The Effect of Genetic Complexity on the Time-Course of Ribonucleic Acid-Deoxyribonucleic Acid Hybridization

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1. The rate of RNA-DNA hybridization was studied under conditions of RNA excess, with RNA synthesized *in vitro*. The initial rate of the reaction was proportional to the initial RNA concentration. Throughout the observed course of the reaction there was a linear relationship between the reciprocal of the amount of RNA hybridized/ μ g. of DNA and the reciprocal of time. The slope of the reciprocal plot was inversely proportional to the initial RNA concentration. 2. A comparison was made of the hybridization of DNA from *Escherichia coli* and from bacteriophages T4 and λ with homologous RNA. The initial rate of hybridization was inversely proportional to the genetic complexity of the hybridizing system. The slope of the reciprocal-time plot was directly proportional to genetic complexity. These results are interpreted to indicate that the rate of hybridization reflects the mean concentration of the various unique RNA species in a preparation.

Specific complex-formation (hybridization) between denatured DNA and complementary RNA can be brought about by the process of annealing at high temperature and high salt concentration (Hall & Spiegelman, 1961; Schildkraut, Marmur, Fresco & Doty, 1961). The RNA-DNA complex is resistant to digestion by ribonuclease under prescribed conditions (Nygaard & Hall, 1964). The species specificity of hybrid formation indicates that only specific sequences of complementary base pairs can form hybrid duplexes.

Nygaard & Hall (1964) showed that the initial rate of hybrid formation is proportional to the RNA concentration. In most biological studies, however, the extent of complex-formation is measured when the reaction is well advanced. It is therefore important to study complex-formation beyond the initial period. In the present paper it is shown that the rate of complex-formation throughout the course of the reaction is a function of the initial RNA concentration. This is an extension of the results of Gillespie & Spiegelman (1965). Further evidence is presented which indicates that the rate is determined by the concentration of each unique RNA sequence. A preliminary report of this work has been given (Bishop, 1968).

MATERIALS AND METHODS

Ribonuclease A (three-times crystallized), deoxyribonuclease ('ribonuclease-free') and papain were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6. Sar-

torius nitrocellulose membrane filters (MF-50, 30 cm. diam.) were from V.A. Howe Ltd., London W.11. Sephadex SE-50 was from Pharmacia (G.B.) Ltd., London W.13. Unlabelled ribonucleoside triphosphates were from Pabst Laboratories, Milwaukee, Wis., U.S.A. [^{14}C]ATP and [^{32}P]P_i were from The Radiochemical Centre, Amersham, Bucks. Spray-dried cells of *Micrococcus lysodeikticus* were obtained from Cambrian Chemicals Ltd., Croydon. Frozen cells of *Escherichia coli* M.R.E. 600 were kindly provided by the Medical Research Council Microbial Products Division, Microbiological Research Establishment, Porton, Wilts. Visking dialysis tubing was obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex. This was treated with 64% (w/v) ZnCl₂ for 15 min. at room temperature to make it permeable to oligonucleotides, and then washed thoroughly in distilled water. After immersion in 0.1 M-HCl for 5 min. it was boiled in 0.01 M-EDTA and finally washed again in several changes of distilled water (R. Frame, personal communication).

Preparation of DNA. DNA was prepared from bacteriophages T4 and λ by the method of Mandel & Hershey (1960) and dialysed against 0.01 M-NaCl-0.01 M-tris-HCl buffer, pH 7.5, at 0°. The *E. coli* cells (1 part) were suspended in 5 parts (by wt.) of buffer A (0.01 M-EDTA-0.1 M-NaCl-0.01 M-tris-HCl, pH 7.5 at 0°) and 10% (w/v) sodium dodecyl sulphate was added to give a final concentration of 2%. Within a few minutes at 45° the cells had lysed, and the solution was diluted with an equal volume of buffer A. β -Mercaptoethanol (final concn. 0.01 M) and then papain (heated at 71° for 30 min.; final concn. 25 μ g./ml.) were added. The solution was kept at 37° until it had become clear (2-4 hr.), and then it was extracted twice with an equal volume of water-saturated redistilled phenol. After each extraction the phenol was removed by centrifugation. DNA was spooled from the aqueous phase after the addition of an equal volume of ethanol and redissolved in a volume of

buffer A equal to that used initially to suspend the cells. The solution was treated for 1 hr. at 37° with 50 µg. of ribonuclease/ml. (previously heated for 10 min. at 98°), sodium dodecyl sulphate (final concn. 1%) and then papain (final concn. 10 µg./ml.) were added and incubation was continued for 2 hr. The solution was extracted twice with phenol, and the DNA spooled from the aqueous phase after addition of an equal volume of ethanol. The DNA was again dissolved in buffer A and the digestion with ribonuclease and papain was repeated, followed by extraction with phenol and spooling. Finally the DNA was dissolved in buffer A and dialysed against several changes of 0.01 M-NaCl-0.01 M-tris-HCl buffer, pH 7.5 at 0°, with ZnCl₂-treated dialysis tubing.

³²P-labelled bacteriophage and bacteria were grown in MS broth (Davis & Sinheimer, 1963) containing 0.5-1 mCi of [³²P]P_i/ml. ³²P-labelled DNA was prepared from suitable mixtures of labelled and unlabelled bacteriophage or bacteria.

Preparation of c-RNA.* RNA polymerase (fraction VI) was purified from spray-dried *Micrococcus lysodeikticus* by the method of Nakamoto, Fox & Weiss (1964). Enzyme activity was assayed by the method of Nakamoto *et al.* (1964) except that the [¹⁴C]ATP concentration was 0.4 mM and T4 DNA was used as primer. Several concentrations of DNA were used and activity was calculated at the optimum DNA concentration. Acid-insoluble radioactivity was measured as described by Bollum (1959). RNA was synthesized quantitatively in a 10 ml. reaction mixture of the same constitution, usually for 30 min. at 30°. The amounts of DNA and enzyme were adjusted to give a product/primer ratio between 1.5 and 2. The radioactive precursor was [¹⁴C]ATP (1 mCi/m-mole or about 1100 c.p.m./nmole). At the end of the reaction MgCl₂ (final concn. 5 mM) and then deoxyribonuclease (final concn. 25 µg./ml.) were added and the mixture was incubated for 15 min. at 37°. Then 2 M-NaCl was added to give a final concentration of 0.15 M and then sodium dodecyl sulphate (final concn. 0.5%). After 3 min. at 37° the mixture was extracted with water-saturated phenol at room temperature. The phenol phase was separated by centrifugation and re-extracted with 0.1 M-NaCl. The combined aqueous phases were extracted with phenol, and RNA was precipitated from the resulting aqueous phase with 2 vol. of cold ethanol. After 30 min. at 0° the RNA was collected by centrifugation and dissolved in 1-2 ml. of water. This was adjusted to 0.3 M-NaCl-0.01 M-sodium acetate buffer, pH 5, by using 0.11 vol. of a ten-times concentrated solution, and passed through a 1.6 cm. × 20 cm. column of Sephadex SE-50 developed with the same solvent at room temperature. This procedure separates RNA from contaminating oligodeoxyribonucleotides and ribonucleotides. The column eluate was neutralized with 3 M-tris base and stored frozen. The specific radioactivity was calculated from the *E*₂₆₀ and radioactivity of the RNA solution. In each case this was very close to that expected from the specific radioactivity of the [¹⁴C]ATP substrate.

RNA-DNA hybridization. Nitrocellulose membrane filters were loaded with 1.5 µg. of ³²P-labelled alkali-denatured DNA in 6×SSC as described by Gillespie & Spiegelman (1965), and washed with 50 ml. of 2×SSC.

* Abbreviations: c-RNA, RNA synthesized *in vitro* on a DNA template by using purified RNA polymerase. SSC, 0.15 M-NaCl-0.015 M-sodium citrate, pH 7.5.

One DNA-carrying membrane and one blank washed membrane were rolled together, inserted into a 5 mm. × 45 mm. glass tube, and dried for 2 hr. in a vacuum desiccator at room temperature and then for 2 hr. in a vacuum oven at 80°.

RNA solutions were made up in 2×SSC containing 100 µg. of rat liver cytoplasmic RNA/ml. as an inhibitor of non-specific adsorption. Then 1 ml. of RNA solution was added to each membrane-containing tube, and the tubes were tightly stoppered and incubated in a 65° paraffin bath. The annealed membranes were removed and treated for 1 hr. at 65° in 20 ml. of 2×SSC, chilled, washed with 500 ml. of 2×SSC at room temperature, treated with 20 ml. of 2×SSC containing 20 µg. of ribonuclease/ml., again washed with 500 ml. of 2×SSC and thoroughly dried. ¹⁴C-labelled RNA and ³²P-labelled DNA radioactivities were simultaneously measured by means of a two-channel liquid-scintillation counter. ¹⁴C radioactivity was excluded from the ³²P channel, and 8% of the ³²P radioactivity appeared in the ¹⁴C channel. Contamination of the blank filter by RNA was always less than 0.05% of the RNA input. The blank values were deducted from the experimental values, and the amounts of RNA and DNA on each filter were calculated by reference to the determined specific radioactivities.

Complexity of DNA. The term 'complexity' has been defined by Wetmur & Davidson (1968). The values used here for bacteriophage λ DNA are from MacHattie & Thomas (1964) and those for bacteriophage T4 and *E. coli* DNA are taken from Wetmur & Davidson (1968).

RESULTS

In the following experiments denatured DNA was hybridized with complementary RNA (c-RNA; Nakamoto *et al.* 1964) synthesized *in vitro* on a homologous DNA template (from bacteriophages λ and T4, and *E. coli*) with RNA polymerase purified from *Micrococcus lysodeikticus*. c-RNA was used in preference to cellular RNA because the latter consists mainly of ribosomal RNA. This does not hybridize with bacteriophage DNA, and hybridizes with only a small part of *E. coli* DNA. The concentration of messenger RNA cannot be measured directly in RNA preparations from cells. c-RNA contains no ribosomal RNA sequences when synthesized on bacteriophage DNA templates and only a very small amount when synthesized on a template of *E. coli* DNA (Robinson, Hsu, Fox & Weiss, 1964). In this respect it is equivalent to purified messenger RNA.

The denatured DNA was immobilized on nitrocellulose membrane filters by the method of Gillespie & Spiegelman (1965). The assumption is made that this prevents renaturation of DNA during annealing. The c-RNA is partially self-complementary (Geiduschek, Nakamoto & Weiss, 1961), and no method is known which will prevent it from self-annealing while allowing it to hybridize with DNA. The effect on the experimental results of self-annealing of RNA is considered in the Discussion section.

Effect of RNA concentration on the rate of hybridization. A constant amount of bacteriophage T4 DNA was annealed for different times with four

Fig. 1. Effect of initial RNA concentration on the time-course of RNA-DNA hybridization. (a) Conventional plot; (b) reciprocal plot. Hybridization is expressed as $\mu\text{g. of RNA hybridized}/\mu\text{g. of DNA}$. Each membrane carried about $1.5 \mu\text{g. of } ^{32}\text{P}$ -labelled DNA. The c-RNA was labelled with $[1-^{14}\text{C}]\text{ATP}$. The c-RNA concentrations were: $\times$, $5.33 \mu\text{g./ml.}$; $\circ$, $9.06 \mu\text{g./ml.}$; $+$, $13.3 \mu\text{g./ml.}$; $\bullet$, $26.6 \mu\text{g./ml.}$

different concentrations of c-RNA. At the lowest concentration the RNA/DNA input ratio was 4. It is shown below (Fig. 2) that 40% of bacteriophage T4 DNA hybridizes with a saturating amount of homologous c-RNA. Thus the lowest ratio of RNA to hybridizable DNA used in this experiment was 10. It is therefore very likely that all RNA sequences are in excess of the complementary DNA sequence at all four c-RNA concentrations. The results are shown in Fig. 1. Fig. 1(a) shows a conventional plot of amount of hybrid formed against time. The amount of hybrid formed is expressed as $\mu\text{g. of RNA hybridized}/\mu\text{g. of DNA recovered}$. This eliminates errors caused by variable recovery of DNA. The initial rate of the reaction, approximated by the 1 hr. points shown in Fig. 1(a), increased as the initial c-RNA concentration was increased. Nygaard & Hall (1964) annealed bacteriophage T4 DNA in liquid with messenger RNA, and found the initial rate of hybridization to be linearly proportional to the initial RNA concentration. In the present case the proportionality was not quite linear (Table 1, ratio R/h). This can be attributed to inaccurate estimation of the initial rate, and does not suggest a difference between the two methods of hybridization.

After longer times of annealing the four reactions converge (Fig. 1a), and it is clear that the course of each is a complex function of the initial RNA concentration. It was found that all the results for each initial c-RNA concentration could be represented by a straight line, by making a plot of the reciprocal of hybridization against the reciprocal of time (Fig. 1b). The standard errors of regression lines, calculated by the method of least squares, show that straight lines represent the data adequately (Table 1, column K_1). The relationship between amount of hybridization (r) and time (t) can therefore be written: $1/r = K_1/t + K_2$, where K_1 and K_2 are constants for a given initial RNA concentration. The slope of the line, K_1 , is found to be linearly inversely proportional to the initial c-RNA concentration. This is shown by the constancy of the product of the two values (Table 1, K_1h). K_1h then defines an approximate constant, K_1^1 , with a mean value of $43 \mu\text{g. of RNA} \times \mu\text{g. of DNA} \times \text{hr.} \times (\mu\text{g. of$

Table 1. *Effect of the initial c-RNA concentration on the time-course of RNA-DNA hybridization*

c-RNA synthesized on a template of bacteriophage T4 DNA was annealed with bacteriophage T4 DNA. The experiment is that described in Fig. 1.

Concn. of c-RNA ($\mu\text{g./ml.}$) (h)	Proportion of DNA hybridized at 1 hr. (R)	Ratio R/h	Slope of reciprocal plot ($\pm$ s.e.m.) [$\mu\text{g. of DNA} \times \text{hr.} \times (\mu\text{g. of RNA}$ hybridized) $^{-1}$] (K_1)	K_1h
5.3	0.091	0.017	7.65 ± 0.72	40.6 ± 3.8
9.1	0.132	0.014	4.64 ± 0.21	42.2 ± 1.9
13.3	0.159	0.012	3.33 ± 0.30	44.3 ± 4.0
26.6	0.217	0.008	1.69 ± 0.21	45.0 ± 5.3

hybridized RNA) $^{-1} \times \text{ml.}^{-1}$ from the data given in Table 1. The intercepts of the lines do not differ significantly from each other, and this suggests the development of an equation, $1/r = K_1/h + K_2$, describing all four lines. This experiment thus shows a linear relationship between K_1 and t within the limits of the experiment. More far-reaching generalizations are not possible, as will be shown below.

Effect of RNA complexity on the rate of hybridization. The complexity of DNA from a virus or bacterium is the amount of DNA, in daltons, in a single genetic complement (Wetmur & Davidson, 1968). At a given DNA concentration, the concentration of each unique sequence is inversely

proportional to the complexity. The second-order rate constant of DNA renaturation has been found also to be inversely proportional to the complexity (Marmur & Doty, 1961; Wetmur & Davidson, 1968; Britten & Kohne, 1966, 1968; Thrower & Peacocke, 1968), showing that the rate of DNA renaturation is determined by the absolute concentration of the unique sequences.

The following experiments were undertaken to see whether the rate of RNA-DNA hybridization is affected in the same way by complexity. The experiments are possible because c-RNA reflects to a considerable degree the complexity of the DNA from which it is transcribed (see the Discussion section).

DNA was extracted from bacteriophages λ and T4 and from *E. coli*, and c-RNA homologous with each was prepared with *Micrococcus* RNA polymerase. To estimate the extent of transcription, each DNA was annealed for 16 hr. with different concentrations of homologous c-RNA. The curve of concentration-dependence, like the time-curve, can be represented by a single straight line. In this case the reciprocal of hybridization is plotted against the reciprocal of the c-RNA concentration (Fig. 2). Thus, with the same symbols as before, $1/r = K_3/h + K_4$, where K_3 and K_4 are constants of concentration-dependence. Assuming that the relationship continues to be linear beyond the highest c-RNA concentration used, when $h = \infty$, $K_3/h = 0$ and $K_4 = 1/r_s$, where r_s represents the amount of hybridization at infinite RNA concentration. Since r represents the amount of RNA hybridized/unit of DNA, the maximum hybridization of each DNA species is given by the reciprocal of the intercept (Fig. 2).

The three intercepts (Fig. 2) are not significantly different, showing that the same proportion (close to 0.4) of each DNA species would hybridize with a saturating amount of homologous c-RNA. This allows two conclusions to be drawn which simplify subsequent calculations. (1) Making the reasonable assumption that the equal hybridization of the three types of DNA means that they are equally

Fig. 2. Reciprocal plot of the hybridization of ^{32}P -labelled DNA from *E. coli* (+), bacteriophage T4 (O) and bacteriophage λ (●) with homologous c-RNA. Annealing was carried out for 16 hr. in each case. Other conditions were as for Fig. 1.

Table 2. Effect of complexity on the concentration-dependence of hybridization

DNA from bacteriophage λ , bacteriophage T4 and *E. coli* was annealed for 16 hr. at 65° with various concentrations of homologous c-RNA. The data are taken from Fig. 2.

Source of DNA	10 ⁻⁸ × Complexity of DNA (daltons) (C)	Slope of reciprocal plot (± s.e.m.) [μg. of DNA × μg. of RNA × (μg. of RNA hybridized) ⁻¹]	Ratio K ₃ /C
		(K ₃)	
Bacteriophage λ	0.3	5.42 ± 1.11	18.1 ± 3.7
Bacteriophage T4	1.3	7.44 ± 0.71	5.7 ± 0.5
<i>E. coli</i>	25	47.4 ± 0.7	1.9 ± 0.03

Fig. 3. Effect of complexity on the time-course of RNA-DNA hybridization. ³²P-labelled DNA from *E. coli* (+), bacteriophage T4 (o) and bacteriophage λ (●) was annealed for various times with homologous c-RNA, 12.4, 7.7 and 8.0 $\mu\text{g./ml.}$ respectively. Other conditions were as for Fig. 1. (a) Conventional plot; (b) reciprocal plot.

transcribed, the experiment shows that the complexities of the c-RNA preparations are in the same ratios as the complexities of the corresponding DNA preparations. (2) For each DNA species, a

given amount of hybridization, r , represents the same degree of approach to the saturation value, r_s .

The slope of the regression line, K_3 , is evidently different for each type of DNA and c-RNA (Fig. 2). The relationship between K_3 and complexity is shown in Table 2. K_3 increases with complexity, but the proportionality is non-linear: as the complexity increases, the ratio of K_3 to complexity decreases. The time-course of hybridization of the three homologous systems was studied (Fig. 3). The initial rates of hybridization, estimated approximately by the 1 hr. value for bacteriophage λ and bacteriophage T4 DNA, and by one-half of the 2 hr. value for *E. coli* DNA, are inversely proportional to complexity (Table 3). The proportionality is nearly linear, as shown by the constancy of the product $C \times R$. For an 80-fold difference in complexity, there is a 2.6-fold change in the product $C \times R$.

Reciprocal plots of the data are shown in Fig. 3(b). The slopes of these plots (K_1) increase with increasing complexity. For the purpose of this experiment the concentrations of the three types of c-RNA should ideally be the same. Instead, the concentrations varied from 7.7 $\mu\text{g./ml.}$ to 12.4 $\mu\text{g./ml.}$ These are sufficiently close that it seems appropriate to use the linear relationship between K_1 and initial c-RNA concentration demonstrated for bacteriophage T4 DNA-c-RNA hybridization. Values of K_1^1 are shown in Table 3. K_1^1 is proportional to complexity and the proportionality is nearly linear, as shown by the ratio K_1^1/C . The 80-fold difference in complexity between bacteriophage λ DNA and *E. coli* DNA is associated with a 1.3-fold difference in the ratio, and the 20-fold difference between bacteriophage T4 DNA and *E. coli* DNA with a 1.9-fold difference. However, for a 4-fold difference in complexity between bacteriophage λ DNA and bacteriophage T4 DNA the difference in ratio is 2.5-fold. This rather large divergence may be related to the difference in G + C content between bacteriophage λ (49%) and bacteriophage T4 (34%) DNA. The annealing temperature used was optimum for the bacteriophage T4 system, and therefore probably slightly suboptimum for the bacteriophage λ system. The effect of high G + C content on the renaturation of DNA is to increase the rate of the reaction (Thrower & Peacocke, 1968; Wetmur & Davidson, 1968), rather than to decrease it as observed here.

On the basis of a linear relationship between K_1^1 and C the data of Table 3 may be treated as three separate estimates of $K_{11}^1 = K_1^1/C$, with an average of 73. To remove the concentration term, let $K_{11} = K_{11}^1/h$. Taking 10 $\mu\text{g./ml.}$ as an integral c-RNA concentration close to the mean of the three concentrations used, $K_{11} = 7.3$ for a c-RNA concentration of 10 $\mu\text{g./ml.}$ Hence $1/r = 7.3C/t + K_2$.

Table 3. *Effect of complexity on the time-course of hybridization*

The kinetic data are taken from Fig. 3.

Source of DNA	$10^{-8} \times$ Complexity of DNA (daltons) (<i>C</i>)	Approx. initial rate (proportion of DNA hybridized) (<i>R</i>)	<i>C</i> $\times$ <i>R</i>	Slope of reciprocal plot ($\pm$ S.E.M.) [$\mu\text{g. of DNA} \times \text{hr.} \times (\mu\text{g. of RNA}$ hybridized) $^{-1}$] (<i>K</i> ₁)	Ratio <i>K</i> ₁ / <i>C</i>
Bacteriophage λ	0.3	0.16	0.05	3.77 ± 0.48	101 ± 13
Bacteriophage T4	1.3	0.1	0.13	7.00 ± 0.37	41 ± 2
<i>E. coli</i>	25	0.005	0.13	157.4 ± 6.5	78 ± 3

Table 4. *Experimental rate constants*

The data are taken from Figs. 2 and 3. Reciprocal rate constants are calculated from the slopes of the reciprocal plots on the assumption that the instantaneous rate of hybridization at any time is directly proportional to the proportion of DNA remaining single-stranded at that time.

Source of DNA	Slope of concentration plot $\times$ time (<i>K</i> ₃ <i>t</i>) [$\mu\text{g. of DNA} \times \mu\text{g. of RNA} \times \text{hr.} \times$ ($\mu\text{g. of RNA hybridized}$) $^{-1}$]	Slope of time-plot $\times$ concentration (<i>K</i> ₁ <i>h</i>) [$\mu\text{g. of DNA} \times \mu\text{g. of RNA} \times \text{hr.} \times$ ($\mu\text{g. of RNA hybridized}$) $^{-1}$]
Bacteriophage λ	86.7 ± 17.8	30.2 ± 4.0
Bacteriophage T4	119.0 ± 11.4	53.9 ± 2.8
<i>E. coli</i>	758.4 ± 11.2	1951.8 ± 80.6

DISCUSSION

The linear relationships described here between the reciprocal of amount of hybridization and the reciprocal of time, and between the reciprocal of amount of hybridization and the reciprocal of the initial RNA concentration, are suggestive of a second-order dependence of hybridization on both time and RNA concentration. As pointed out above, in the experiments described here the c-RNA appears to be in considerable excess, so that its concentration will be little affected by the amount of RNA which becomes hybridized with DNA. Consequently a first-order dependence on c-RNA concentration is expected. A number of simple models of hybridization have been considered to see whether they generate the observed dependency on c-RNA concentration and time. Of these, only one produced the type of curve observed. The rate equation of this model is $dr/dt = k_2(r_s - r)^2h$, where k_2 is a second-order rate constant and the other symbols have the same meaning as before. Upon integration, with the condition $r = 0$ at $t = 0$, the equation $1/r = 1/k_2htr_s^2 + 1/r_s$ is obtained, and putting $1/k_2r_s^2 = K_1^1$ and $1/r_s = K_2$ we obtain the equation used here to describe the time-course experiments. However, if the rate equation is correct, the same integral should describe the concentration-dependence experiments. That is, $1/k_2r_s^2 = K_1^1 = K_1h = K_3t$. Table 4 shows a comparison of K_1h and K_3t for the three homologous hybridizing systems, from which

it is clear that K_1h does not equal K_3t . The reciprocal method remains a convenient way of analysing the time- or concentration-dependence of hybridization; but it is not based on any plausible kinetic model.

In these experiments certain factors are not completely under control, such as the extent of transcription during c-RNA synthesis, the quality of the membrane filters and the presence of self-complementary RNA. However, the three DNA templates were shown to be equally reactive with homologous c-RNA, strongly suggesting equality of transcription. Under our experimental conditions, the amount of hybridization appears to fall short of the degree of complementarity of the DNA and RNA by a factor of at least 2 (J. O. Bishop, F. W. Robertson & M. Melli, unpublished work). Thus the 40% hybridization of the three DNA templates observed here suggests at least 80% transcription of the template. Geiduschek, Snyder, Colvill & Sarnat (1966) have shown that in the presence of Mg^{2+} RNA polymerase from *E. coli* transcribes a restricted part (the early sequences) of T4 DNA. Our results (J. O. Bishop, F. W. Robertson & M. Melli, unpublished work) show that in the presence of Mn^{2+} RNA polymerase from *Micrococcus* transcribes both early and late sequences of T4 DNA with a high degree of symmetry.

The second uncontrolled factor in these experiments is the use of membrane filters. A comparison of the values for K_1h given in Tables 1 and 4 for bacteriophage T4 DNA-RNA hybridization shows

the sort of variation observed in experiments carried out some weeks apart. The differences in complexity (up to 80-fold) are, however, very much greater than these experimental variations.

The c-RNA preparations used here contained self-complementary RNA. Up to 40% ribonuclease-resistance was developed in each by self-annealing different concentrations, and this is the best estimate available of the withdrawal of self-complementary RNA. Thus 60% of the RNA is unaffected by RNA-RNA hybridization. The 40% that does self-anneal does so with a time-course very similar to that of RNA-DNA hybridization (J. O. Bishop, unpublished work), so that its effective concentration for hybridization is close to half its actual concentration. This indicates that the overall effective concentration of the c-RNA is about 80% of the actual concentration. If the ribonuclease-resistance developed during self-annealing grossly underestimates the amount of RNA-RNA hybridization, the effective concentration might be as low as 50% of the actual concentration.

The results presented permit the interpretation that the time-course of the hybridization reaction is approximately a linear function of the complexity of the DNA and RNA. Since the RNA is in excess, and the amount of DNA is kept constant, it is the complexity of the RNA alone that is under study. Increasing complexity decreases the rate of hybridization throughout the reaction. Like the results of DNA-renaturation studies, these results show that the rate of the reaction reflects the concentration of unique nucleotide sequences. With DNA renaturation, for each DNA the frequency of all sequences is equal. It is not clear whether the frequency of different sequences is the same in a c-RNA preparation. If they are different, the rate of the hybridization reaction will reflect the average concentration.

It is interesting to extrapolate the findings described here to hybridizing systems even more complex than that of *E. coli*. Mammalian DNA contains sequences that are reiterated and sequences (the majority) that appear to be unique (Britten & Kohne, 1966, 1968; Wetmur & Davidson, 1968). On the model of ascites-cell DNA, the complexity of the non-reiterated sequences of mammalian DNA is about 600 times greater than that of *E. coli* DNA (see Wetmur & Davidson, 1968). Taking the relationship $1/r = 7.3C/t + K_2$ and letting $K_2 = 3$, we obtain $1/r = 110000/t + 3$ for the non-reiterated fraction of mammalian DNA at a c-RNA concentration of $10 \mu\text{g./ml.}$, and this gives $t = 110000r/(1 - 3r)$. Thus at a c-RNA concentration of $10 \mu\text{g./}$

ml., which would correspond to an RNA concentration *in vivo* of 0.2–1 mg./ml., the hybridization of half of the hybridizable DNA sequences ($r = 0.15$) would take 30000 hr., or about $3\frac{1}{2}$ years. Although the grounds for the operation are less strong, it is probably true to say that even at a c-RNA concentration of 1 mg./ml. half-hybridization would require about 320 hr., or about 13 days.

Church & McCarthy (1968) observed rapid hybridization of mouse DNA with cellular RNA and suggested that this is due to imperfect base-pairing of the RNA and DNA. Melli & Bishop (1969) have shown directly that the non-reiterated fraction of rat liver DNA does not hybridize with up to $20 \mu\text{g.}$ of c-RNA/ml. Evidently Church & McCarthy were observing the hybridization of RNA with the reiterated DNA fraction, and their results agree well with those of Britten & Kohne (1966, 1968), who showed that imperfect base-pairing occurs in the renaturation of reiterated mammalian DNA.

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