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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 $1.82 ml \mu g^{-1} h^{-1}$ ($1601 mol^{-1} s^{-1}$) and $0.023 h^{-1}$ ($6.4 \times 10^{-6} s^{-1}$) respectively, giving a ratio $k_2/k_1 = 0.013 \mu g ml^{-1}$. After 24h annealing the dissociation constant was estimated to be $0.114 \mu g ml^{-1}$, and by extrapolation to infinite time, $0.047 \mu g ml^{-1}$. 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, 1969*a*).

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, 1969*a*; 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, 1969*a*). 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.6, U.K.; Sartorius nitrocellulose membrane filters (MF-50, 30cm 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-^{14}C]uracil and [^{32}P]P_i were from The Radiochemical Centre, Amersham, Bucks., U.K.

In all experiments the DNA was labelled with ^{14}C and the rRNA with ^{32}P. 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 125μCi of [^{14}C]uracil/l and purified as described by Bishop (1969*a*).

rRNA was labelled by growing cells in a low-phosphate medium containing 2mCi of [^{32}P]P_i/100ml. The cells were washed and grown for two generations in unlabelled medium. The cells were disrupted at 0°C by grinding with 2 parts by weight of alumina, and extracted with 5 vol. of 0.1M-NaCl–10mM-$MgCl_2$–10mM-tris–HCl buffer, pH7.5. Debris was removed by centrifugation for 10min at 10000*g*, and the supernatant was treated for 2min at 37°C with 25μg of deoxyribonuclease/ml. The ribosomes were collected by centrifugation for 50min at 45000 rev./min in the Spinco no. 50 rotor at 0°C, and dissolved in 0.1M-NaCl–10mM-tris–HCl buffer, pH7.5; 0.05 vol. of 10% sodium dodecyl sulphate was added and the solution was shaken with an equal volume of water-saturated phenol for 20min 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 0.1M-NaCl–10mM-tris–HCl buffer, pH7.5, and adjusted to 1–2mg/ml. Then 1.5ml of this solution was layered on top of a 30ml linear 5–20% (w/v) sucrose gradient and centrifuged for 20h 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 10mM-$MgCl_2$–10mM-tris–HCl buffer, pH7.5, treated with deoxyribonuclease, extracted with phenol and passed through a column of Sephadex SE-50 as described by Bishop (1969*a*).

For DNA–RNA-hybridization experiments, DNA was denatured, the solution adjusted to 6×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×SSC. After annealing, the membranes were removed and immediately held for 5min in 2×SSC at 65°C to minimize non-specific hybridization. Each membrane was washed with 250ml of 2×SSC, treated for 20min at 37°C with 10ml of 2×SSC containing 500μg of ribonuclease, washed again with 250ml of 2×SSC and dried and its radioactivity was measured in a liquid-scintillation counter, set to exclude ^{14}C counts from the ^{32}P channel and with a 5% cross-count of ^{32}P counts in the ^{14}C 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 260nm (taking 20 as the extinction of a 1mg/ml solution of DNA and 24 as that of a 1mg/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 deducted from the corresponding experimental values.

Experimental approach

The symbols used are defined as follows:

H, RNA concentration (μg/ml);
h, initial RNA concentration (μg/ml);
D, initial amount of DNA present (μg/ml of solution);
R_s, initial amount of complementary DNA present (μg/ml of solution);
R, amount of DNA hybridized with RNA at any time (μg/ml of solution);
R_0, amount of DNA hybridized with RNA at zero time in the measurement of the back reaction (μg/ml of solution);
k_1, rate constant of the forward reaction (mlμg^{-1}h^{-1});
k_2, rate constant of the back reaction (h^{-1});
K, dissociation constant (μg ml^{-1}).

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

$$[\text{RNA}][\text{DNA}] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [\text{DNA–RNA hybrid}]$$

At equilibrium, $k_1 (h-R)(R_s-R) = k_2 R$, and the dissociation constant, $K = k_2/k_1 = (h-R)(R_s-R)/R$. The special condition that generates the reciprocal relationship (Lavallé & de Hauwer, 1968) is that the RNA is in substantial excess, so that $h-R \approx h$. Then $1/R = (K/hR_s) + (1/R_s)$. In the plot of $1/R$ against $1/h$ the intercept should equal $1/R_s$ and the slope should equal K/R_s.

The initial rate of the forward reaction is given by $dR/dt = k_1 h R_s$. For values of t approaching zero, $k_1 = R/R_s ht$. In practice, k_1 was calculated from the initial slope of the plot of R/R_s against t, which is equal to $k_1 h$. The initial rate of the back reaction was measured from the loss of hybrid when preformed hybrid was incubated

* Abbreviation: SSC, 0.15M-NaCl–15mM-sodium citrate, pH7.5 (standard saline citrate).

at 65°C. The initial rate is $dH/dt = k_2 R_0$, from which $k_2 = H/R_0 t$. 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 (μg/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, R_s and $R_s - R$) 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_s. Three experiments are available for analysis and these give satisfactory agreement. In each case the value was obtained by extrapolating either to infinite RNA concentration (Bishop *et al.* 1969) or to infinite time (Bishop, 1969*a*). The experiment shown in Fig. 1 gives a value of 0.00362 μg/ml. A time-course experiment described by Bishop (1969*b*) with 8 μg of DNA/1 ml sample and 1 μg of rRNA/ml gives 0.00364, and a further time-course experiment with 15 μg of DNA/1 ml sample and 5 μg of rRNA/ml gave 0.00363. The proportion is here taken to be the mean of these determinations, 0.00363, and therefore $R_s = 0.00363 \times D$. This is in reasonable agreement 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×SSC was poured over them, and the membranes were gently agitated. At intervals membranes were withdrawn and placed in 2×SSC at 65°C for 5 min, then chilled and finally washed at room temperature in the usual way. The results of three experiments 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 $\mu g^{-1} h^{-1}$, with a mean of 1.82 ml $\mu g^{-1} h^{-1}$.

Measurement of k_2. DNA-carrying and blank membranes were incubated for 90 min with 16.7 μg of rRNA/ml in 2×SSC at 65°C, then kept for 5 min in 2×SSC at 65°C, then chilled, and washed by filtration at room temperature with 250 ml of 2×SSC/membrane. Each membrane was next placed separately in 25 ml of 2×SSC at 65°C and agitated gently. Membranes were removed at intervals, treated with ribonuclease and again washed with 250 ml of 2×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.

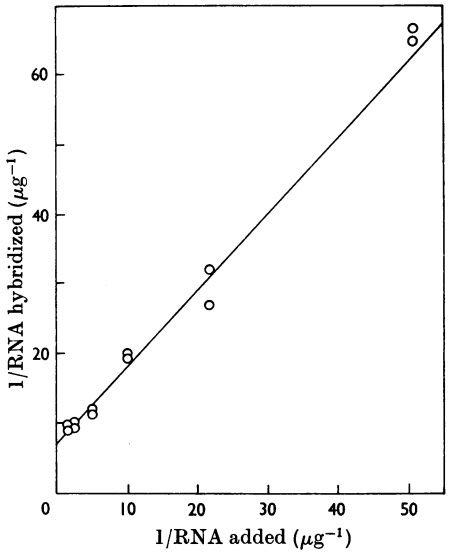

Fig. 1. Hybridization of rRNA with *E. coli* DNA. Portions (1 ml) of RNA solution (0.02–0.67 μg/ml) were annealed for 40 h at 65°C with two DNA-carrying membranes and one blank membrane. The average amount of DNA/membrane was 19.1 μg. To obtain the amount of RNA hybridized the values for the two parallel membranes 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./μg and 53100 c.p.m./μg.

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 ribonuclease might be important. The values of k_2 estimated from Fig. 3 are 0.036, 0.021 and 0.012 h^{-1} with a mean of 0.023 h^{-1} (6.4×10^{-6} s^{-1}).

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

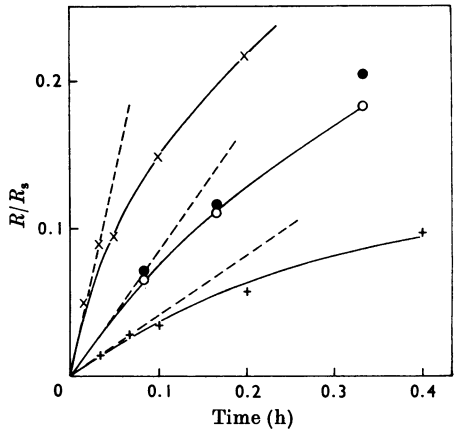

Fig. 2. Measurement of k_1. The ordinate shows the ratio of the amount of RNA hybridized to the saturation value, which is taken to be 0.00363 × the amount of DNA. Each point is the mean of two determinations. The experimental conditions were: Expt. 1, 7.4 μg of DNA/membrane, 1.18 μg of RNA/ml (×); Expt. 2, 7.8 μg of DNA/membrane, 0.238 μg of RNA/ml (+); Expt. 3, 3.6 μg of DNA/membrane, 0.5 μg of RNA/ml either untreated (○) or kept for 24 h at 65°C in 2 × SSC before the experiment (●). The specific radioactivity of the DNA was 205 c.p.m./μg and at the time the experiments were analysed that of the RNA was 64500, 53100 and 46000 c.p.m./μg in Expts. 1, 2 and 3 respectively.

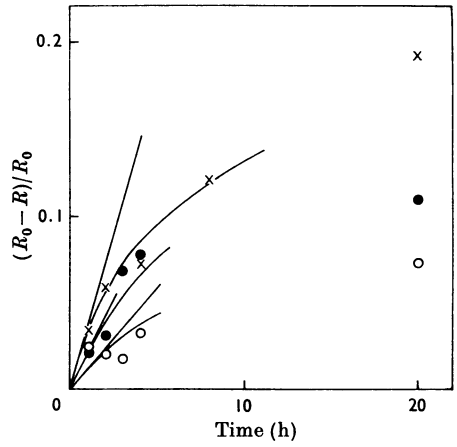

Fig. 3. Measurement of k_2. The ordinate shows the ratio [(RNA hybridized at zero time)−(RNA hybridized after incubation)]/(RNA hybridized at zero time). The values were normalized by calculating the RNA hybridized at zero time from the amount of DNA remaining on the membrane, by using the value found at $t = 0$. The amounts of DNA/membrane at the outset of the experiments were: about 24 μg, specific radioactivity 110 c.p.m./μg (×); 18 μg, specific radioactivity 55 c.p.m./μg (○); 24 μg, specific radioactivity 53 c.p.m./μg (●). The specific radioactivity of the RNA at the time of measurement was 67720 (×), 27200 (○) and 19400 (●) c.p.m./μg. Each point is the mean of either two or three parallel determinations.

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, 1969*b*) 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 (24 h) 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 (1969*b*). About 60 μg of *E. coli* DNA was annealed with (*a*) 1 μg and (*b*) 0.5 μg of rRNA/ml for different times up to 24 h. 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, 1969*b*). 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

Table 1. *Test for inactivation of complementary DNA during dissociation*

DNA-carrying and blank membranes were annealed for 90 min at 65°C with 16.7 μg of rRNA/ml in 2 × SSC, held for 5 min in 2 × SSC at 65°C and then washed with 2 × SSC. Some samples were then treated with ribonuclease and washed as usual (line 1). The remainder were held for 20 h at 65°C in 2 × SSC and some were treated with ribonuclease and washed (line 2). The remainder were reincubated with 16.7 μg of rRNA/ml for 90 or 180 min, then held for 5 min in 2 × SSC at 65°C, washed, treated with ribonuclease and again washed. Results are given as the means ±S.E.M. of three membranes.

Conditions	Hybrid found (μg of RNA/mg of DNA) Expt. 1	Expt. 2
Annealed for 90 min	3.38±0.03	3.20±0.06
Dissociated for 20 h	3.01±0.05	2.96*
Reannealed for 90 min	3.29±0.02	2.96±0.05
Reannealed for 180 min	—	3.13±0.05

* Mean of two membranes.

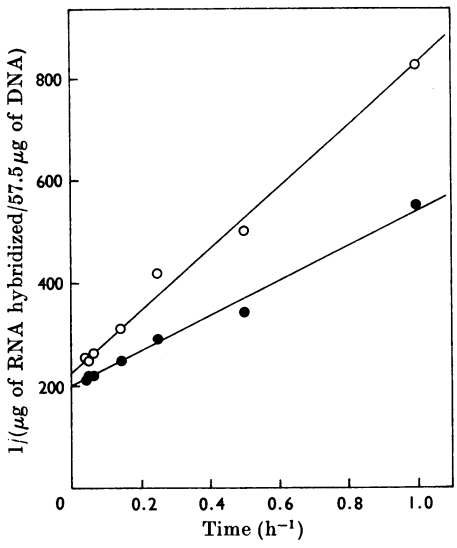

Fig. 4. Extrapolation of the time-course of hybridization to infinite time. The experiment is taken from Bishop (1969*b*). The amount of DNA/sample at zero time was 57.5 μg. One DNA-carrying and one blank membrane were incubated with 1 ml of 2×SSC containing 1 μg (●) or 0.5 μg of rRNA/ml (○). The specific radioactivity of the DNA was 114 c.p.m./μg and of the RNA, 3380 c.p.m./μg.

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 $K = (h-R)(R_s-R)/R$, $K = 0.104$ (*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 2×SSC. Nevertheless it is possible that the rate of hybridization is significantly affected. To test this point, rRNA at 2 μg/ml in 2×SSC 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 23×10^6 daltons. The value obtained in the present paper for rRNA is due to a total chain length of $10^6 + 5 \times 10^5 = 1.5 \times 10^6$ daltons. We expect the rate constant to be inversely proportional to the chain length (Wetmur & Davidson, 1968; Britten & Kohn, 1966; Bishop, 1969*a*). Normalizing to a length of 1.5×10^6 daltons, we find for the bacteriophage T2 experiments $k_1 = 800$ l 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

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 $0.023/1.82 = 0.013\,\mu g\,ml^{-1}$. By direct estimation, by using $K = (h-R)(R_s-R)/R$, $K = 0.114\,\mu g\,ml^{-1}$ after 24h of annealing and $K = 0.047\,\mu g\,ml^{-1}$ 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 24h of annealing the reaction is still in progress. This result emphasizes that the equilibrium interpretation is not applicable when annealing times of about 24h 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 24h. 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 $0.66\,\mu g/ml$ 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_s. The slope of the line is 1.15 and $R_s = 0.14$. 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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