Examination of an Equilibrium Interpretation of Deoxyribonucleic Acid–Ribonucleic Acid Hybridization Data

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1. When a constant amount of denatured DNA is annealed for a constant time with a series of different RNA concentrations, it is often observed that the reciprocal of the amount of RNA hybridized is linearly proportional to the reciprocal of the RNA concentration. This may be explained by assuming that an equilibrium is set up between free RNA and DNA on the one hand and DNA–RNA hybrid on the other. The hybridization of *Escherichia coli* DNA and ribosomal RNA was used to test this proposition. Rate constants were estimated from the initial rates of the forward and back reactions and compared with direct estimates of the dissociation constant. 2. The rate constants of the forward and back reactions were estimated to be $1.82 \times 10^3 \text{mol}^{-1} \text{l}^{-1} \text{s}^{-1}$ and $0.023 \text{h}^{-1}$ ($6.4 \times 10^{-4} \text{s}^{-1}$) respectively, giving a ratio $k_d/k_1 = 0.013 \mu g \text{ml}^{-1}$. After 24h annealing the dissociation constant was estimated to be $0.114 \mu g \text{ml}^{-1}$, and by extrapolation to infinite time, $0.047 \mu g \text{ml}^{-1}$. 3. It is concluded that (a) equilibrium greatly favours the hybrid complex, (b) equilibrium is not established in 24h, (c) the equilibria that were directly estimated are incompatible either with the measured rates of the forward and back reactions or with the simple formulation of the reaction that was adopted, and finally (d) for these reasons the equilibrium interpretation of the linear reciprocal relationship is unsatisfactory.

Although it is some years since DNA–RNA hybridization was first described (Hall & Spiegelman, 1961; Schildkraut, Marmur, Fresco & Doty, 1961) the basic kinetics of the reaction are not yet completely understood. Nygaard & Hall (1964) showed that the initial rate of the reaction is determined by the product of the RNA concentration and the DNA concentration, and this suggests that the reaction rate is proportional to the collision frequency of complementary RNA and DNA sequences. Later stages of the hybridization reaction, however, seem to be more complex (Bishop, 1969a).

An experimental procedure that is often used is to anneal a constant amount of DNA (usually immobilized on nitrocellulose membrane filters) with different amounts of RNA. The result is a 'saturation' curve: equal successive increments in the initial RNA concentration produce progressively smaller increments in the amount of DNA–RNA hybrid formed. It is regularly observed that the reciprocal of the amount of DNA–RNA hybrid formed in this type of experiment is linearly proportional to the reciprocal of the initial RNA concentration (Lavallé & de Hauwer, 1968; Bishop, 1969a; Bishop, Robertson, Burns & Melli, 1969).

Bishop et al. (1969) made a detailed study of the use of this reciprocal relationship in analysing DNA–RNA hybridization data.

There are two models of DNA–RNA hybridization that generate the linear reciprocal relationship. (1) The first proposes that the rate of formation of hybrid depends on the first power of the RNA concentration and the second power of the DNA concentration (Bishop, 1969a). This was shown not to be a sufficient explanation. (2) Under certain conditions, and assuming that the rate of hybrid formation is proportional to the product of the RNA and DNA concentrations, the reciprocal relationship is predicted if an equilibrium is established between free RNA and DNA–RNA hybrid (Lavallé & de Hauwer, 1968). This model is very attractive, but it has not yet been critically examined. The experiments described below were designed to test it. The results show that an equilibrium state may eventually be established, but that this is not a satisfactory explanation of the linear reciprocal relationship.

The test system was the hybridization of *Escherichia coli* rRNA with *E. coli* DNA. Fig. 1 shows that the test system exhibits the usual linear relationship between the reciprocal of the initial
RNA concentration and the reciprocal of the amount of RNA hybridized. This system offers the advantage that rRNA can readily be obtained in highly purified form. Thus the amount of complementary RNA added in each experiment may be accurately measured. Only a small proportion of E. coli DNA is able to hybridize with rRNA, but this proportion may be estimated by means of saturation experiments (Yankofsky & Spiegelman, 1962). The amount of RNA hybridized can be measured at the end of the reaction. From these values the dissociation constant and the rate constants of the forward and back reactions may be estimated.

MATERIALS AND METHODS

Ribonuclease A (three times crystallized), deoxyribonuclease (ribonuclease-free) and papain were obtained from Sigma (London) Chemical Co. Ltd., London S.W.5, U.K.; Sartorius nitrocellulose membrane filters (MF-50, 30 cm diam.) were from V. A. Howe Ltd., London W.11, U.K.; Sephadex SE-50 was from Pharmacia (G.B.) Ltd., London W.13, U.K.; [2-14C]uracil and [32P]P1, were from The Radiochemical Centre, Amersham, Bucks., U.K.

In all experiments the DNA was labelled with 14C and the rRNA with 32P. Both DNA and RNA were prepared from E. coli K12, strain M.R.E. 600.

DNA was labelled by growing cells in glucose-casamino acids medium containing 125 μCi of [14C]uracil and purified as described by Bishop (1969a).

rRNA was labelled by growing cells in a low-phosphate medium containing 2 mCi of [32P]P1/100 ml. The cells were washed and grown for two generations in unlabelled medium. The cells were disrupted at 0°C by grinding with 2 parts of weight of alumina, and extracted with 5 vol. of 0.1 M NaCl-10 mM MgCl2-10 mM-tris-HCl buffer, pH 7.5. Debris was removed by centrifugation for 10 min at 10000g, and the supernatant was treated for 2 min at 37°C with 25 μg of deoxyribonuclease/ml. The ribosomes were collected by centrifugation for 50 min at 45000 rev./min in the Spinco no. 50 rotor at 0°C, and dissolved in 0.1 M NaCl-10 mM-tris-HCl buffer, pH 7.5; 0.05 vol. of 10% sodium dodecyl sulphate was added and the solution was shaken with an equal volume of water-saturated phenol for 20 min at room temperature. The aqueous phase was re-extracted with phenol and again collected by centrifugation. RNA was precipitated by adding 2 vol. of ethanol and collected by centrifugation. The pellet was dissolved in 0.1 M NaCl-10 mM-tris-HCl buffer, pH 7.5, and adjusted to 1-2 mg/ml. Then 1.5 ml of this solution was layered on top of a 30 ml linear 5-20% (w/v) sucrose gradient and centrifuged for 20 h at 25000 rev./min and 0°C in the Spinco SW25 rotor. Fractions containing rRNA were pooled and the RNA was precipitated by adding 2 vol. of ethanol. The RNA was dissolved in 10 mM MgCl2-10 mM-tris-HCl buffer, pH 7.5, treated with deoxyribonuclease, extracted with phenol and passed through a column of Sephadex SE-50 as described by Bishop (1969a).

For DNA-RNA hybridization experiments, DNA was denatured, the solution adjusted to 6 x SSC* and the DNA loaded on membrane filters, as described by Gillespie & Spiegelman (1965). The membranes were annealed at 65°C with RNA dissolved in 2 x SSC. After annealing, the membranes were removed and immediately held for 5 min in 2 x SSC at 65°C to minimize non-specific hybridization. Each membrane was washed with 250 ml of 2 x SSC, treated for 20 min at 37°C with 10 ml of 2 x SSC containing 500 μg of ribonuclease, washed again with 250 ml of 2 x SSC and dried and its radioactivity was measured in a liquid-scintillation counter, set to exclude 14C counts from the 32P channel and with a 5% cross-count of 32P counts in the 14C channel. The amounts of DNA and RNA present were calculated from the specific radioactivities of the DNA and RNA. These were measured from the extinctions of the solutions at 260 nm (taking 20 as the extinction of a 1 mg/ml solution of DNA and 24 as that of a 1 mg/ml solution of rRNA) and from the radioactivities of samples dried on membrane filters. In all experiments blank (washed and dried) membranes were taken through all the procedures in parallel with the DNA-carrying membranes. The blank RNA values were deduced from the corresponding experimental values.

Experimental approach

The symbols used are defined as follows:

\[ H, \text{ RNA concentration (μg/ml); } \]
\[ h, \text{ initial RNA concentration (μg/ml); } \]
\[ D, \text{ initial amount of DNA present (μg/ml of solution); } \]
\[ R_0, \text{ initial amount of complementary DNA present (μg/ml of solution); } \]
\[ R, \text{ amount of DNA hybridized with RNA at any time (μg/ml of solution); } \]
\[ k_1, \text{ rate constant of the forward reaction (ml/μg·h⁻¹); } \]
\[ k_2, \text{ rate constant of the back reaction (h⁻¹); } \]
\[ K, \text{ dissociation constant (μg ml⁻¹); } \]

The equilibrium reaction, which predicts a linear reciprocal relationship between RNA concentration and formation of hybrid (see Lavallé & de Hauser, 1968), is defined as follows:

\[ [\text{RNA}][\text{DNA}] \xrightarrow{k_1} [\text{DNA–RNA hybrid}] \xrightarrow{k_2} [\text{RNA}] \]

At equilibrium, \( k_1 (h - R)(R_0 - R) = k_2 R \), and the dissociation constant, \( K = k_2/k_1 = (h - R)(R_0 - R)/R \). The special condition that generates the reciprocal relationship (Lavallé & de Hauser, 1968) is that the RNA is in substantial excess, so that \( h - R \approx h \). Then \( 1/R = (K/R_0) + 1/R_0 \). In the plot of \( 1/R \) against \( 1/h \) the intercept should equal \( 1/R_0 \) and the slope should equal \( K/R_0 \). The initial rate of the forward reaction is given by \( dR/dt = k_1 h R_0 \). For values of \( t \) approaching zero, \( k_1 = R/R_0 h \). In practice, \( k_1 \) was calculated from the initial slope of the plot of \( R/R_0 \) against \( t \), which is equal to \( k_1 \). The initial rate of the back reaction was measured from the loss of hybrid when preformed hybrid was incubated

* Abbreviation: SSC, 0.15 M NaCl-15 mM sodium citrate, pH 7.5 (standard saline citrate).
at 65°C. The initial rate is \( \frac{dH}{dt} = k_2 R_0 \), from which
\( k_2 = \frac{H}{R_0 t} \). Since \( H \) may be assumed to equal \( R_0 - R \)
under the conditions of the experiment, \( k_2 = (R_0 - R)/ R_0 t \). \( k_2 \) was calculated from the initial slope of the plot of \( (R_0 - R)/ R_0 \) against \( t \).

In all the experiments the DNA was immobilized on
membrane filters and the RNA was in solution. The
RNA concentration (\( \mu g/ml \)) is known, but the effective
concentration of the DNA is not known. However, in
the expressions that define \( K \) and \( k_1 \), membrane-bound
components (\( R_0 \), \( R_0 \) and \( R_0 - R \) appear above and below
the line. If we make the plausible assumption that the
effective concentrations of equivalent amounts of the two
membrane-bound components are the same, we can take
the measured rate constants to be correct in absolute
terms, even if arbitrary units are used in representing
DNA and hybrid. The rate constant of the back reaction
is, of course, concentration-independent.

RESULTS
Measurement of \( R_0 \). Three experiments are
available for analysis and these give satisfactory
agreement. In each case the value was obtained by
extrapolating either to infinite RNA concentra-
tion (Bishop et al. 1969) or to infinite time (Bishop,
1969a). The experiment shown in Fig. 1 gives a
value of 0.00362 \( \mu g/ml \). A time-course experiment
described by Bishop (1969b) with 8 \( \mu g \) of DNA/1ml
sample and 1 \( \mu g \) of rRNA/ml gives 0.00364, and
a further time-course experiment with 15 \( \mu g \) of
DNA/1ml sample and 5 \( \mu g \) of rRNA/ml gave
0.00363. The proportion is here taken to be the
mean of these determinations, 0.00363, and there-
fore \( R_0 = 0.00363 \times D \). This is in reasonable agree-
ment with published values (Gillespie & Spiegelman,
1965; Attardi, Huang & Kabat, 1965; Kennell,
1968).

Measurement of \( k_1 \). DNA-carrying and blank
membranes were heated to 65°C, rRNA at 65°C in
2 \( \times \) SSC was poured over them, and the membranes
were gently agitated. At intervals membranes were
withdrawn and placed in 2 \( \times \) SSC at 65°C for 5 min,
then chilled and finally washed at room tempera-
ture in the usual way. The results of three experi-
ments carried out at different RNA concentrations
are shown in Fig. 2. The three values of \( k_1 \) were
found to be 1.45, 2.32 and 1.70 \( ml/\mu g \times h \), with a
mean of 1.82 \( ml/\mu g \times h \).

Measurement of \( k_2 \). DNA-carrying and blank
membranes were incubated for 90 min with 16.7 \( \mu g \)
of rRNA/ml in 2 \( \times \) SSC at 65°C, then kept for
5 min in 2 \( \times \) SSC at 65°C, then chilled, and washed
by filtration at room temperature with 250 ml of
2 \( \times \) SSC/membrane. Each membrane was next
placed separately in 25 ml of 2 \( \times \) SSC at 65°C and
agitated gently. Membranes were removed at
intervals, treated with ribonuclease and again
washed with 250 ml of 2 \( \times \) SSC. The RNA and
DNA that remained were then measured in the
usual way. There was a progressive loss of DNA
from the membranes, but this was compensated for
by calculating \( (R_0 - R)/ R_0 \) for each membrane
on the basis of DNA recovery. With this method
of calculation it is assumed that neither hybridized
nor non-hybridized DNA is preferentially lost.
This assumption does not significantly affect the
value of \( k_2 \), however, because in the first 2 h of
incubation the loss of DNA was insignificant.

The results of three experiments are shown in
Fig. 3. The variation between different experiments
was rather large. The reason for this is not known,
but clearly when minute amounts of DNA–RNA
hybrid are incubated for rather long periods of
time even a very slight contamination by ribo-
nuclease might be important. The values of \( k_2 \)
estimated from Fig. 3 are 0.036, 0.021 and 0.012 \( h \)
with a mean of 0.023 \( h \) (6.4 \( 10^{-6} \) s).

To determine whether the release of RNA from
the hybrid involved inactivation of the DNA,
membranes that had been annealed with RNA and
dissociated for 20 h were reannealed with RNA.
The results of two experiments (Table 1) indicate

Fig. 1. Hybridization of rRNA with E. coli DNA. Por-
tions (1 ml) of RNA solution (0.02–0.67 \( \mu g/ml \)) were
annealed for 40 h at 65°C with two DNA-carrying
membranes and one blank membrane. The average amount
of DNA/membrane was 19.1 \( \mu g \). To obtain the amount
of RNA hybridized the values for the two parallel mem-
branes should be added together. Instead, the value for
each membrane was multiplied by two so that parallel
membranes could be shown separately. The specific
radioactivities of DNA and RNA were respectively 205
c.p.m./\( \mu g \) and 53100 c.p.m./\( \mu g \).
that the DNA is not inactivated completely. The findings do not exclude the possibility that some inactivation occurs.

**Measurement of K.** The direct measurement of \( K \) presents particular problems. When the initial RNA concentration is high, the 'equilibrium' value is indistinguishable from that for saturation of the complementary DNA sequences. When the initial RNA concentration is sufficiently low to fail to saturate the DNA, the reaction between RNA and DNA is very slow (Bishop, 1969b) and the attainment of equilibrium is less certain. Therefore, two questions were asked, which are primarily of practical interest. (1) Is the dissociation constant that may be calculated after an arbitrary annealing time (24h) similar to the ratio \( k_2/k_1 \)? (2) Is the dissociation constant that can be calculated after extrapolating the time-course of hybridization to infinite time similar to the ratio \( k_2/k_1 \)?

An experiment that is suitable for this analysis was described by Bishop (1969b). About 60 \( \mu \)g of \( E. coli \) DNA was annealed with (a) 1 \( \mu \)g and (b) 0.5 \( \mu \)g of rRNA/ml for different times up to 24h. The complementary fraction of the DNA was measured separately by using a greater excess of RNA and was found, in this case, to be 0.0030 (Bishop, 1969b). This low value is probably due to the occurrence of self-annealing while the membrane filters were being loaded with DNA. In this experiment loss of DNA from the filters was again
observed (8% in 24h). This was compensated for by normalizing the amount of hybrid observed at each time to the amount of DNA present at zero time. In this case the assumption that neither hybridized nor non-hybridized DNA is preferentially lost is important. The amounts of RNA hybridized after 24h were (a) 0.154 µg/57.5 µg of DNA and (b) 0.129 µg/57.5 µg of DNA. From $K = (h-R)/(R-R)/R$, $K = 0.104$ (a) and 0.124 (b), with a mean of 0.114 µg/ml.-1.

The hybridization at infinite time was estimated from the same results by plotting the reciprocal of the RNA hybridized/57.5 µg of DNA against the reciprocal of time (Fig. 4). The extrapolated values were (a) 0.164 µg/57.5 µg of DNA and (b) 0.149 µg/57.5 µg of DNA, giving $K = 0.041$ (a) and 0.054 (b), with a mean of 0.047 µg/ml.-1.

**Stability of the RNA.** Attardi et al. (1965) and Bonner, Kung & Bekhor (1967) have shown that rRNA is degraded, but not destroyed, by annealing overnight in 2xSSC. Nevertheless it is possible that the rate of hybridization is significantly affected. To test this point, rRNA at 2 µg/ml in 2xSSC was incubated for 24h at 65°C in the presence of blank washed membrane filters. The initial rate of hybridization of this RNA was then compared with that of an identical solution that had meanwhile been kept frozen. The result is shown in Fig. 2. The effect of preincubation was to increase slightly the rate of hybridization.

**DISCUSSION**

**Measured value of $k_1$.** The value of 1.82 ml µg⁻¹ h⁻¹ is equivalent to 160 l (mol of RNA nucleotide)⁻¹ s⁻¹. It is of interest to compare this with the value observed by Nygaard & Hall (1964) in their study of hybridization in solution between bacteriophage T2-specific RNA and bacteriophage T2 DNA. Their apparent value of $k_1$ was 10 l (mol of DNA nucleotide)⁻¹ s⁻¹. Correcting this to allow for the fact that only 30% of the DNA appeared to be reactive (Nygaard & Hall, 1964), $k_1 = 33.3$ l (mole of nucleotide)⁻¹ s⁻¹. This 30% of the T4 DNA corresponds to a total nucleotide-chain length of 23 x 10⁶ daltons. The value obtained in the present paper for rRNA is due to a total chain length of 10⁶ + 5 x 10⁵ = 1.5 x 10⁶ daltons. We expect the rate constant to be inversely proportional to the chain length (Wetmur & Davidson, 1968; Britten & Kohn, 1966; Bishop, 1969a). Normalizing to a length of 1.5 x 10⁶ daltons, we find for the bacteriophage T2 experiments $k_1 = 800$ 1 mol⁻¹ s⁻¹, i.e. five times greater than the value for rRNA. Some of the difference between these values may be due to differences in the salt concentration and in the lengths of the RNA molecules (Wetmur & Davidson, 1968). It is likely, however, that the main effect is due to the fact that the bacteriophage T2 experiments were carried out with both reactants dissolved directly in solution, whereas in the rRNA experiments the DNA was immobilized on membrane filters.

**Significance of $k_2$.** Several different mechanisms could explain the breakdown of DNA–RNA hybrid. One such mechanism is the separation of the hybrid complex into its two original components. Alternatively the breakdown might be the result of the destruction of either or both components. If the hybrid is separating into its two original components, an equilibrium must eventually be established. Under the conditions used for the equilibrium experiment, namely with a comparative excess of RNA, even if the breakdown of hybrid is due to the destruction of RNA, we would expect an apparent equilibrium condition to be reached, since $k_2$ is so much smaller than $k_1$. Again, if the breakdown is accompanied by the destruction or inactivation of the hybridized DNA, an apparent equilibrium would be found, as long as the bulk of the DNA is unaffected: since $R_0$ for each point of each experiment is calculated by multiplying the amount of DNA recovered by a constant factor, $R_0$ would be overestimated. In the above experiments the possibility that DNA inactivation

![Fig. 4. Extrapolation of the time-course of hybridization to infinite time. The experiment is taken from Bishop (1969b). The amount of DNA/sample at zero time was 57.5 µg. One DNA-carrying and one blank membrane were incubated with 1 ml of 2xSSC containing 1 µg (•) or 0.5 µg of rRNA/ml (○). The specific radioactivity of the DNA was 116 c.p.m./µg and of the RNA, 3380 c.p.m./µg.](image-url)
contributes to the breakdown of the hybrid has not been excluded.

Equilibrium analysis of hybridization. The value of $K$ obtained from the ratio $k_2/k_1$ is $0.023/1.82 = 0.013\mu\text{g ml}^{-1}$. By direct estimation, by using $K = (h-R)/(R_0-R)/R$, $K = 0.114\mu\text{g ml}^{-1}$ after 24 h of annealing and $K = 0.047\mu\text{g ml}^{-1}$ by extrapolation to infinite time. The direct estimates are thus respectively 9 times and about 4 times the ratio of the rate constants. The lack of agreement shown by the first direct estimate is not surprising, because even after 24 h of annealing the reaction is still in progress. This result emphasizes that the equilibrium interpretation is not applicable when annealing times of about 24 h are used.

The discrepancy between $k_2/k_1$ and the value of $K$ obtained by extrapolation is more difficult to understand. Three alternative explanations may be suggested. (1) The linear extrapolation may be insufficiently accurate. (2) The complementary DNA may be inactivated during the breakdown of hybrid. (3) The formulation of the reaction proposed by Lavallé & de Hauwer (1968) may not correctly describe the reaction when it is close to equilibrium. It is not yet possible to distinguish between these possibilities. However, it is important to emphasize that the results described in the present paper seriously weaken the equilibrium interpretation proposed by Lavallé & de Hauwer (1968), whatever the correct interpretation of the discrepancy. If the first interpretation is correct, it will mean that equilibrium is not reached even at annealing times greatly in excess of 24 h. If either the second or third explanation is correct, it will mean that the equilibrium condition cannot be analysed in the manner proposed.

The equilibrium interpretation was proposed as a means of explaining the linearity of the plot of $1/R$ against $1/h$ (Lavallé & de Hauwer, 1968). However, it is not a sufficient explanation. Linear reciprocal plots are observed in experiments terminated after short annealing periods, and where it is proved that equilibrium is not reached (Bishop et al. 1969). Again, under conditions that might be expected to approach equilibrium, straight-line reciprocal plots may be observed although the special conditions required by the equilibrium hypothesis are not fulfilled. Fig. 1 provides an example. Here $h$ varies from 0.02 to 0.66 $\mu\text{g ml}^{-1}$ so that the condition $(h-R) \sim h$ is never fulfilled, although it is approached at the highest RNA concentrations. Nevertheless, the fit of the straight line is very good. If we suppose that this is because the condition $(h-R) \sim h$ is approximated sufficiently, then the slope of the line should equal $K/R_0$. The slope of the line is 1.15 and $R_0 = 0.14$. Therefore $K = 0.16$, which differs from the ratio $k_2/k_1$ by the factor 12, and from the extrapolated value of $K$ by the factor 3. By this direct test the linear plot is not compatible with the equilibrium interpretation.

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