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Kinetics of Ribonucleic Acid–Deoxyribonucleic Acid Membrane Filter Hybridization†

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ABSTRACT: The kinetics of hybridization of RNA to DNA immobilized on membrane filters were examined. It was found that hybridization binding curves could not be described in terms of a single forward and a single reverse rate constant for the formation and dissociation of the hybrid. Detection of a new form of hybrid provided additional evidence that the adsorption process was more complex. This new hybrid form, although stable to conditions which removed nonspecific hybrid, was more readily dissociated and more sensitive to RNase treatment than the final hybrid form. The amount of this less stable hybrid bound to the filters was inversely related to the amount of stable hybrid bound. Furthermore, the unstable hybrid could be converted into stable RNase-resistant hybrid under hybridization conditions. From these results we conclude that the unstable hybrid is a direct intermediate in hybrid formation and that both the formation and the conversion of the intermediate are rate determining in the kinetics of the overall reaction.

The kinetics and the mechanism of association of single strands of nucleic acid when both strands are in solution have been widely studied (Wetmur and Davidson, 1968; Craig *et al.*, 1971; Porshke and Eigen, 1971). While it has been found that the mechanism of association is complex, intermediates in the reaction do not accumulate, and thus the reaction appears to have only one kinetically significant step.

DNA–RNA hybridization on membrane filters have also been assumed to be closely approximated by the kinetics of a one-step adsorption reaction (Perry *et al.*, 1964; Mangiarotti *et al.*, 1968; Lavallé and De Hauwer, 1968; Kennell, 1968). However, this assumption has not been precisely tested, and the actual mechanism of strand association in membrane filter hybridization remains obscure.

For example, the annealing of RNA to membrane filter bound DNA is generally agreed to reach an apparent steady state after 24 hr (at 66° and 0.33 M sodium). However, Bishop (1970) has shown that the dissociation constant measured after 24 hr does not equal either the equilibrium constant calculated from the ratio of the reverse to the forward rate constant for the reaction or the equilibrium constant calculated by extrapolating the dissociation constant to infinite time. The simplest explanation for this discrepancy is that, after 24 hr, hybridizations have not reached equilibrium. An alternative explanation is that Bishop's rate constants are distorted by the presence of fast and slow reacting DNA sites. Still another alternative is that this discrepancy reflects a more complex kinetic mechanism for membrane filter hybridizations. To examine these alternatives, we investigated in detail the kinetics of membrane filter hybridizations.

For these investigations we used as a model system the hybridization of bacterial ribosomal RNA (rRNA) to total bacterial DNA. This system offers the advantages of natural polynucleotides with little internal redundancy of sequences (Fellner, 1971). Furthermore, the polynucleotides are easy to isolate in highly pure radioactive form. While there are three distinct ribosomal RNA species in our hybridizations (23S, 16S, and 5S), the RNA species and their DNA sites are present in the ratios of 1:1:1, and thus the hybridization can be considered in terms of a single RNA species (Avery and Midgely, 1969).

Our investigations showed that the kinetics of membrane filter hybridizations were not those expected of a one-step nonequilibrium adsorption process. We found no evidence of fast and slow reacting DNA sites which could explain our results. However, we detected a second, less stable form of DNA–RNA hybrid. The properties of this new form of hybrid suggest that it is a direct kinetic intermediate in the hybridization reaction.

Methods

DNA Isolation. All of the DNA used in these hybridizations was labeled with tritiated thymidine. Cultures of *Bacillus cereus* T were grown in YP medium (4 g/l. of Bacto-peptone (DIFCO), 0.5 g/l. of yeast extract (DIFCO)) containing 5 mCi/l. of [^{3}H]thymidine (New England Nuclear Corp., 6.7 Ci/mmol). DNA (final specific activity of 4.3×10^4 dpm/μg) was isolated from stationary phase cultures by a modification of the method of Marmur (1961) in which redistilled phenol saturated with 0.05 M Tris buffer, pH 7.5, was used for deproteinization.

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RNA Isolation. An overnight culture of *B. cereus* T grown in a low phosphate medium (Spiegelman, 1972) was used to inoculate low phosphate medium containing 120 mCi/l. of carrier free $^{32}PO_4$ (New England Nuclear Corp.). The culture was grown until an A_{600} of 0.45 on a Beckman DB spectrophotometer was reached, and then potassium phosphate, pH 7.0, was added to the culture to a final concentration of 0.1 M. After growth for one generation, the cells were harvested by centrifugation, resuspended in magnesium acetate buffer, pH 5.0, and disrupted by one pass through a French Pressure Cell (Aminco) at 20,000 psi. Ribosomes were obtained from the pellet of a differential centrifugation at 105,000*g* for 90 min. The RNA was purified by phenol extraction and MAK column chromatography (Spiegelman *et al.*, 1973[1]). Unlabeled RNA was obtained in a similar manner.

Hybridization Methods. The filter method of hybridization was used (Gillespie and Spiegelman, 1965). The hybridization buffer (f30SS) was based on that of Bonner *et al.* (1967) and contained 0.3 M NaCl–0.03 M sodium citrate, 30% (v/v) stabilized formamide (Fisher), and 0.4% (w/v) USP grade sodium dodecyl sulfate. Hybridizations were carried out in paraffin oil sealed vials containing 1 ml of f30SS. DNA was immobilized on Schleicher and Schuell type B6 membrane filters (Gillespie and Spiegelman, 1965) which were cut to 13 mm diameter circles containing approximately 0.5 μg of DNA. Retention of the DNA by the membrane filters was always greater than 98%. All of the hybridizations were carried out at 37° with vigorous shaking.

All RNA used in the hybridizations was degraded by heating as an aqueous solution in a boiling water bath for 5 min and then chilling in ice immediately before preparing the incubation mixtures. This treatment results in a population of RNA molecules with an average size of about 10^2 nucleotides as measured by electrophoresis on 2.5% polyacrylamide gels (Spiegelman, 1972; Spiegelman *et al.*[1]).

Both the total amount of RNA hybridized to the filter and hybrid which was resistant to RNase were measured. After incubation, the reaction was quenched by immersing the filters in 0.3 M NaCl, 0.03 M sodium citrate, pH 7.0 (2 × SSC[2]) at room temperature and then washing each filter once on each side with 25 ml of 2 × SSC under suction. Ribonuclease (RNase) resistant hybrid was determined by the method of Gillespie and Spiegelman (1965). An identical procedure was used to measure the total amount of hybrid, except that the RNase incubation was omitted.

The amount of hybrid on the filter was calculated from the ratio of [^{32}P]RNA to [^{3}H]DNA radioactivity. The filters were washed with 1 ml of distilled water to remove excess salt (Spiegelman, 1972; Spiegelman *et al.*[1]), dried, and dissolved in 1 ml of ethyl acetate. Ten milliliters of toluene scintillator containing 4 g/l. of 2,5-diphenyloxazole (Packard) and 0.05 g/l. of 1,4-bis[2-(5-phenyloxazolyl)]benzene (Packard) was added to each dissolved filter and the radioactivity measured with a Packard Tri-Carb scintillation counter. Parallel sets of filters without DNA were used as blanks to correct for nonspecific association of [^{32}P]RNA to the membrane itself.

Results

Evaluation of Hybridizations as a Nonequilibrium One-Step Reaction. If membrane filter hybridizations occur by a one-step reaction as do liquid associations, then they should be adequately described by the mechanism

$$\text{RNA} + \text{DNA} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{hybrid} \qquad (1)$$

If, as indicated by Bishop (1970), the reaction has not reached equilibrium, data relating the degree of hybridization to the input concentration of RNA should be described by the time-dependent form of a Langmuir adsorption curve (Laidler, 1965), using the measured values for the rate constants k_{on} and k_{off}. The rate constant for formation (k_{on}) was determined from the initial linear slope of the time course shown in Figure 1a, yielding a value of 0.142 ml/(μg hr). The rate constant for dissociation (k_{off}) was measured from the slope of the decay curve (Figure 1b) and equaled 0.0022 hr^{-1}.

In Figure 2 the theoretical hybridization binding curve based on the measured rate constants is compared in the form of a double reciprocal plot with data from several hybridization experiments carried out for 20 hr. The theoretical curve is

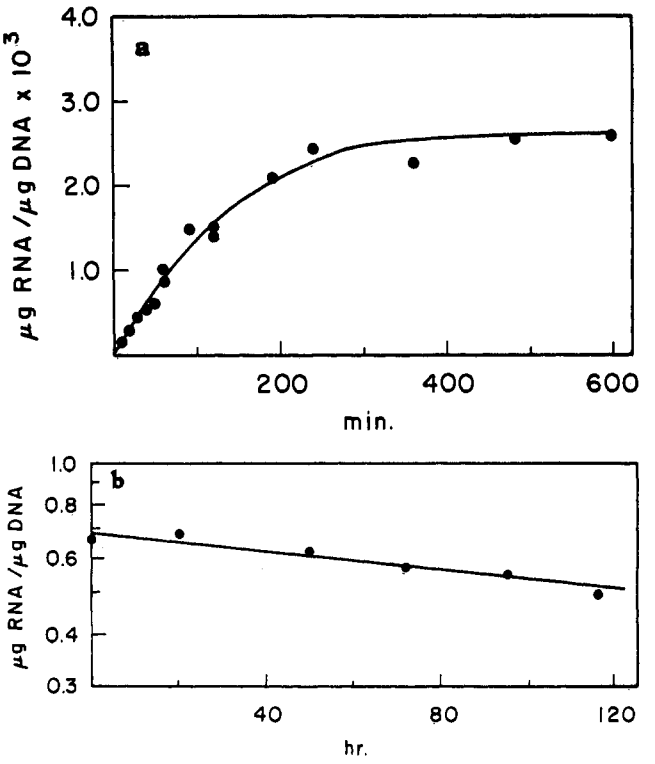

FIGURE 1: Measurement of the rates of formation and dissociation of the RNA–DNA hybrid. (a) Kinetics of formation of RNA–DNA hybrid on membrane filters. Labeled rRNA (2 μg/ml) was hybridized to ^{3}H labeled DNA in f30SS at 37°. At intervals, sets of filters were removed and the amount of RNase-resistant RNA bound was measured. The specific activity of the RNA was 6 × 10^6 cpm/μg. Each point of the graph represents the average of three DNA membranes. The initial rate of formation of the hybrid was used to measure the rate constant, k_{on}. (b) The dissociation of RNA hybridized to DNA filters. DNA filters were hybridized with 2 μg/ml of ^{32}P-labeled RNA in f30SS at 37°. After 20 hr two sets of six filters were used to measure the amount of RNase-resistant hybrid. The remaining filters were rinsed, blotted, and returned to hybridization buffer containing 10 μg/ml of unlabeled rRNA. At intervals, filters were removed from the second incubation and the amount of RNase-resistant hybrid remaining was measured. Each determination of the amount of RNA remaining bound to the filter is the average of four filters. The rate of dissociation, k_{off}, was determined from the slope of the dissociation curve.

[1] Spiegelman, G. B., *et al.* (1973), manuscript in preparation.

[2] Abbreviation used is: SSC, standard saline–citrate.

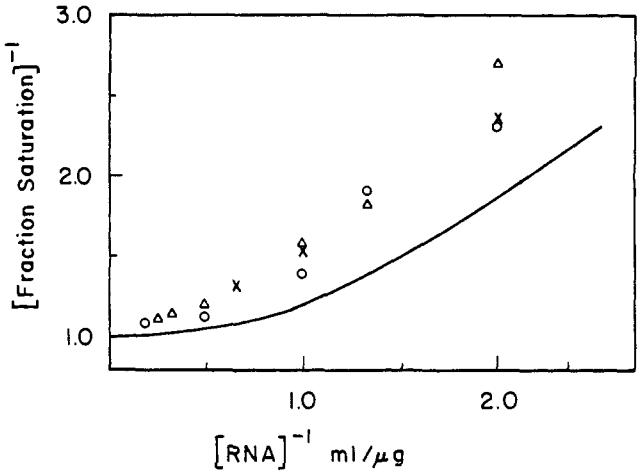

FIGURE 2: Comparison of saturation hybridization data with a theoretical nonequilibrium, one-step adsorption curve. Several saturation curves for the hybridization of rRNA to DNA were measured after 20 hr of incubation at 37° in f30SS. The data are presented in the form of a double reciprocal plot. The maximum saturation level was determined by extrapolation of the saturation curves; the extent of hybridization is expressed as a per cent of the maximum saturation level. A theoretical curve (——) was calculated from the nonequilibrium form of a one-step binding reaction as discussed in the text using the measured rate constants, k_{on} = 0.145 ml/(μg hr) and k_{off} = 0.0025 hr^{-1}.

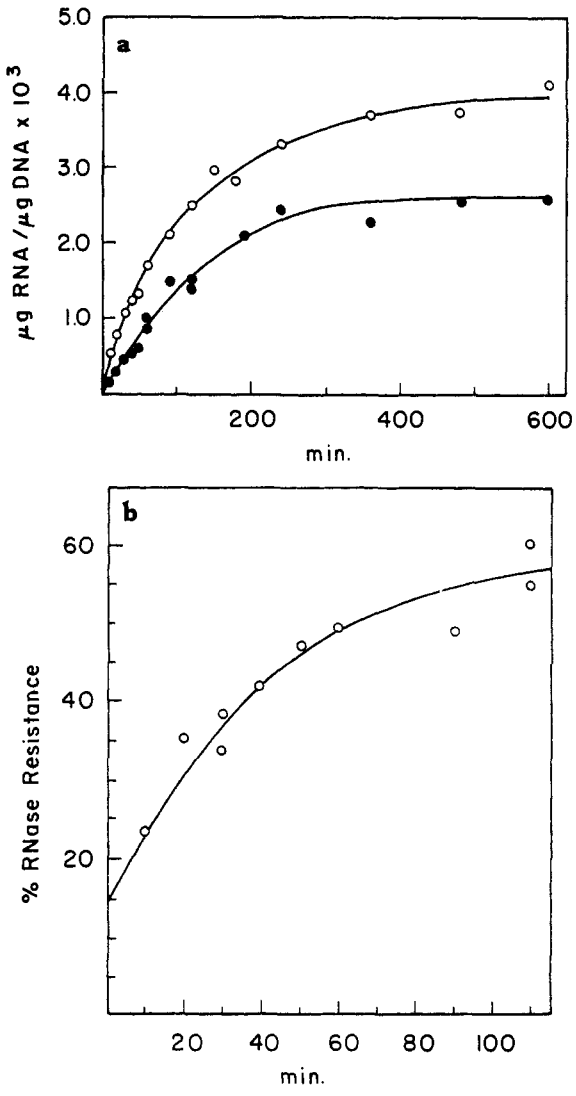

FIGURE 3: Rate of formation of hybrid in the presence and absence of RNase treatment. (a) Hybrid formation with 2 μg/ml of labeled RNA was measured in the presence (●) and absence (○) of RNase treatment, as described in Methods. (b) The degree of RNase resistance was calculated as the ratio of RNase-resistant hybrid to total hybrid formed, as measured in part a.

nonlinear, as expected for a nonequilibrium condition. While the data for the 20-hr saturation experiments are also nonlinear, the data points do not fall on the theoretical curve.

This lack of correspondence between the theoretical curve and the saturation data far exceeds experimental error (8%); a fit of the data could be obtained either by decreasing the value of k_{on} by a factor of 10 or by raising the value of k_{off} by a factor of 10. Since measurement of k_{off} did not appear to be subject to much experimental error, only a systematic error could cause an underestimation of k_{off}. Such an error, which would result in an abnormal stabilization of duplexes, seemed unlikely to us; thus, systematic errors in the measurement of k_{on} were sought.

One possible explanation for a systematic overestimation of k_{on} is that the RNase treatment used to eliminate regions of RNA not in heteroduplex (Gillespie and Spiegelman, 1965) might distort the early kinetics of the reaction. If, at the beginning of the hybridization reaction, a subfraction of the RNA binds more rapidly and with significantly higher RNase resistance (*i.e.*, more complete duplex formation), the initial slope determination of k_{on} would be too high. Such a situation could be detected by taking the ratio of RNase-resistant hybrid to total hybrid formed during a time course (Figure 3a). The total hybrid can be directly measured in the absence of RNase treatment by washing the membranes extensively with 2 × SSC, so that essentially all nonspecifically bound RNA is removed. Under the conditions used for these washings only molecules which are hybridized for approximately 15 or more continuous nucleotides are retained (Niyogi and Thomas, 1967; Niyogi, 1969). Thus, molecules which are retained meet requirements for specificity (McCarthy and Church, 1970).

As seen in Figure 3b, the RNase resistance of bound RNA has a low initial value which can be extrapolated to 15% at zero time. This resistance increases to approximately 65% as the hybridization proceeds. This final value is in agreement with other measurements of RNase resistance of hybrids formed on filters using rRNA (Gillespie and Spiegelman, 1965; Fry and Artman, 1969). Since the initial RNase resistance is low, it is extremely unlikely that the measured value of k_{on} reflects selective binding of high RNase resistant molecules.

Characterization of a Rapidly Dissociating Class of Specific Hybrids. A one-step binding mechanism for membrane filter hybridization predicts that the degree of RNase resistance will remain constant throughout hybridization. In the data presented in Figure 3, it does not. The predominance of bound RNA with low RNase resistance at the beginning of hybridization suggests that there may be a class of specifically bound RNA molecules which are hybridized for only a small portion of their length. These molecules, although stable to washing procedures which remove all nonspecifically associated RNA, should dissociate much more rapidly than molecules hybridized over a greater portion of their length.

A more rapidly dissociating class of specific hybrid duplexes

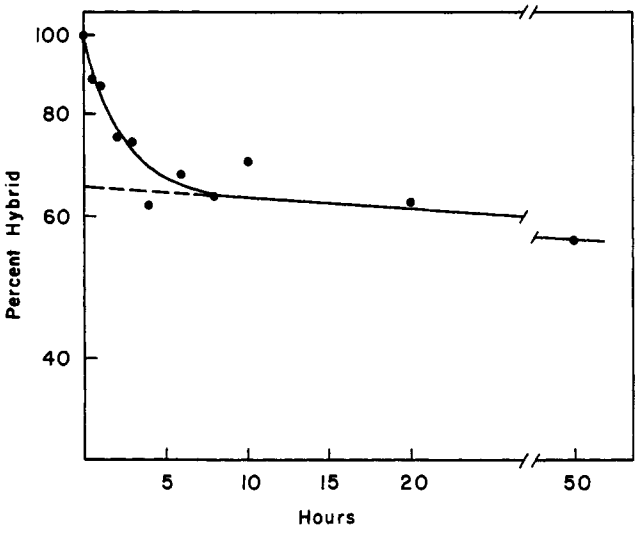

FIGURE 4: Dissociation of RNA–DNA hybrid measured without RNase treatment. ^{32}P-Labeled total rRNA (2 μg/ml) was hybridized to ^{3}H-labeled DNA for 2 hr in f30SS at 37°. The filters were then placed into f30SS buffer containing 5 μg/ml of unlabeled rRNA. At intervals, sets of six filters were removed and the amount of total hybrid remaining was measured. The data were normalized by setting the initial amount of hybrid equal to 100%. The dotted line is an extrapolation of the slow dissociation part of the curve.

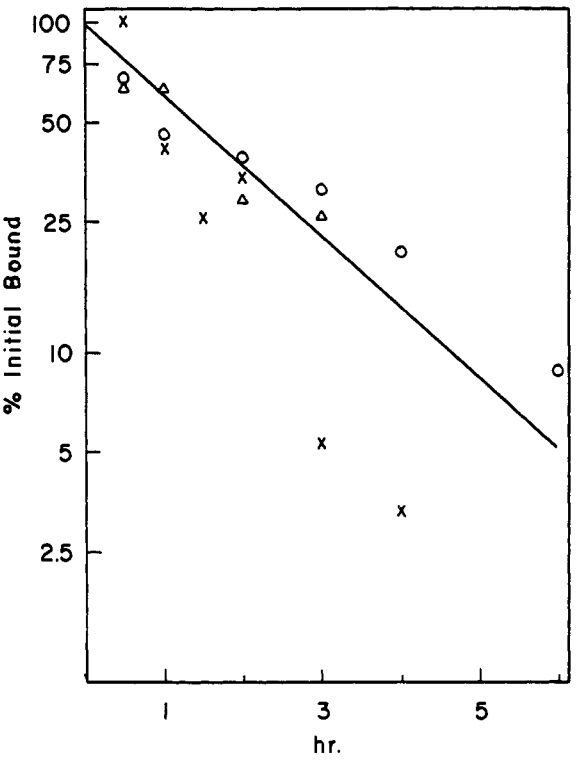

FIGURE 5: Rate of dissociation of the unstable hybrid. The rate of dissociation of the unstable hybrid was measured by subtracting the slow rate of decay (dotted line, Figure 4) from total the rate during the first 10 hr of the reaction. Experimental conditions are the same as in Figure 4. Three independent measurements were made (○, △, ×). Each set of data was normalized to 100% initially bound. The slope for the rapid dissociation is -0.49 hr^{-1}.

could be detected by a measurement of the dissociation of total bound RNA in the absence of RNase treatment. The time course of dissociation of total specific hybrid (Figure 4) is biphasic when plotted semilogarithmically, suggesting that there are at least two classes of dissociating RNA molecules. The rapid initial rate of decay can be resolved by subtracting the contribution of the slow rate seen after 20 hr (dotted line, Figure 4) from the total decay curve. Three determinations of the fast rate showed linear kinetics when plotted semilogarithmically (Figure 5). While the slopes of the fast decay curves were somewhat variable in several experiments, the half-life of the rapidly dissociating hybrid is approximately 1.5 hr.

If this class of rapidly dissociating hybrids were binding nonspecifically, or were a contaminant of the RNA preparation binding to nonribosomal cistrons, then the amount of this unstable hybrid would be independent of the saturation of the ribosomal RNA sites. The relation between the amount of unstable RNA and the saturation level was tested by measuring the amount of unstable hybrid as a function of RNA concentration after a 20-hr hybridization incubation. The amount of rapidly dissociating hybrid can be estimated by extrapolating the slow linear slope of the dissociation curve back to zero time (as in Figure 4). The amount of the fast component is then the difference between the total bound RNA at zero time and the point of extrapolation. In Figure 4 approximately 35% of the RNA bound is rapidly dissociating.

For each measurement, two sets of membrane filters were hybridized for 20 hr. One set of filters was used to measure the amount of total RNA bound. The second set of filters was washed free of unbound labeled RNA and placed in f30SS containing 10 μg/ml of unlabeled RNA for 20 hr. As seen above, after the second 20-hr incubation the amount of hybrid remaining on the filter represents approximately 95% of the stable RNA which had been formed. Correcting the amount of hybrid remaining on the second set of filters by 5% gives a measurement of the amount of stable hybrid present at the start of the 20-hr dissociation. Subtraction of the corrected stable hybrid from the total hybrid should yield a measurement of the amount of rapidly dissociating hybrid.

In Table I, the amount of rapidly dissociating hybrid is measured as a function of both RNA concentration and the amount of total hybrid bound. The amount of unstable hybrid decreased as the DNA was saturated with the stable form. Such data strongly indicate that the rapidly dissociating RNA is neither nonspecifically bound nor a contaminating RNA species, but rather competes for the same sites on the DNA as the ribosomal RNA species.

The rapidly dissociating hybrids which apparently occupy rRNA might be very short RNA pieces (12–20 nucleotides), which would be displaced by larger RNAs as the saturation level is approached. Such rapidly dissociating hybrids, composed of short RNA molecules, should have high RNase resistance since the greater part of each molecule would be in heteroduplex. We tested this possibility by measuring RNase activity during the first 20 hr of the dissociation reaction. Membrane filters were hybridized for 2 hr with 2 μg/ml of radioactive rRNA and then removed, rinsed, blotted, and placed into f30SS containing 10 μg/ml of unlabeled RNA at 37°. At intervals, parallel sets of filters were removed. One set was used to determine the total amount of radioactive RNA remaining (curve a, Figure 6) and the second set was used to measure the amount of RNase-resistant radioactive RNA bound (curve b, Figure 6).

As seen in Figure 6, two processes occur simultaneously in the second incubation. During the first 10 hr of the dissocia-

TABLE I: Stable and Unstable RNA–DNA Hybrid Formation after 20 Hr of Hybridization as a Function of RNA Concentration.[a]

	RNA Input (μg/ml)					
	0.10	0.25	0.50	0.75	1.50	3.00
Unstable hybrid formed (μg of RNA/μg of DNA × 10^3)	0.048	0.047	0.041	0.038	0.028	0.011
Stable hybrid formed (μg of RNA/μg of DNA × 10^3)	0.131	0.205	0.264	0.295	0.340	0.352

[a] Parallel sets of DNA filters were incubated at 37° for 20 hr in f30SS containing various concentrations of ^{32}P labeled rRNA. The amount of stable and unstable hybrid was measured by the procedure described in the text.

tion there is a 34% loss of total bound material (curve a). At the same time there is an increase of 31% in RNase-resistant hybrid (curve b). After 10 hr, both curves decrease at the rate previously found for the slow decay of total hybrid after 20 hr of dissociation (Figure 1b). Exactly analogous conversions were obtained in cases where the second incubation contained either 1 μg/ml or no unlabeled RNA. Since the increase in RNase-resistant hybrid takes place in the presence of a vast excess (10 μg/ml) of unlabeled RNA, the increase must represent further hybridization of RNA molecules already partially hybridized. Furthermore, the half-life of decay of total hybrid and the half-life of increase of RNase resistant hybrid are identical. Thus, it seems that the two processes are acting on the same pool of hybrids; that is, the unstable hybrid is the same species as that converted to a more RNase-resistant form. Finally, it should be noted that the increase in RNase resistance observed here is analogous to the change in RNase resistance in the overall hybridization reaction (Figure 3).

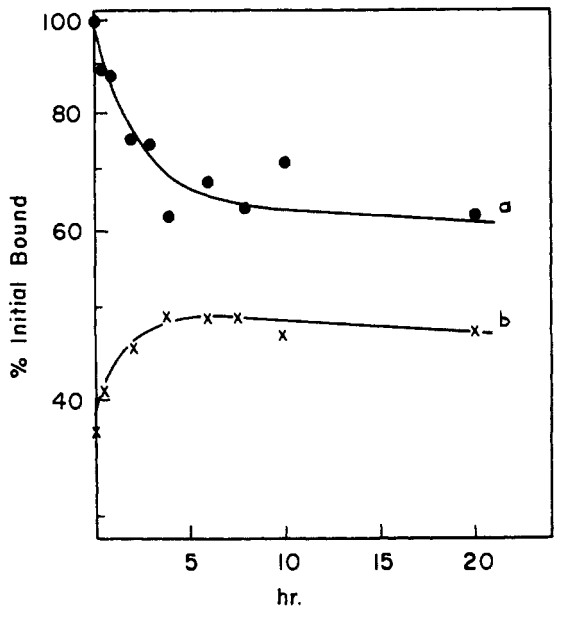

FIGURE 6: Chase experiment. DNA membranes and blank filters were hydridized for 2 hr with 2 μg/ml of radioactive rRNA in f30SS at 37°. The filters were then transferred to fresh f30SS containing 10 μg/ml of unlabeled rRNA. At intervals, parallel sets of filters were removed from the second incubation. One set was washed and then the total hybrid (without RNase treatment) remaining was measured (curve a). The second set was RNase treated and then the remaining RNase resistant hybrid determined (the chase curve, curve b). The data were normalized so that the total amount of hybrid on the non-RNase treated sample was 100% initially.

Evaluation of Membrane Filter Hybridization as a Two-Step Reaction. The data presented in Figures 3, 4, and 6 suggested that there exists a class of RNA–DNA hybrids which are specifically bound but only partially hybridized. This class of hybrids can either dissociate to free RNA or hybridize further, resulting in a more stable hybrid. The detection of this class of hybrid suggested that membrane filter hybridization might be kinetically a two-step reaction which can be described by the mechanism

$$\text{RNA} + \text{DNA} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{X} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \text{H} \qquad (2)$$

where the unstable class of hybrid is the intermediate stage in the reaction (X) and the stable class of hybrid is the final stage (H).

If membrane filter hybridization does involve a significant intermediate step, it should be possible to calculate the various rate constants of eq 2 from the dissociation and hybridization curves of Figure 6. These constants should generate a theoretical saturation curve which describes the measured saturation data. In addition, measurement of the formation of both the intermediate and the final stable hybrid form should show that the intermediate is found prior to the stable hybrid according to classical precursor–product relationships.

In considering the hybridization as a two-step process, it becomes necessary to reexamine the manner in which the amount of stable hybrid (H_{obsd}) was measured above. In a two-step mechanism it is no longer sufficient to determine the amount of stable hybrid by extrapolating the slope of the slow decay part of the dissociation curve, as was illustrated in Figure 4. The data in Figure 6 show that during the dissociation experiment some unstable hybrid continues to hybridize. Consequently, H_{obsd} as measured by extrapolation, is an overestimate of the amount of stable hybrid actually present at the start of the dissociation experiment (H_0). H_0 can be related to H_{obsd} by solving the kinetic equations for the decay of both stable and unstable hybrid (which depend on H_0) for the intercept used to measure H_{obsd}. The difference between H_{obsd} and H_0 depends on the values of the rate constants k_2, k_3, and k_4 of eq 2. For the constants determined by the curve fitting procedure below, H_{obsd} is nearly equal to $((k_2 + k_3)/k_2)H_0$. Since H_{obsd} is greater than H_0, the observed amount of unstable hybrid (X_{obsd}) will be less than the true amount of unstable hybrid (X_0). X_{obsd} will underestimate X_0 by the same amount as H_{obsd} exceeds H_0, since ($H_{obsd} + X_{obsd}$) must equal ($H_0 + X_0$).

Values for the rate constants k_2, k_3, and k_4 were determined by curve fitting, using the data in Figure 6. The appropriate kinetic equations describe the mechanism

TABLE II: First Approximation for Rate Constants k_2, k_3, and k_4.[a]

Assumed Relationship between k_2 and k_3	Calculated Constants (hr^{-1})		
	k_2	k_3	k_4
$k_2 = k_3$	0.24	0.24	0.01
$k_2 = 10k_3$	0.40	0.04	0.004
$k_2 = 0.1k_3$	0.04	0.40	0.06

[a] Values of k_2, k_3, and k_4 were calculated from rate equations for a two-step adsorption process by first assuming a relationship between k_2 and k_3. The sum of these three constants ($k_2 + k_3 + k_4$) is approximately equal to the rapid dissociation rate (0.50 hr^{-1}), while k_2k_4 is approximately equal to the slow dissociation rate (0.0025 hr^{-1}).

$$(H_0)_R \underset{k_3}{\overset{k_4}{\rightleftharpoons}} (X_0)_R \xrightarrow{k_2} RNA_{free} \qquad (3)$$

where $(H_0)_R$ and $(X_0)_R$ are the RNase-resistant fractions of stable and unstable hybrids, respectively. The equations used are similar to those for consecutive first-order reactions (Benson, 1960). In using the equations two alterations had to be made. First, since the data were measured in terms of H_{obsd} and not H_0, the appropriate expressions relating those two quantities were substituted into the equation. Secondly, factors had to be included to account for the use of RNase treatment. This was accomplished by assuming that each of the two hybrid forms had a constant RNase resistance. The RNase resistance of unstable hybrids was measured by extrapolating the curve in Figure 3b to zero time (15%) since it is assumed that the very first hybrid formed would be of the intermediate unstable class. The RNase resistance of the stable hybrid was measured by the long time values in Figure 3a (65%), since it was assumed that little unstable hybrid would be present after a long incubation.

By assuming different relationships between k_2 and k_3 (Table II), curves were generated and then compared to curve b, Figure 6, which had been normalized to an initial value of 1.0. As seen in Figure 7, the comparison shows clearly that k_2 must be nearly equal to k_3 and approximately equal to 0.25 hr^{-1}.

To measure the rate constant k_1, the rates of formation of both stable and unstable hybrids were measured. DNA membranes were hybridized with 2 μg/ml of radioactive RNA. At intervals two sets of filters were removed. One set was used to measure the total amount of hybrid bound ($H_{obsd} + X_{obsd}$ or $H_0 + X_0$) and the other set of filters was placed in a dissociation reaction (f30SS, 37°, 10 μg/ml of unlabeled rRNA, for 20 hr) to measure the amount of observed stable hybrid (H_{obsd}). The values of H_{obsd} and X_{obsd} obtained from the experiment were corrected to values H_0 and X_0 as described above. The value for k_1, taken from the initial slope of the curve for the formation of the unstable hybrid, is 0.3 ± 0.1 ml/(μg hr). The corrected time course data in Figure 8 confirm one prediction of the hypothesis of the existence of an intermediate hybrid in the reaction: the kinetics of the two forms do follow a precursor–product relationship.

Using the rate constants calculated for the two-step adsorption mechanism, theoretical saturation curves were generated. The kinetic equations used for these curves are similar to those published by Nossell and Ninham (1970) for multisite adsorption. These curves describe the reaction

$$RNA + DNA \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (X_0)_R \underset{k_4}{\overset{k_3}{\rightleftharpoons}} (H_0)_R \qquad (4)$$

where $(X_0)_R$ and $(H_0)_R$ are the RNase-resistant fractions of X_0 and H_0. Therefore, use of these equations requires inclusion of the RNase-resistance factors for the curve fitting procedure described above. A theoretical curve, based on the calculated rate constants, is presented in Figure 9. The fact that the saturation data are well described by two-step adsorption curves is further evidence for the complex hybridization mechanism.

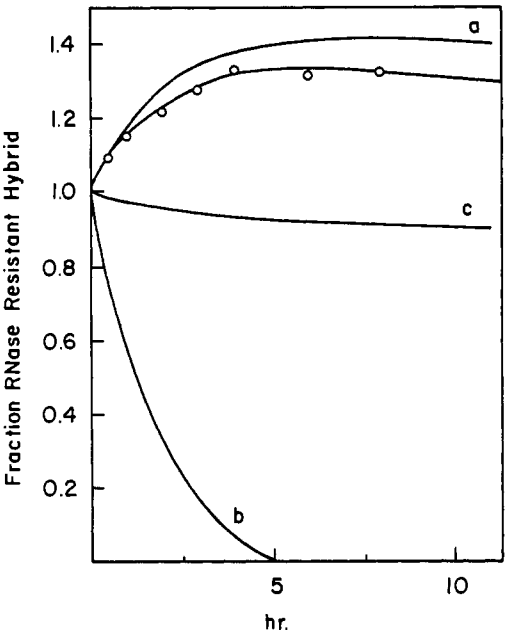

FIGURE 7: Comparison of theoretical and measured chase curves. Theoretical chase curves for a two-step reaction were calculated and compared with curve b in Figure 6 (O–O). In curve a, $k_2 = k_3$; in curve b, $k_2 = 0.1k_3$; and in curve c, $k_2 = 10k_3$. The values of the rate constants in each case are given in Table II. The RNase resistance of the intermediate form was taken from Figure 3 as 15% and the RNase resistance of the stable hybrid was taken at 65%. The initial amounts of H_0 and X_0 were calculated by extrapolation of the slow dissociation curve to give H_{obsd} and X_{obsd} and these values converted to H_0 and X_0, as described in the text.

Discussion

The measured kinetics of ribosomal RNA hybridization to DNA immobilized on membrane filter do not conform to the generally assumed theoretical one-step mechanism. After examining several possible sources of systematic error in the measurement of either the forward or reverse rate constant, we concluded that the hybridization process must be more complex, with more than one kinetically significant step. Several experiments produced data which suggested the existence of an intermediate hybrid formed during hybridization. This intermediate hybrid is stable to procedures which remove nonspecifically bound RNA (Niyogi and Thomas, 1967), but dissociates 200 times more rapidly than "normal" hybrid under hybridization conditions. Since the rate of dissociation of double stranded nucleic acids with a

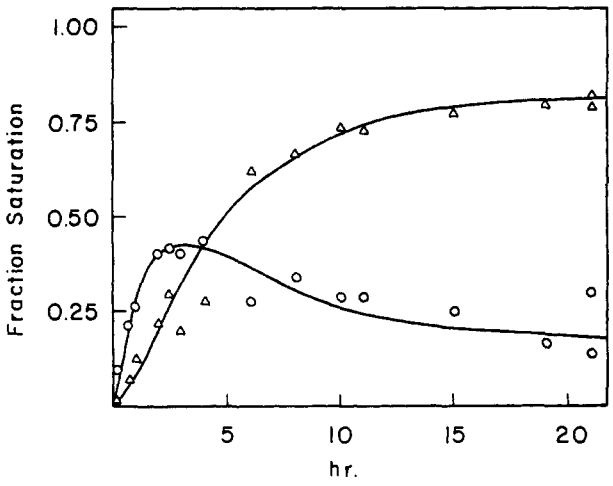

FIGURE 8: Relation of the appearance of the unstable and stable hybrids during RNA–DNA membrane filter hybridization. The kinetics of hybrid formation were measured with 2 μg/ml of labeled RNA in f30SS at 37°. At each time point, one set of filters was removed, washed, and counted to measure total RNA ($H_0 + X_0$) bound. A second set of filters was transferred to fresh hybridization buffer containing 10 μg/ml of unlabeled RNA for 20 hr. The amount of hybrid remaining on this second set of filters, corrected by 5% decay of the slowly dissociating hybrid during 20 hr, represents H_{obsd}. Applying the conversion equation of $H_0 \cong H_{obsd}[k_2/(k_2 + k_3)]$, the amounts of H_0 and X_0 were then determined for each point.

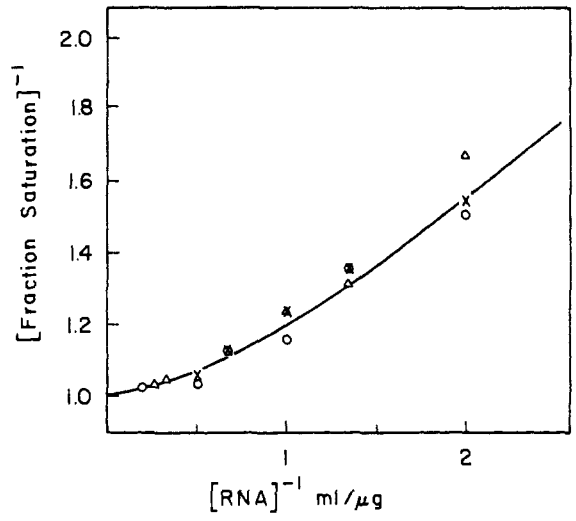

FIGURE 9: Comparison of measured saturation data and a theoretical two-step saturation curve. Saturation data are plotted as in Figure 2 in a double reciprocal form. The theoretical adsorption curve (——) based on two kinetically significant steps was calculated using the measured rate constants: $k_1 = k_2 = k_3 = 0.25$ hr^{-1} and $k_4 = 0.01$ hr^{-1}. As the saturation data were determined using RNase treatment, the theoretical curve was calculated by assuming the RNase resistance for the unstable hybrid (X_0) and for the stable hybrid (H_0) equaled 15 and 65%, respectively.

molecular weight of less than 10^5 is limited by the number of hydrogen bonds to be broken (Cohen and Crothers, 1971), the less stable form of hybrid is apparently bound to the DNA by fewer nucleotide pairs. This conclusion was supported by measurements of RNase resistance of hybrid formed as a function of time during hybridization. Rapidly dissociating hybrid accumulates early in the time course; the RNase resistance of this initially bound RNA is about 15%. The value for RNase resistance of hybrid measured after 20 hr of incubation is approximately 65%. The rapidly dissociating hybrid form apparently binds to the same DNA sites as the stable hybrid form, since the formation of increasing amounts of the stable hybrid is accompanied by a decrease in the amount of the intermediate form. In the early part of the hybridization reaction, the formation of the intermediate actually precedes the appearance of the stable hybrid and the time course of the level of rapidly dissociating hybrid is that observed for an intermediate formed in a two-step reaction. Finally, the most direct evidence for the existence of an intermediate during hybridization was found in a "chase" experiment (Figure 6), in which membrane filters containing a large proportion of rapidly dissociating RNA accumulate more RNase-resistant hybrid during further incubation under hybridization conditions.

In the course of characterizing the apparent intermediate hybrid form, a number of alternative interpretations were considered and tested. A simple alternative is that the apparent intermediate is an artifact derived from the heterogeneity of the RNA used in the hybridization. For example, variation in the sizes or the base composition of some of the fragmented ribosomal RNA used in these experiments could give rise to subclasses which exhibit "fast" or "slow" kinetics. Thus, a class of short oligonucleotides would be expected to dissociate more rapidly from a hybrid form, since the rate of dissociation of the hybrid is determined primarily by the number of hydrogen bonds to be broken (Cohen and Crothers, 1971). However, the minimum number of base pairs necessary to maintain hybrid stability would than represent a greater fraction of the lengths of the short oligonucleotides, so that shorter RNA fragments in hybrid form would be expected to have a very high RNase resistance. In fact, the more rapidly dissociating hybrid has a much lower RNase resistance. The hybrid formed initially also exhibits low RNase resistance. It also seems unlikely that differences in the base composition of various fragments could have a significant effect, since Lee and Wetmur (1972) have shown that the rate of formation of the ribonucleic acid duplex poly(A)–poly(U) differs less than 5% from the rate of hybrid formation from poly(G) and poly(C). Finally, alternatives based on heterogeneity effects of the RNA cannot account for the observed conversion of the intermediate hybrid to the sable hybrid form during subsequent hybridization in the presence of excess unlabeled RNA. We conclude, therefore, that the unstable hybrid formed in the hybridization of *B. cereus* T ribosomal RNA to membrane-bound DNA is a true intermediate on the pathway from free RNA and DNA to a stable RNA–DNA heteroduplex and that the unstable hybrids are bound by fewer nucleotides.

The results obtained with membrane filter hybridizations are different from nucleic acid strand associations measured in liquid. Studies on renaturation of DNA in solution (Wetmur and Davidson, 1968; Wetmur, 1970) and RNA–DNA duplex formation in solution (Pörschke, 1968; Craig *et al.*, 1971 Pörschke and Eigen, 1971) indicate there are several steps in these associations: initial collision (nucleation); stabilization of the initial collision site by reversible formation of a small number of base pairs adjacent to the initial collision site (formation of the helix nucleus); and subsequent helix formation by a very rapid, nearly irreversible, "zippering." In liquid hybridizations the conversion of the helix nucleus to a stable hybrid form is much more rapid than its dissociation to free RNA. Thus the overall association appears to

be essentially a one-step process. With the DNA immobilized on a membrane filter, however, an intermediate stage accumulates. In this case the conversion of the intermediate to stable hybrid occurs at rate comparable to its dissociation to free RNA.

The intermediate in the work reported here appears to be analogous to the more transient stage depicted as the rate limiting step in the liquid hybridization studies. In DNA–DNA renaturation experiments, the helix nucleus has been calculated to be between 10 and 20 base pairs (Wetmur and Davidson, 1968). The intermediate reported here, which was found to have an RNase resistance of 15%, is probably bound for 10–20 nucleotides, calculating from the average nucleotide number of the RNA polymers used in these experiments (10^2). This value corresponds well to the minimum length needed both for stability of the interaction under hybridization conditions (Niyogi and Thomas, 1967; Niyogi, 1969) and for specificity of interaction (McCarthy and Church, 1970) as well as the estimated size of the stabilized helix nucleus described by Wetmur and Davidson (1968).

The rate of formation of the intermediate in membrane filter hybridizations is virtually identical with the rate of formation of the helix nucleus in the studies of RNA–DNA duplex formation in liquid. Nygaard and Hall (1964) measured the overall rate constant for liquid hybridization of T2 RNA to T2 DNA in 2 × SSC at 60° as 20 l./mol of nucleotide per second. The best fit values for k_1 in the present study (0.2–0.4 ml/(μg hr)) are 18–36 l./mol of nucleotide per second. The conditions used in these experiments (f30SS buffer at 37°) are equivalent (Bonner *et al.*, 1967; McConaughy *et al.*, 1969). The two rate constants may be compared directly, as correction factors to account for differences in the size of the RNA and genetic complexity of the DNA give nearly equivalent values (Wetmur and Davisdon, 1968; Bishop, 1969; Wetmur, 1970). Thus, the rates of formation of the intermediate or the helix nucleus are different by no more than a factor of 2. The similarity of these rate constants supports the idea that the formation of the intermediate in membrane filter hybridizations is a step analogous to the formation of the helix nucleus.

The major difference between liquid and membrane filter hybridizations therefore appears to rest with the rate of conversion of an intermediate form to the final stable hybrid form. The rate constant for the addition of a single base pair at the end of a growing helix, k_f^0, has been estimated to be about 10^5 sec^{-1} (Craig *et al.*, 1971) in liquid under moderate salt and temperature conditions. If the conversion of the intermediate to the final hybrid form is only the process of "zippering" approximately 50 nucleotides, k_3 (0.25 hr^{-1}) would give an estimate of k_f^0 of less than 10^{-2} sec^{-1}, a factor of seven orders of magnitude of difference. Such a discrepancy cannot be attributed to the slight difference in viscosity of the two hybridization buffers or the length of the strands annealed. In conjunction with the difference in the zippering rates, it should be noted that Nygaard and Hall (1964) found no change in the RNase resistance of hybrid formed in solution or during the dissociation of the hybrid.

We believe that there are two parameters which might account for the departure of membrane filter hybridizations from single step kinetics. First, the hybridization reaction may be sensitive to the RNA species used. If the RNA molecules contain a significant degree of secondary structure, it may be necessary to break intramolecular hydrogen bonds within the RNA chains before an RNA–DNA heteroduplex can be completed. If a partially hybridized RNA chain still contained an internal duplex region of about ten base pairs, continued zippering would first require denaturation of this region.

Ribosomal RNA used in these studies and in those of Bishop (1970) has been shown to contain 50–70% base pairing (Gould and Simpkins, 1969; Gratzer and Richards, 1971), with an average of four–ten base pairs in individual loops (Gould and Simpkins, 1971). An estimate of the rate of denaturation of such a region may be found from k_2, the dissociation of an eqivalent number of base pairs forming an RNA–DNA heteroduplex. If this were the rate limiting step in further zippering of a partially hybridized RNA chain, then the rate constant for the zippering step, k_3, would be equal to k_2 as was observed. In the study of RNA–DNA hybridization in solution (Nygaard and Hall, 1964) the RNA used was isolated from *Escherichia coli* infected with the phage T2. The degree of intramolecular base pairing of this RNA is not known. However, the secondary structure of several messenger-like RNAs from RNA bacteriophages has been shown to be extensive (Steitz, 1969; Adams *et al.*, 1969).

An equally plausible explanation for the accumulation of a partially hybridized RNA is that the DNA, when immobilized on a membrane filter, may be restricted so that the annealing process is significantly slowed. The decrease in the rate of hybridization when the membrane filter technique is employed has been noted in several cases. For example, Kennell (1971) noted that, in the hybridization of both *E. coli* RNA and T4 RNA to their homologous DNAs, the association constant for the reaction was higher when both strands were free in solution than when the DNA were immobilized on a filter. A similar observation has been made for DNA–DNA hybridizations (McCarthy and McConaughy, 1968). Thus, it appears that the immobilization of the DNA on a membrane filter may alter the kinetics of hybridization.

The manner in which denatured DNA is fixed to nitrocellulose membrane filters is not well understood. There is evidence that the denatured DNA forms aggregates which are then trapped by the membranes (Gillespie, 1968; Phillips, 1968). However, it is not clear whether the strands are fixed only at a few points or whether significant portions of each molecule are held in fixed positions. It seems likely that the DNA strands are entangled forming loops and coils, and that this entanglement may restrict free diffusion of strands. In such a state, the rate of formation of significant lengths of heteroduplex might be limited in part by the rate of reorientation of the DNA strands or by the rate of breakage of bonds between the DNA and the membrane. Partial hybrids equivalent to the unstable type reported here would be expected to accumulate if the rate of nucleation were equal to the rate of DNA strand release. Since the partial hybrids complex only a small length of DNA, their rate of formation would probably not be slowed by the immobilization of the DNA strands.

If membrane filter hybridizations do involve a necessary release of the DNA from its attached state, then one would predict that extensive hybrid formation should cause loss of DNA from the membrane filter. Such loss has been noted in the saturation of homologous DNA with RNA made *in vitro* using T4 (Melli and Bishop, 1969) and SV40 (Hass *et al.*, 1972) DNA templates. In the latter case, as the saturation level increased above 80%, DNA complexed with RNA was released from the membrane. At such high saturation levels, some of the strands which are only fragments of the total genome should be totally heteroduplex. If the formation

of heteroduplex with a region of DNA requires detachment of that region from the filter, completely hybridized strands should be released. At lower saturation levels, most DNA strands will have long regions not in the heteroduplex and thus will remain attached to the filter (Hass *et al.*, 1972). Thus, in the case of hybridization of rRNA, which is homologous to only about 0.4% of the genome, loss of hybridized DNA would not be expected. Nevertheless, the observations that the immobilization of DNA on the filter is altered by heteroduplex formation suggests that, conversely, filter hybridization might be significantly affected by the association of DNA strands with the membrane. We consider these facts to be strong supporting evidence that the accumulation of an intermediate of hybridization arises from constraint of the DNA strands on membrane filters.

From the experiments reported here it is clear that neither an equilibrium (Bishop, 1970) nor a nonequilibrium equation for RNA–DNA hybridization in terms of a single kinetically significant step can describe the hybridization of bacterial ribosomal RNA to DNA immobilized on a membrane filter. Hybridization in this system leads to the accumulation of partially hybridized forms which exhibit the classical properties of an intermediate in a reaction in which at least two steps are kinetically important. Whether the extreme reduction in the rate of annealing of the rest of a partially hybridized RNA is due wholly or in part to the secondary structure of the RNA or to the constraints of immobilizing DNA on the membrane awaits further investigation.

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