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Kinetics of Ribonucleic Acid–Deoxyribonucleic Acid Membrane Filter Hybridization†

George B. Spiegelman,‡ James E. Haber, and Harlyn O. Halvorson*

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 de-

† From the Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, Massachusetts 02154. *Received March 30, 1972.* Supported by a grant from the U. S. Public Health Service (GM-18904-01) and by funds from the Rosenstiel Basic Medical Sciences Research Center.

‡ National Institutes of Health Predoctoral Trainee. Present address: Department of Microbiology, University of Washington, Seattle

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

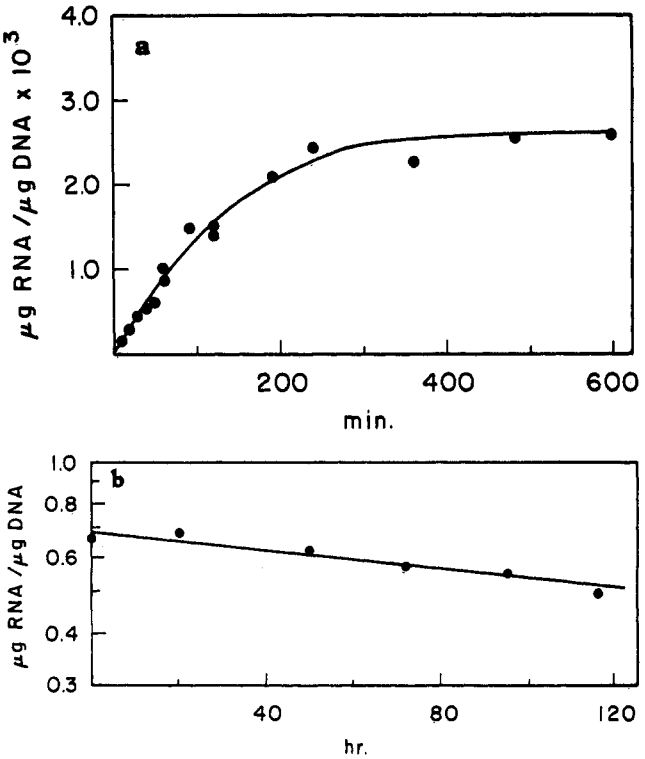

FIGURE 1: Measurement of the rates of formation and dissociation of the RNA–DNA hybrid. (a) Kinetics of formation of RNA–DNA hybrid on membrane filters. Labeled rRNA (2 μg/ml) was hybridized to ^{3}H labeled DNA in f30SS at 37°. At intervals, sets of filters were removed and the amount of RNase-resistant 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.

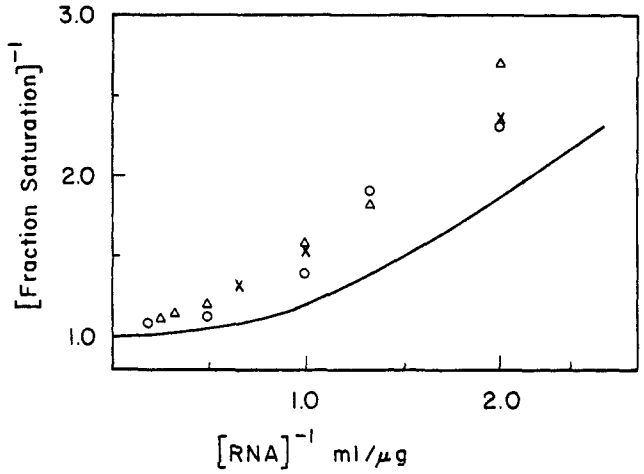

FIGURE 2: Comparison of saturation hybridization data with a theoretical nonequilibrium, one-step adsorption curve. Several saturation curves for the hybridization of rRNA to DNA were measured after 20 hr of incubation at 37° in f30SS. The data are presented in the form of a double reciprocal plot. The 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}.

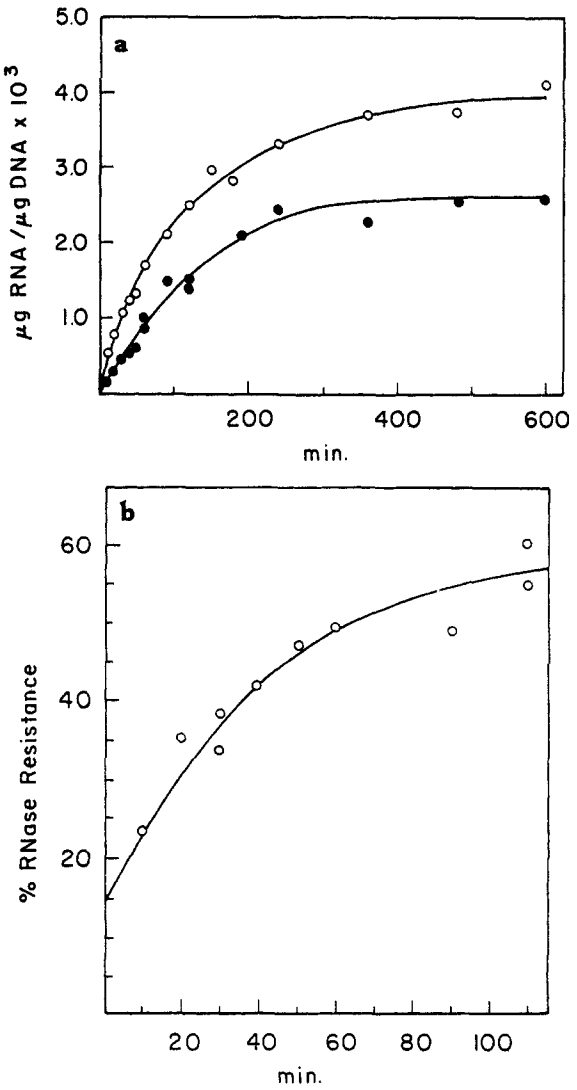

FIGURE 3: Rate of formation of hybrid in the presence and absence of RNase treatment. (a) Hybrid formation with 2 μg/ml of labeled RNA was measured in the presence (●) and absence (○) of RNase treatment, as described in Methods. (b) The degree of RNase resistance was calculated as the ratio of RNase-resistant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

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.

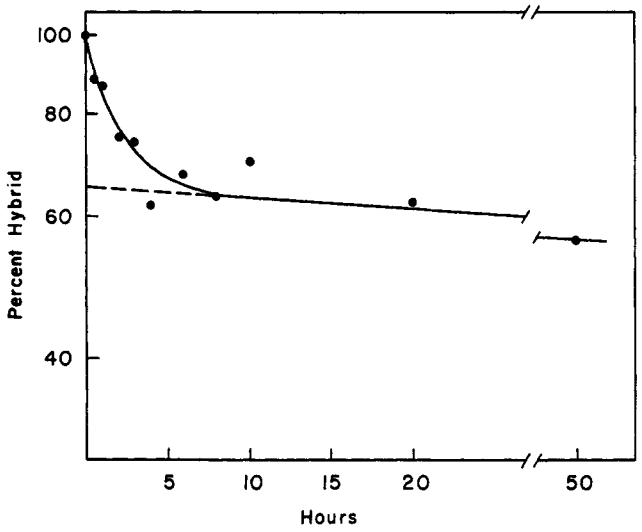

FIGURE 4: Dissociation of RNA–DNA hybrid measured without RNase treatment. ^{32}P-Labeled total rRNA (2 μg/ml) was hybridized to ^{3}H-labeled DNA for 2 hr in f30SS at 37°. The filters were then placed into f30SS buffer containing 5 μg/ml of unlabeled rRNA. At intervals, sets of six filters were removed 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.

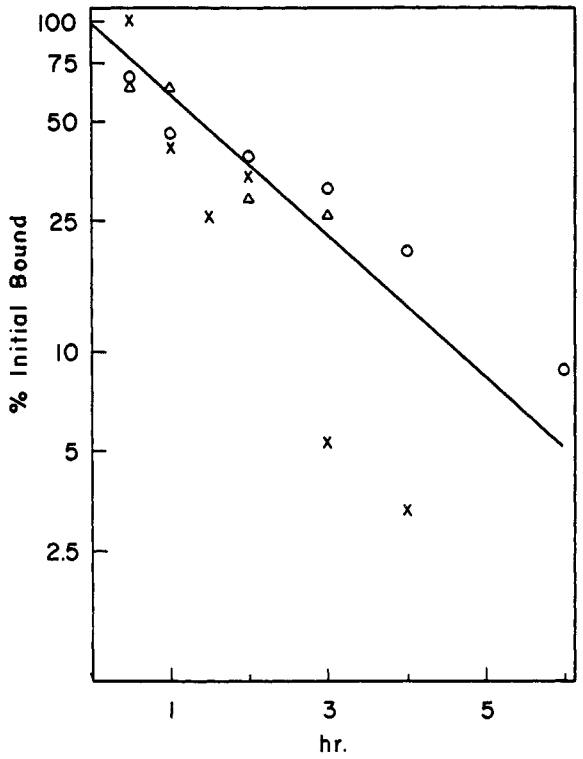

FIGURE 5: Rate of dissociation of the unstable hybrid. The rate of dissociation of the unstable hybrid was measured by subtracting the slow rate of decay (dotted line, Figure 4) from total the rate during the first 10 hr of the reaction. Experimental conditions are the same as in Figure 4. Three independent 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 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

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).

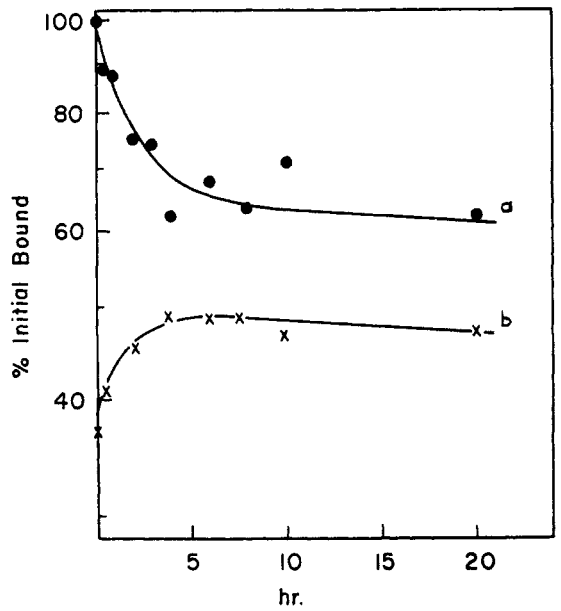

FIGURE 6: Chase experiment. DNA membranes and blank filters were hydridized for 2 hr with 2 μg/ml of radioactive rRNA in f30SS at 37°. The filters were then transferred to fresh f30SS containing 10 μg/ml of unlabeled rRNA. At intervals, parallel sets of filters were removed from the second incubation. 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

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

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