1 (w/w). Ordinate shows percentage of RNA hybridized, normalized to 100% maximum.

V. A. Howe and Co. Ltd., London W.11) was carried out as described by Pigott & Midgley (1968). In each case, one DNA-bearing filter containing either 100 µg. or 200 µg. of DNA was incubated with a suitable concentration of RNA in 1 ml. of hybridization medium (Pigott & Midgley, 1968) placed in a stoppered scintillation vial. To ensure that, in all experiments, the interaction of RNA and DNA had proceeded to equilibrium between hybridized and non-hybridized fractions, a time-course was followed of the hybridization of (16s + 23s) *B. subtilis* rRNA at a DNA/RNA ratio 400:1 (w/w). In each vial, one DNA-bearing filter with 200 µg. of DNA was incubated in 1 ml. of hybridization medium containing 0.5 µg. of RNA. Fig. 1 shows that the routine incubation period of 16 hr. used was adequately long to ensure the establishment of equilibrium between hybridized and non-hybridized RNA, even with the lowest concentrations of RNA used.

Where necessary, filters containing DNA-RNA hybrids were incubated with pancreatic ribonuclease (EC 2.7.7.16) (free from deoxyribonuclease; British Drug Houses Ltd., Poole, Dorset) to remove non-specifically bound RNA (Gillespie & Spiegelman, 1965). In all experiments, a standard concentration of enzyme was used (0.5 µg./ml.).

Assay of RNA and DNA. DNA was labelled with [2-¹⁴C]thymidine and RNA was labelled with [5-³H]uracil to allow detection of the nucleic acids by two-channel counting in the Beckman liquid-scintillation counter. The filters, after hybridization of the RNA and washing, were dried and placed in scintillation vials containing 6 ml. of a scintillation solution [6 g. of 2,5-diphenyloxazole and 0.05 g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene/l. of toluene (British Drug Houses, sulphur-free)]. The scintillation vials were placed in the dark for 24 hr. before counting, to minimize inaccuracies due to fluorescence etc. Controls were made up in which blank filters containing no DNA were taken through the hybridization and washing procedures. These corrected for the non-specific binding of RNA to the filters.

Radiochemicals. [5-³H]uracil, sp. radioactivity 1000 mc/m-mole, [2-¹⁴C]thymidine, sp. radioactivity 54.5 mc/m-mole and [³²P]phosphate (as H₃PO₄), sp. radioactivity 5–200 mc/m-mole, were obtained from The Radiochemical Centre, Amersham, Bucks.

RESULTS

Chromatographic separation of 16s and 23s rRNA species. The method of Mandell & Hershey (1960)

Fig. 2. Column chromatography of ³H-labelled rRNA from *B. subtilis* on methylated serum albumin-coated kieselguhr (MAK), with a non-linear elution gradient of NaCl–0.02M-sodium phosphate buffer, pH 6.7 (see the text). The figure shows only that part of the chromatogram where the rRNA fractions were eluted. Fraction size 3 ml.

was used. Unfractionated ³H-labelled RNA from *B. subtilis* was absorbed on to a column (1 cm. × 10 cm.) of kieselguhr coated with methylated bovine serum albumin, in 0.6M-NaCl–0.02M-sodium phosphate buffer, pH 6.7. The 16s and 23s fractions of the rRNA were present in the mixture in a 1:2 weight ratio (initially checked by sucrose-density-gradient sedimentation). The rRNA was eluted by the following solutions in a five-chambered mixing vessel: 0.6M-, 0.65M-, 0.74M-, 0.8M- and 1.0M-NaCl in 0.02M-sodium phosphate buffer, pH 6.7. Fig. 2 shows that the separation of 16s rRNA from 23s rRNA was almost complete in this elution gradient. The weight ratio of the 16s and 23s rRNA species obtained by chromatography maintained its 1:2 value.

To check that the column was fractionating the rRNA species according to their sedimentation coefficients, a sample of the pooled fractions from each peak was analysed by sucrose-density-gradient sedimentation. Fig. 3 shows that the resolution of rRNA species by the column method of Mandell & Hershey (1960) is sufficient to separate completely the 16s and 23s fractions. No more than 1–2% of the material isolated as 23s rRNA on the column could be considered as a contaminant from the 16s fraction. No material isolated with the 16s rRNA by the column sedimented at 23s. As 16s rRNA is derived from 30s ribosomal subunits, and 23s rRNA from 50s subunits, it is likely that the RNA specimens have been derived wholly from different ribosomal subunits (Midgley, 1965).

Competition of 16s and 23s rRNA species in hybridization. ³H-labelled rRNA was isolated from *B. subtilis* 168 grown in glucose-casamino acids-salts medium in the presence of [5-³H]uracil. It

Fig. 3. Sucrose-density-gradient sedimentation of ^3H -labelled *B. subtilis* RNA fractions from the MAK column (Fig. 1) + unlabelled *E. coli* total ribosomal RNA. 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 buffer, 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 centrifuge. Each fraction contained 13 drops. (a) ^3H -labelled *B. subtilis* RNA (peak I on MAK columns, Fig. 1) + unlabelled *E. coli* rRNA; (b) ^3H -labelled *B. subtilis* RNA (peak II on MAK columns, Fig. 1) + unlabelled *E. coli* rRNA. ●, E_{260} ; ○, ^3H radioactivity in RNA.

Fig. 4. Competition of ^3H -labelled *B. subtilis* 16s rRNA with unlabelled 16s or 23s rRNA for hybridization sites on DNA. The DNA/ ^3H -labelled 16s RNA ratio was 660:1 (w/w) throughout. ○, ^3H -labelled 16s RNA against unlabelled 16s RNA; ●, ^3H -labelled 16s RNA against unlabelled 23s RNA.

was then fractionated on the protein-coated kieselguhr column, as described by Mandell & Hershey (1960). Unlabelled rRNA was similarly fractionated. The ^3H -labelled 16s or 23s rRNA species were then hybridized with ^{14}C -labelled DNA in the presence of unlabelled RNA competitors. Fig. 4 showed that whereas the unlabelled 16s rRNA would compete with labelled 16s rRNA for DNA-hybridization sites, there was no cross-

competition between labelled 16s and unlabelled 23s rRNA. The converse experiments with ^3H -labelled 23s rRNA and unlabelled 16s rRNA gave the equivalent results. Thus, in agreement with Yankofsky & Spiegelman (1963), Oishi & Sueoka (1965) and Smith *et al.* (1968), it appears that 16s and 23s rRNA species of *B. subtilis* hybridize specifically to distinct DNA sites without cross-competition.

Base composition of 16s and 23s rRNA. ^{32}P -labelled rRNA was prepared from cultures of *B. subtilis* grown exponentially in the modified tris-phosphate-glucose medium described by Pigott & Midgley (1968) with the additional supplements described in the Materials and Methods section. To a 500 ml. culture, 2 mC of [^{32}P]phosphate was added and the culture was allowed to incorporate the isotope over several hours' exponential growth. After the usual extraction with phenol, the labelled rRNA species were fractionated on the protein-coated kieselguhr column (Mandell & Hershey, 1960). The fractions corresponding to 16s and 23s rRNA were pooled, precipitated in ice-cold 5% (w/v) trichloroacetic acid and filtered on to Oxoid membrane filters. Portions of each filter were cut off and hydrolysed by KOH as described in the Materials and Methods section [see Midgley (1962)]. Enough unlabelled *E. coli* rRNA was added to permit analysis of the mixture by isotope dilution and column chromatography, as

Table 1. *Nucleotide base compositions of B. subtilis 16s and 23s rRNA*

Determinations are to a standard error of $\pm 1.5\%$.

Specimen	Nucleotide composition (moles/100 moles)			
	CMP	AMP	GMP	UMP
16s rRNA	22.9	26.5	29.9	20.7
RNA from 30s ribosomes*	22.3	26.5	29.6	21.6
23s rRNA	22.3	26.2	32.2	19.3
RNA from 50s ribosomes*	22.5	23.2	32.0	19.3

* Values as given by Midgley (1962).

described by Midgley (1962) and Pigott & Midgley (1968). Table 1 shows that the nucleotide base compositions of 16s and 23s rRNA were slightly, through measurably, different. The values agree well in each case with those given by Midgley (1962), which are included in Table 1 for comparison.

Theoretical hybridization curves. (a) General considerations. When a specimen of RNA is hybridized with denatured homologous DNA at a concentration such that all the RNA could, in theory, be bound to specific accepting sites, the reaction does not go to completion in 24 hr. (Pigott & Midgley, 1968). In practice, the process appears to approach an equilibrium between bound and non-hybridized RNA after incubation for about 12 hr. in the usual hybridization medium (Kennell, 1968; Pigott & Midgley, 1968). The system can thus be considered as one obeying the classical Law of Mass Action, since the initial rate of hybridization of RNA and DNA appears to be concentration-dependent (Nygaard & Hall, 1964; Kohne, 1968). In a complex system such as this, other factors may cause a considerable deviation from the classical case, but it is instructive initially to follow the course of DNA-RNA hybridization mathematically and then to see how closely the experimental curves obey those theoretically predicted.

One inconsistent deviation from the expected curves appears to be based largely on the nature of the DNA used for the formation of hybrids upon cellulose nitrate membrane filters. If special attention is paid to the preparation of DNA of the highest purity and molecular weight, the efficiency of formation of hybrids is consistently high. However, relatively sheared or impure DNA specimens have an adverse effect on the efficiency of hybrid formation with RNA, and this factor must be taken into account when any quantitative measurements of hybridization are undertaken. Ways in which this anomaly may be taken into account are discussed below.

(b) The saturation of specific DNA sites by

excess of RNA. Let us consider the case in which increasing amounts of a purified specimen of RNA are offered to a fixed amount of denatured DNA, from a homologous source, for hybridization. Let us first propose that the RNA specimen consists of products from several genes, and that they are present in the same relative weight ratio as that of the regions of DNA from which they were originally transcribed. Thus, after any addition of RNA, each of the appropriate DNA sites will be affected equally, so that for purposes of hybridization we can treat the specimen as a single RNA species.

In the hybridization medium, at equilibrium:

$$K_{\text{equil.}} = [\text{DNA}_{\text{free}}][\text{RNA}_{\text{free}}]/[\text{Hybrid}] \quad (1)$$

where $K_{\text{equil.}}$ is the dissociation constant for the equilibrium between non-hybridized RNA and DNA ($[\text{RNA}_{\text{free}}]$ and $[\text{DNA}_{\text{free}}]$) and DNA-RNA hybrids. Let us now propose that the equilibrium is not disturbed by competition from DNA-DNA interactions, owing to the prior immobilization of all the available DNA as denatured single strands on an inert support.

Let the amount of RNA offered in the hybridization be X weight units and let an amount Y be hybridized to the DNA. If the amount of DNA present is D weight units, we will suppose that an amount μD is capable of specifically binding the RNA. Thus eqn. (1) becomes:

$$K_{\text{equil.}} = (X - Y)(\mu D - Y)/Y \quad (2)$$

or:

$$Y^2 - Y(K_{\text{equil.}} + \mu D + X) + X\mu D = 0$$

Dividing throughout by $(\mu D)^2$, this becomes:

$$\left(\frac{Y}{\mu D}\right)^2 - \frac{Y}{\mu D} \left(\frac{K_{\text{equil.}}}{\mu D} + 1 + \frac{X}{\mu D}\right) + \frac{X}{\mu D} = 0 \quad (3)$$

Now, from eqn. (1) we can define $K_{\text{equil.}}$ as the concentration of specific DNA not hybridized when the concentrations of hybridized and unhybridized RNA are equal. If this is a fraction β of the total specific DNA regions, then the concentration of unhybridized DNA in these conditions is $\beta\mu D$, i.e. $\beta = K_{\text{equil.}}/\mu D$. Further, let us define $\mu D/X$ as a factor C . Then, from eqn. (3):

$$\left(\frac{Y}{\mu D}\right)^2 - \frac{Y}{\mu D} \left(\beta + 1 + \frac{1}{C}\right) + \frac{1}{C} = 0$$

or:

$$\frac{Y}{\mu D} = \frac{(\beta + 1 + 1/C) \pm [(\beta + 1 + 1/C)^2 - 4/C]^{1/2}}{2} \quad (4)$$

Fig. 5. Theoretical hybridization curves for RNA and homologous DNA, derived from eqns. (4) and (5) (see the text). It was assumed that the RNA sample contains 10 species of the same molecular weight from 10 dissimilar DNA cistrons, and that only 1% by weight of the total DNA is capable of forming hybrids. Curves were obtained by assuming values for β of 0, 0.01, 0.1, 0.2 and 0.4. (a) Hybridization curves for various values of β , also assuming that all RNA species are present in the RNA samples in the same weight ratios as the DNA cistrons from which they were transcribed. (b) Hybridization curves for values $\beta = 0, 0.01$ or 0.1 , also assuming that one-third of the RNA hybridizes to five DNA cistrons, and two-thirds hybridizes independently to a further five cistrons. Within each group of RNA species, the individual species are present in the same weight ratio as the DNA cistrons hybridizing with them.

If, however, β is small, indicating a small $K_{\text{equil.}}$, then ignoring β in the above equations:

$$\left(\frac{Y}{\mu D} - 1\right)\left(\frac{Y}{\mu D} - \frac{1}{C}\right) = 0 \quad (5)$$

In Fig. 5(a), a plot of $Y/\mu D$ against $1/C$ is shown for a set of values of β in the range 0–0.40. This shows a series of hybridization curves with weight of RNA hybridized/100 wt. units of DNA as ordinate and RNA/DNA weight ratio as abscissa. Only when $K_{\text{equil.}} = 0$ does a straight line of increasing RNA hybridization occur at sub-saturating amounts of RNA offered. However, when $K_{\text{equil.}}/\mu D \leq 0.01$, the hybridization curve is a close approximation to two straight lines (derived from eqn. 5), giving the solutions:

$$Y/\mu D = 1/C \text{ or } 1$$

with no more than 10% inaccuracy in the area round the inflexion point of the curve for $K_{\text{equil.}} = 0$.

Again, only when $K_{\text{equil.}} = 0$ does the point or area of most rapid change of slope in the hybridization curves accurately represent the amount of DNA specifically binding to the RNA, as indicated by a perpendicular dropped to the abscissa. In all other cases, this projection gives an overestimate, the degree of inaccuracy increasing with increasing values of β .

Overestimations of this kind are not confined to the effects of equilibrium between free and hybridized RNA molecules. If the individual RNA species within the purified specimen are present in weight ratios different from those of the specific DNA regions to which they hybridize, then a curve similar to that shown in Fig. 5(b) will be obtained, assuming $K_{\text{equil.}}/\mu D \leq 0.1$. If the weight ratios of the individual RNA species are not extremely different from the weight ratios of the corresponding DNA regions, this will also lead to non-linearity in the early part of the hybridization curve, followed by the true saturation plateau. Again, if $(K_{\text{equil.}}/\mu D) \leq 0.01$, the transition points in the slope of the hybridization curve are rounded, but approximate closely to the curves obtained by assuming $K_{\text{equil.}} = 0$.

However, whatever the value of $K_{\text{equil.}}$ in this case, point A in Fig. 5(b) gives a value from the abscissa that overestimates the percentage of DNA responsible for the transcription of all the RNA species. Finally, if some RNA molecules are present in amounts that are very small compared with the weight fraction of the DNA hybridizing with them, the graph will be further complicated by a prolonged small positive slope in the hybridization curve after the other sites have been saturated, and this, in addition to any effects of equilibrium, may lead to an underestimate of the true value of the saturation plateau for the RNA. In this case, a plot of $1/[\text{RNA hybridized}]$ against $1/[\text{RNA input}]$ will be needed to find the true value, for the equation:

$$\frac{1}{Y} = \frac{1}{X} \cdot \frac{K_{\text{equil.}}}{\mu D} + \frac{1}{\mu D} \quad (6)$$

holds when there is a very great excess of input RNA (X) over DNA binding sites (μD) and thus $X - Y \approx X$. For this special case, the reciprocal plot extrapolates to give the value of $1/\mu D$ when $1/X = 0$, giving a straight line of slope $K_{\text{equil.}}/\mu D$.

Summarizing, therefore, we can be sure that if equilibrium phenomena and the relative amounts of RNA species in a mixture are the only factors involved in the determination of the progress of hybridization curves, the 'saturation plateau' should accurately represent the percentage of the DNA capable of accepting the RNA as a correct

hybrid. Additional complications, such as the physical state of the immobilized DNA, the formation of incorrect hybrids and the instability of correct hybrids during treatment with ribonuclease, may distort the curves further.

(c) Estimations using the efficiency of RNA hybridization with DNA. One of the disadvantages of the analytical methods with excess of RNA is that, if eqn. (6) is to hold, the amounts of RNA that must be added to the DNA are so large that only a small fraction of the RNA input is capable of hybridization. Practically, the non-specific contribution of spuriously bound RNA, which is sensitive to digestion with ribonuclease, becomes comparable with the amounts of correctly formed DNA-RNA hybrids. This enforces the use of treatment with ribonuclease to ensure that the amount of non-specific background hybridization is small. Any uncertainty in the effectiveness of the treatment with ribonuclease can have a considerable effect on the accuracy of the experimental points. We also have to be certain that the definition of true hybrids as completely ribonuclease-resistant is rigidly correct in the conditions employed. For example, the intimate contact of the denatured DNA strands with the support and the probability of such disturbing factors as DNA-strand overlapping may also cause a proportion of the RNA hybrids associated with the correct DNA sites to be unstable and easily removed by the enzyme or by the washing procedures. We have therefore considered hybridizing the RNA in conditions in which it is not greatly in excess of the available DNA binding sites, so that the most important experimental points are determined when most of the RNA offered is hybridized. In this case, the contributions of the ribonuclease-sensitive hybrids resistant to the washing procedures are small, and treatment with the enzyme is no longer necessary to remove large fractions of incorrectly bound RNA.

Let us again take the simple case of the hybridization of a purified RNA specimen in which individual species are present in weight ratios equal to the weight ratios of the DNA regions to which they hybridize. Using the symbols for the derivation of the saturation-hybridization curve, then from eqn. (2), dividing throughout by X^2 :

$$\left(\frac{Y}{X}\right)^2 - \frac{Y}{X} \left(\frac{K_{\text{equil.}}}{X} + \frac{\mu D}{X} + 1 \right) + \frac{\mu D}{X} = 0 \quad (7)$$

or, by substituting for $K_{\text{equil.}}$ and $\mu D/X$:

$$\left(\frac{Y}{X}\right)^2 - \frac{Y}{X} (\beta C + C + 1) + C = 0 \quad (8)$$

or:

$$\frac{Y}{X} = \frac{(\beta C + C + 1) \pm [(\beta C + C + 1)^2 - 4C]^{1/2}}{2} \quad (9)$$

If βC is small, indicating a small $K_{\text{equil.}}$, then from eqn. (8):

$$\left(\frac{Y}{X} - C\right) \left(\frac{Y}{X} - 1\right) \simeq 0 \quad (10)$$

In Fig. 6(a), a plot of Y/X against C is shown for three values of β . This graph thus represents a series of curves with the [RNA hybridized]/[RNA input] ratio as ordinate and the DNA/RNA weight ratio as abscissa. When $K_{\text{equil.}}$ and thus $\beta = 0$, the hybridization curves become two straight lines obeying the solutions for Y/X from eqn. (10). Just as with hybridization-saturation curves, if $K_{\text{equil.}}/\mu D \leq 0.01$, the curve is a close approximation to the case when $\beta = 0$, with a maximum 10% error near the inflexion point. However, the early part of the hybridization curve, at saturating RNA concentrations, has virtually the same slope for all values of $\beta \leq 0.01$, so that a projection of this line to that given by $Y/X = 1$ again intersects at A,

Fig. 6. Theoretical hybridization curves for RNA and homologous DNA, derived from eqns. (9) and (10) (see the text). The RNA sample is constituted as described in the legend of Fig. 5. (a) Curves obtained when the RNA sample is constituted as in Fig. 4(a), with values for β of 0-0.4. (b) Curves obtained for RNA constituted as in Fig. 4(b), with values for β of 0 and 0.01.

the DNA/RNA ratio indicating the amount of DNA responsible for the formation of hybrids. If $\beta \leq 0.1$, this is no longer true, as the extrapolated slope from low DNA/RNA ratios (saturating RNA concentrations) cuts the line given by $Y/X = 1$ at DNA/RNA ratios that give a progressively greater underestimation of the percentage of DNA taking part in the hybridization as the value assumed for β becomes greater.

If some species of RNA are present in the sample in weight ratios that are different from those of the corresponding DNA regions, then a curve of the type shown in Fig. 6(b) will be observed for values of $\beta \leq 0.01$. At low DNA/RNA ratios, the curve is identical with that obtained in the same region of Fig. 6(a) ($Y/X = C$), but when the minor RNA species in the mixture no longer saturate their specific DNA sites, a change in slope will occur [point (b)] to a line given by $Y/X = \alpha C$, where α is the fraction of the specific DNA hybridizing with the major RNA species. Full hybridization of all the RNA sample is first observed after point (c) (given by $Y/X = 1$) where the DNA sites capable of binding the major species are no longer saturated with RNA. If a line (b)–(a) is drawn as an extrapolation of the early part of the hybridization curve, this cuts the line given by $Y/X = 1$ at point (a), allowing us to find the fraction of specific DNA (μD) involved in binding all the RNA. Point (c), the minimum DNA/RNA ratio giving full hybridization of the RNA, is obviously an underestimate of the value given by (a). If a line 0–(d) is drawn from the origin parallel with the line (b)–(c) ($Y/X = \alpha C$), this will cut the line $Y/X = 1$ at (d), corresponding to the fraction of the DNA ($\alpha \mu D$) specific for binding the major fraction of the RNA in the specimen. The difference between this value and the total (μD) gives the fraction of the DNA, $(1 - \alpha)\mu D$, specific for the RNA species present as the minor component by weight. Finally, if we produce the line (b)–(c) to the ordinate, we divide the ordinate between the origin and normalized 100% hybridization in the ratio of the amounts of the RNA species present in the two groups. Thus if $\beta \leq 0.01$, the shapes of the hybridization curves may, in simple cases, provide information about the relative amounts of RNA species hybridizing independently to DNA, in a mixture. If, however, $\beta > 0.01$, this analysis is less accurate, owing to the distortions in all parts of the curve caused by mass-action effects. Therefore one of the tests for the effects of mass-action upon the curves is whether the estimations from extrapolations of hybridization-efficiency curves at low DNA/RNA ratios to the line of constant maximum RNA hybridization at very high ratios agree with those obtained by the saturation of the DNA by excess of RNA. Examination of Figs. 5(a) and 6(a) shows that if the value of

Fig. 7. Hybridization of *B. subtilis* total (●), 16s (▲) or 23s (○) rRNA to DNA.

β is such that the hybridization curves are seriously affected by mass-action effects, a determination, by hybridization-efficiency analysis, of the percentage of DNA hybridizing with an RNA specimen, gives an underestimate compared with the value obtained from saturation curves. Thus we can define a value for β as being < 0.01 only in the case when these two analyses give the same results. We will show that the value of β in our experiments is small enough to be ignored as a factor influencing hybridization curves at the concentrations of RNA and DNA used.

Experimental hybridization curves. Increasing quantities of ^3H -labelled total, 16s or 23s rRNA from *B. subtilis* were hybridized with a fixed amount of ^{14}C -labelled DNA on cellulose nitrate filters. After incubation for 17 hr. at 66° , the hybrids were washed and incubated with pancreatic ribonuclease (free from deoxyribonuclease) to remove unstably bound RNA (Gillespie & Spiegelman, 1965), as described in the Materials and Methods section. Fig. 7 shows the results obtained.

Whereas the percentage of DNA specific for the binding of the total (16s + 23s) *B. subtilis* rRNA was $0.46 \pm 0.02\%$, that specific for 16s and 23s rRNA alone was $0.15 \pm 0.02\%$ and $0.30 \pm 0.02\%$ respectively. The 'plateau' of saturation of the DNA with RNA was flat in each of the three cases. The results confirm those of Oishi & Sueoka (1965) and Smith *et al.* (1968), in that the percentages of the DNA of *B. subtilis* capable of binding the 16s and 23s rRNA species bear a 1:2 weight relationship. Also, (16s + 23s) rRNA binds to an amount of DNA equal to the sum of the percentages independently binding each species.

Efficiency of hybridization of rRNA. Similar experiments were carried out in which the efficiency of hybridization of RNA was measured over a wide range of DNA/RNA ratios. In contrast with the previous experiments, the bulk of the experimental

points were obtained in conditions in which a high proportion of the rRNA offered was hybridized.

To demonstrate that ribonuclease-sensitive hybrids form a negligible proportion of the total hybridized RNA in our conditions, two sets of DNA-containing filters were made up. To both, various amounts of RNA were added, so that between 20% and 90% of the rRNA was apparently hybridized. One set of filters was washed extensively without the additional treatment with ribonuclease and the other was incubated with the enzyme as described by Gillespie & Spiegelman (1965), in addition to the other steps. The decrease in the percentage of RNA bound was, in each case, only 1–2%, as compared with untreated controls. Most of this spurious background binding could be ascribed to RNA bound to the filter material itself, since 1–2% of input RNA was bound to the filters containing no DNA and was also sensitive to ribonuclease. Thus, a suitable control was set up to take account of this small background binding, without the need for recourse to treatment with ribonuclease of filters bearing true ribonuclease-resistant hybrids.

Figs. 8(a), 8(b) and 8(c) show the course of hybridization of a fixed amount of DNA with decreasing amounts of total, 16s or 23s rRNA. In each case, a reasonably linear increase in the percentage of RNA hybridized occurs with each added increment of DNA. With total rRNA (Fig. 8c), there is a rapid change of slope in the curve at DNA/RNA ratios (w/w) of 215:1 ($\pm 10:1$). After this ratio has been reached, the DNA sites are no longer saturated with RNA, and thereafter 90–95% of the input RNA will hybridize. This curve may be compared with the theoretical curve in Fig. 6(a). The DNA/RNA ratio of 215:1 thus determines the percentage of DNA specific for total (16s+23s) rRNA, but is only accurate if K_{equil} for hybridization is small and the RNA from each group of specific DNA sites distinguishable by hybridization is produced at the same overall rate. In any other case, the DNA/RNA ratio at which maximum hybridization is first achieved is an underestimate of this percentage (Fig. 6b). A value of $1/215 \times 100 = 0.46\%$ is obtained by analysis of hybridization-efficiency curves. This may be compared with the value of 0.45% obtained from the 'saturation plateau' in conditions in which RNA is added in excess to a fixed amount of DNA (Fig. 7). Similarly, the DNA/RNA ratios at which the specific DNA sites for 16s and 23s rRNA appear to be just saturated are 660/1 and 315/1 respectively. These values correspond to percentages of 0.15% and 0.32% of the DNA specific for the 16s and the 23s fractions respectively. Again, these values are almost identical with those obtained by saturation of DNA with RNA. In all cases, the hybridization-

Fig. 8. Hybridization of *B. subtilis* total, 16s and 23s rRNA with DNA. As in Fig. 7, all specimens also contained a small proportion of mRNA. (a) 16s RNA. (b) 23s RNA. (c) Total (16s+23s) RNA.

efficiency curves do not extrapolate to the origin. This phenomenon is ascribed to mRNA, which is also labelled and is eluted with the rRNA fractions from the columns. From the hybridization curve for total rRNA (+mRNA) (Fig. 8c), it appears that 2–3% of the total RNA is efficiently hybridized at very low DNA/RNA ratios, a characteristic of mRNA (Pigott & Midgley, 1968). The slight curvature in the early part of the hybridization curve is less easy to understand. We believe that it is best explained by (a) difficulties in accurate correction for non-specific adsorption of RNA to the filters at low DNA/RNA ratios (the fraction of RNA binding in this way to the filters tends to rise with increasing amounts of RNA) and (b) the hybridization properties of the small labelled mRNA fraction over the range of low DNA/RNA ratios. As shown below, the hybridization of rRNA free from mRNA gives more linear plots, with hybridization curves extrapolating through the origin (Fig. 9). However,

Fig. 9. Hybridization of a mixture of *B. subtilis* 16s and 23s rRNA with DNA. This RNA had been purified from mRNA by the method of Pigott & Midgley (1968).

the linearity of most of the hybridization curve, even in the presence of mRNA, and the extremely close agreement between estimations obtained by analyses using both approaches confirms that the DNA sites specific for the rRNA are present in the same weight ratio as their products in the RNA fractions. Thus, each group of DNA sites distinguishable by hybridization produces rRNA at the same rate. The curves also show little perturbation caused by equilibrium factors at these concentrations of RNA and DNA.

Analyses of mixtures of 16s and 23s rRNA. Mixtures of ^3H -labelled 16s and 23s rRNA, free from mRNA (Pigott & Midgley, 1968) were made up in a 16s/23s weight ratio 2:1. This mixture was then hybridized with denatured DNA and hybridization-efficiency curves were produced. Fig. 9 shows the results obtained. Since the weight ratio of the 16s and 23s RNA is not the same as that of the DNA regions hybridizing with them, it should be possible to analyse the hybridization curve in the way described in Fig. 6(b). Since $K_{\text{equil.}}$ is probably small enough to be ignored as an important factor, an analysis of Fig. 9 confirms this. An extrapolation of the early part of the hybridization curve cuts the line representing 88% hybridization (the maximum in this particular set of experiments) at a DNA/RNA ratio 205:1 ($\pm 15:1$). If, in Fig. 9, a line is drawn from the origin parallel to the second part of the hybridization curve, it cuts the line $Y/X=1$ (at 88% hybridization) at a DNA/RNA ratio of 780:1, showing (a) that 16s rRNA is present in excess of 23s rRNA, compared with the relative availability of their specific DNA sites (see Fig. 6b and its interpretation), and (b) that 0.13% of the DNA is specifically capable of binding the 16s rRNA fraction. Finally, if the slope of the hybridization curve just before the attainment of maximal RNA hybridization is produced to the ordinate, it divides the ordinate between 0 and 100%

normalized hybridization in the approximate ratio of 1:2. This strongly supports the theoretical analyses, and indicates that since equilibrium factors affect the curves only slightly, simple mixtures of RNA fractions may be analysed to determine the proportions of each material hybridizing independently with different DNA sites.

DISCUSSION

In the present paper we have shown that the denatured DNA of *B. subtilis*, immobilized on a cellulose nitrate membrane filter, will combine with homologous rRNA with very high efficiency to form specific DNA-RNA hybrids. We have confirmed that the 16s and 23s rRNA species hybridize, without cross-competition, to different specific DNA regions (Yankofsky & Spiegelman, 1963; Oishi & Sueoka, 1965; Mangiarotti, Apirion, Schlessinger & Silengo, 1968; Smith *et al.* 1968), but our estimations of the percentage of DNA capable of hybridizing with the rRNA are slightly higher than the results cited by these authors. Of the DNA, 0.45% was specific for total hybridization of rRNA, either by analyses based on saturation of DNA sites by excess of RNA (saturation analysis) or by the use of RNA-hybridization-efficiency curves (hybridization-efficiency analysis). Also, by using both analytical approaches, 0.15% of the DNA was shown to hybridize with 16s rRNA, and 0.30% with 23s rRNA. Taking the molecular weight of *B. subtilis* DNA as 4×10^9 (Eberle & Lark, 1967) and the molecular weights of 16s and 23s rRNA to be the same as those of the corresponding material in *E. coli* (i.e. 5.5×10^5 and 1.1×10^6 , respectively) (Stanley & Bock, 1965), it is calculated that there are 10–11 DNA cistrons each specific for transcription of 16s and 23s rRNA.

As the molecular-weight ratios of 16s and 23s rRNA are maintained in the weight ratios of the DNA to which they hybridize and equal numbers of 16s and 23s rRNA molecules are produced over a given period of cell growth, this system is ideal for the quantitative study of the hybridization of well-defined RNA species to limited regions of the DNA, and allows us to analyse the hybridization of both purified RNA specimens and simple mixtures of the 16s and 23s fractions. The system is further simplified by the probability that the multiple DNA cistrons binding either of the RNA species are so similar in their nucleotide sequences that, for example, any 16s rRNA molecule may hybridize with any one of the 10 or 11 DNA cistrons specific for this fraction (Takanami, 1967; Sugiura & Takanami, 1967; Kohne, 1968).

Our aim in this work was to show that the hybridization of a given RNA sample could be analysed with equal accuracy by methods that do

not involve the usual saturation of DNA sites by a large excess of RNA, with all the attendant practical precautions then needed to validate the results. We have indicated that mass-action effects on the positions of equilibrium between hybridized and non-hybridized RNA molecules at reasonable concentrations of DNA and RNA are small enough to permit an analysis of the efficiency of hybridization of input RNA over a wide range of DNA/RNA ratios (see Kennell, 1968). Our experiments thus differ in that most of the experimental points are obtained without a great excess of RNA present and without the need for treatment of filters by ribonuclease to minimize the number of incorrect hybrids. With *B. subtilis* DNA, the efficiency of hybridization of RNA to DNA is high and most of the available DNA sites are saturated with RNA when a relatively small excess of RNA is added. Further, it seems that the degree of resistance of most of the hybrids to ribonuclease is high, as analyses carried out with or without this treatment with enzyme gave the same results.

However, our experience of the reproducibility of DNA-RNA hybridization processes has warned us that equilibria between hybridized and non-hybridized RNA are affected by factors, other than mass-action, that also play a part in determining how much RNA will bind to a fixed amount of DNA in given circumstances. The individual characteristics of DNA specimens can affect quantitative measurements of the processes of hybridization to a degree far outweighing that expected of simple equilibrium phenomena. For example, we have found that some specimens of *B. subtilis* DNA would accept fewer RNA hybrids per unit mass than others. With these specimens, hybridization-efficiency curves were produced with a maximum of as little as 50% of the input RNA bound at DNA/RNA ratios of 200:1 or more, even though the conditions for hybridization were identical in all known respects with those for other DNA specimens that bound 90-95% of the input RNA. The RNA-saturation curves were similarly affected, showing clearly that factors other than mass-action equilibria were affecting the production of hybrids. Although these factors could be minimized by close attention to the purity and integrity of the DNA, we could not assume that their influence was entirely removed in the best DNA used. However, if all hybridization curves for a given RNA were normalized to give a nominal 100% efficiency of hybridization at DNA/RNA ratios greater than 200:1, all parts of each hybridization curve were superimposed upon each other (R. J. Avery, unpublished work). This indicates that, in addition to any considerations of equilibrium between hybridized and non-hybridized RNA, other factors affect a fairly constant proportion of

the hybrids formed in a given set of conditions. In optimum conditions, the effect of these factors is relatively small, and close approximations are obtained to the expected hybridization curves. Similar inconsistencies have been observed in the hybridization of *E. coli* RNA and DNA on membrane filters (Mangiarotti & Schlessinger, 1967; Pigott, 1967). Therefore, we have termed the maximum proportion of RNA that will bind with any given DNA specimen, with known concentrations of RNA and DNA, as the hybridization-efficiency coefficient, as it seems inaccurate to relate the observed proportions of hybridized and non-hybridized RNA to a classical equilibrium constant.

The behaviour of the rRNA species of *B. subtilis* in hybridization with DNA may be contrasted with that of the corresponding RNA from *E. coli* (Attardi *et al.* 1965; Mangiarotti *et al.* 1968). Whereas the 16s and 23s rRNA from species of the genus *Bacillus* do not compete for binding sites on homologous DNA, this is not true of the corresponding RNA species from *E. coli*. To throw further light on the structure and genetic homologies of *E. coli* rRNA species, we have used the technique of DNA-RNA hybridization described in the present paper. These results are given in the next paper (Avery, Midgley & Pigott, 1969).

R.J.A. thanks the Science Research Council for provision of a grant for a postgraduate studentship. J.E.M.M. is a Senior Research Associate of the Medical Research Council. This work was carried out as part of the work of the Medical Research Council Research Group in the Structure and Biosynthesis of Macromolecules.

REFERENCES

- Attardi, G., Huang, P. C. & Kabat, S. (1965). *Proc. nat. Acad. Sci., Wash.*, **53**, 1490.
- Avery, R. J., Midgley, J. E. M. & Pigott, G. H. (1969). *Biochem. J.* **115**, 395.
- Burton, K. (1956). *Biochem. J.* **62**, 315.
- Cutler, R. G. & Evans, J. E. (1967). *J. molec. Biol.* **26**, 91.
- Doi, R. H. & Igarashi, R. T. (1965). *J. Bact.* **90**, 384.
- Doi, R. H. & Igarashi, R. T. (1966). *J. Bact.* **92**, 88.
- Dubnau, D., Smith, I., Morell, P. & Marmur, J. (1965). *Proc. nat. Acad. Sci., Wash.*, **54**, 491.
- Eberle, H. & Lark, K. G. (1967). *Proc. nat. Acad. Sci., Wash.*, **57**, 95.
- Gillespie, D. & Spiegelman, S. (1965). *J. molec. Biol.* **12**, 829.
- Kennell, D. (1968). *J. molec. Biol.* **34**, 85.
- Kennell, D. & Kotoulas, A. (1968). *J. molec. Biol.* **34**, 71.
- Kirby, K. S. (1964). *Progr. Nucleic Acid Res. molec. Biol.* **3**, 1.
- Kohne, D. E. (1968). *Yearb. Carnegie Instn, Wash.*, **67**, 310.
- Kurland, C. G. (1960). *J. molec. Biol.* **2**, 83.
- McConkey, E. H. & Dubin, D. T. (1966). *J. molec. Biol.* **15**, 102.
- Mandell, J. D. & Hershey, A. D. (1960). *Analyt. Biochem.* **1**, 66.

- Mangiarotti, G., Apirion, D., Schlessinger, D. & Silengo, L. (1968). *Biochemistry*, **6**, 456.
- Mangiarotti, G. & Schlessinger, D. (1967). *J. molec. Biol.* **29**, 395.
- Marmur, J. (1961). *J. molec. Biol.* **3**, 208.
- Midgley, J. E. M. (1962). *Biochim. biophys. Acta*, **61**, 513.
- Midgley, J. E. M. (1965). *Biochim. biophys. Acta*, **95**, 232.
- Midgley, J. E. M. (1968). *Biochem. J.* **108**, 30P.
- Nygaard, A. P. & Hall, B. D. (1964). *J. molec. Biol.* **9**, 125.
- Oishi, M. & Sueoka, N. (1965). *Proc. nat. Acad. Sci., Wash.*, **54**, 483.
- Pigott, G. H. (1967). Ph.D. Thesis: University of Leeds.
- Pigott, G. H. & Midgley, J. E. M. (1966). *Biochem. J.* **101**, 9P.
- Pigott, G. H. & Midgley, J. E. M. (1968). *Biochem. J.* **110**, 251.
- Smith, I., Dubnau, D., Morell, P. & Marmur, J. (1968). *J. molec. Biol.* **33**, 123.
- Spirin, A. S. (1961). *Biokhimiya*, **26**, 511.
- Stanley, W. M. & Bock, R. M. (1965). *Biochemistry*, **4**, 1302.
- Sugiura, M. & Takanami, M. (1967). *Proc. nat. Acad. Sci., Wash.*, **58**, 1595.
- Takahashi, H., Saito, H. & Ikeda, Y. (1967). *Biochim. biophys. Acta*, **134**, 124.
- Takanami, M. (1967). *J. molec. Biol.* **23**, 135.
- Yankofsky, S. A. & Spiegelman, S. (1963). *Proc. nat. Acad. Sci., Wash.*, **49**, 538.