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Rates of formation and thermal stabilities of RNA:DNA and DNA:DNA duplexes at high concentrations of formamide†

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ABSTRACT

The thermal stabilities of RNA:DNA hybrids are substantially greater than those of DNA:DNA duplexes in aqueous electrolyte solutions containing high concentrations of formamide. Association rates to form DNA:DNA duplexes and DNA:RNA hybrids have been measured in these solvents. There is a temperature range in which DNA:DNA rates are negligible and RNA:DNA rates close to optimal.

INTRODUCTION

Aqueous formamide is a useful solvent for studies of the rates of RNA:DNA and DNA:DNA association reactions because the reactions then proceed at a much lower temperature than in pure aqueous solvents (1,2). It has been shown that the T_m for the thermal denaturation of DNA decreases linearly with increasing formamide concentration (2); however there is much less data as to the effects of formamide on the stability of RNA:DNA hybrids.

There is a broad maximum in the temperature dependence of the rate of association of complementary DNA strands, with a center about 25° below the midpoint, $T_{1/2d}$, of the thermal melting transition (3). (We follow Hamaguchi and Geiduschek (4) and denote by $T_{1/2d}$ the midpoint of the thermal melting curve of a duplex polynucleotide measured at ambient temperature (optical assay) and by $T_{1/2i}$ the midpoint of the irreversible (HAP* or ribonuclease assay) melting curve.) In the absence of further information, $T_{1/2d}$-25 is commonly chosen as the optimal temperature for an RNA:DNA reaction. However, Birnstiel et al., (5) have measured the formation rate and thermal dissociation temperature of RNA:DNA hybrids in 50% formamide solvents with the DNA bound to a membrane filter. These quantities were compared to the corresponding rates and thermal dissociation temperatures of DNA:DNA duplexes in solution, as measured by optical absorbance. Their data support the view that RNA:DNA hybrids are stabilized relative to DNA:DNA duplexes by formamide and that the optimum temperature for reassociation is greater than $T_{1/2}$-25°. Schmeckpeper and Kirby (6) have presented evidence that the optimum temperature for RNA:DNA

hybridization decreases only slightly as the concentration of formamide is increased beyond 40% thus suggesting that at high concentrations of formamide, RNA:DNA hybridization will proceed at temperatures near or above the $T_{1/2i}$ of DNA. White and Hogness (7) and Thomas et al. (8) reported recently that when a mixture of single stranded RNA and a longer duplex DNA containing within it a segment complementary to the RNA is incubated in a high formamide solvent at a temperature just below that at which the duplex DNA denatures, the RNA displaces its identical DNA sequence and form a duplex with its complementary sequence. The resulting structure is called an R-loop. These structures form because RNA:DNA hybrids are more stable than DNA:DNA duplexes in high formamide solvents.

These several observations have stimulated us to make a more detailed investigation of the effects of different formamide concentrations on the relative thermal stabilities of RNA:DNA and DNA:DNA duplexes and on the relative rates of RNA:DNA and DNA:DNA association reactions.

MATERIALS AND METHODS

Materials. Materials were purchased as follows: formamide, 99%, Matheson, Coleman and Bell; duplex polynucleotides, Grand Island Biological Co.; Biogel HTP (HAP), Biorad Laboratories; ^{125}I, Amersham-Searle; sodium iothalamate (Angio-Conray), Mallinckrodt Pharmaceutical Co.

Purification of formamide. Commercial formamide had an initial conductivity of 500-600μmho at 20°C and an A_{270} greater than 5. Two liters of this material was crystalized in a large beaker under continuous stirring at 1°C for 36 hrs (9). The crystals were collected by centrifugation at 1000 rpm in a International PR-6 centrifuge for 5 min in 200 ml glass centrifuge bottles fitted with fritted glass partitions in the center. The crystals were remelted and the process repeated 2 additional times. Recovery was generally 50 to 60%. The conductivity of the recrystalized formamide was 1.7μmho, and the absorbance at 270nm was 0.070. The ionic impurity in formamide is probably ammonium formate. An $8x10^{-3}$ M aqueous solution of this salt had a conductivity of 580μmho in the same cell. Formamide has a viscosity about three times that of H_2O. We therefore estimate the electrolyte content of the initial and purified formamide as $2.4x10^{-2}$ M and $7x10^{-5}$ M respectively.

Preparation of RNA and DNA. HeLa 28S and 18S rRNAs were prepared from a postmitochondrial supernatant stored in 80% ethanol (gift of Dr. G. Attardi). RNA was precipitated with ethanol from phenol extracted ribosomes. 18S and 28S fractions were isolated by sucrose gradient centrifugation in NTE buffer, 0.5% SDS. *E. coli* ^{3}H rRNA, a gift from Dr. Richard Deonier, had been

prepared as described (10). The molecular length of this RNA was greater than 1000 nucleotides. Human ^{32}P DNA from RD-114 cells was a gift from Dr. Margery Nicolson. ^{32}P and ^{3}H E. coli DNA were prepared as previously described (10), including HAP chromatography (11) and CsCl buoyant density centrifugation.

DNA for RNA:DNA hybridization was sheared by two passages through a Britten-Davidson press at 50,000 psi to an average single strand length of 450 nucleotides as determined by electron microscopy. DNA for thermal stability measurements was sonicated in 1M NaCl, 0.01 M Tris, 1mM EDTA, pH 7.8, for repeated 30 sec pulses to an average double strand length of 570 nucleotides.

RNA was labeled with ^{125}I by the procedure of Orosz and Wetmur (12), followed by buoyant banding in sodium iothalamate (13). Specific activities in the range of 3 to $6x10^6$ cpm/μg were achieved, with a molecular length of 1000 to 1100 nucleotides as determined by gel electrophoresis in the presence of methylmercury hydroxide (14).

Thermal stability measurements The $T_{1/2i}$'s of RNA:DNA hybrids in the absence of formamide were determined in 0.01 M NaCl, 1mM Tris, pH 7.8, and calculated for 0.3 M NaCl, 0.01 M Tris, 1mM EDTA, pH 7.8, by adding 24° (15). Melting curves of RNA:DNA hybrids in the latter electrolyte with different concentrations of formamide were determined by incubating the hybrids for 5 min at temperature intervals of 5°C. Aliquots were withdrawn, diluted 15 fold with 1xSSC and incubated at 30°C for 1 hr in the presence of 40μg/ml of RNAase A and 5 units/ml of T1 RNAase. Samples were then precipitated with 8% trichloroacetic acid, collected on glass filters (Whatman GFC) and assayed for radioactivity.

The $T_{1/2i}$'s of ^{32}P labeled sonicated DNAs were determined under the same conditions as stated above except that aliquots were diluted 20 fold in 0.12 M NaP buffer. Samples were assayed for the amount of single-stranded and duplex DNA by HAP chromatography at 60°C (16). The columns used were 5 ml syringes each containing 1 ml volume of HAP equilibrated in 0.12 M NaP at 60°C. Samples were loaded on the columns and washed with 12 ml of 0.12 M NaP. Duplex DNA was eluted with 4 mls of 0.4 M NaP. Fractions were mixed with Instagel (Packard Instrument Co.) and radioactivity determined in a Beckman scintillation counter.

The melting curves of synthetic polynucleotides were determined with a Gilford Model 2000 recording spectrophotometer fitted with a linear temperature programmer. The heating rate was 0.5°C/min and absorbance was monitored at 270 nm.

Preparation of rRNA:DNA hybrids E. coli rRNA:DNA hybrids were prepared by hybridizing 300μg of sheared denatured DNA with 12μg of ^{3}H rRNA (specific activity 1.35×10^{4} cts/min/μg) in 1 ml of 50% formamide, 5xSSC, for 5 hrs at 55°C. This represents approximately a 5 fold RNA sequence excess and an incubation rot of 0.67 moles sec liters^{-1}. The mixture was dialyzed against NTE at 4°C for 10 hrs. The solution was then diluted to 3 ml with NTE and adjusted to a final concentration of 1.53 gm/cc with Cs_2SO_4. The solution was centrifuged for 60 hrs at 40KRPM, in a Ti65 rotor. The fractions containing RNA:DNA hybrids were pooled, dialyzed against NTE and stored at -70°.

HeLa 28S or 18S RNA:DNA hybrids were prepared by hybridizing 200μg of sheared denatured DNA with 0.4μg of either ^{125}I labeled 28S or 18S rRNA (specific activity ~ 2×10^{7} cts/min/μg) in 1 ml of 50% formamide, 5xSSC at 58°C for 20 hr. The mixtures were dialyzed and centrifuged to equilibrium as stated above. The RNA sequence excess is approximately 10 fold (uncertainty is due to the exact specific activity of the ^{125}I RNA), and the incubation rot was 8.5×10^{-2}.

DNA excess hybridization. DNA excess hybridization was carried out by mixing 1.2 mg of ^{32}P E. coli sheared DNA (specific activity 30 cpm/μg) with 1μg of ^{3}H rRNA (specific activity 1.35×10^{4} cpm/μg). Assuming 8-10 copies of ribosomal DNA per chromosome this represents approximately a 2-3 fold ribosomal DNA excess. Hybridization was carried out in 80% formamide, 2xSSC. Samples were withdrawn at various times and assayed for hybrid formation and DNA reassociation as stated under thermal stability measurements.

RNA excess hybridization. RNA excess hybridization was performed by mixing ^{3}H E. coli rRNA (specific activity 1.35×10^{4} cpm/μg) with sheared DNA to give an RNA/DNA ratio of 0.031. The reaction volume for each sample was 0.5 ml containing 0.5μg of rRNA and 16μg of DNA in 80% formamide, 2xSSC. This represents a RNA sequence excess of 10 assuming 8-10 rRNA genes per E. coli chromosome of molecular weight 2.5×10^{9} daltons. The final point of each rot curve contained 6μg of rRNA and 16μg DNA in a 0.5 ml sample. Samples were heated to 85°C for 5 min then quickly equilibrated at the temperature used for hybridization. Samples were then diluted 15 fold in 1xSSC at given times and assayed for hybrid formation as stated under thermal stability measurements.

RESULTS

Stability of formamide. Recrystalized formamide was tested for its stability at hybridization temperatures. It should be noted that this batch of

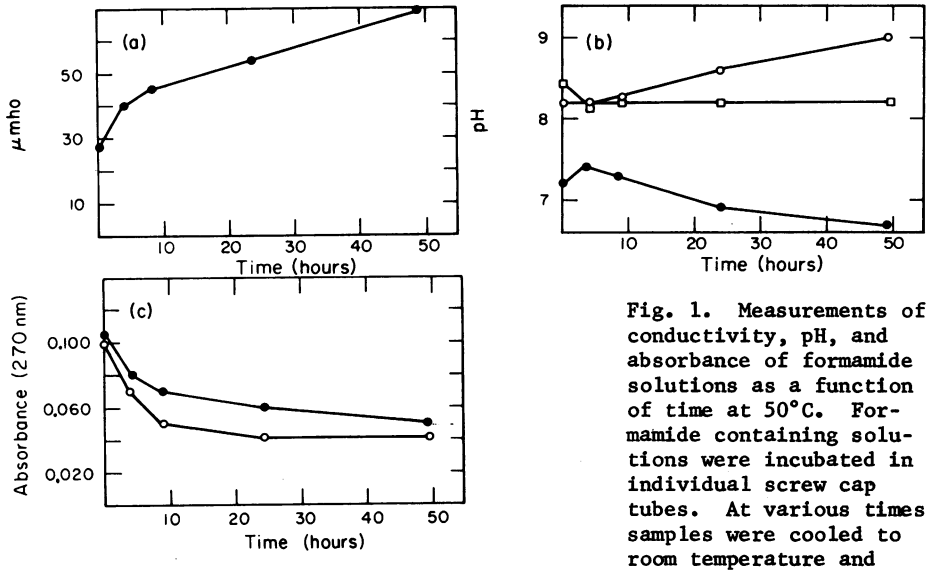

Fig. 1. Measurements of conductivity, pH, and absorbance of formamide solutions as a function of time at 50°C. Formamide containing solutions were incubated in individual screw cap tubes. At various times samples were cooled to room temperature and measurements taken. (a) Measurement of conductivity of a 80% formamide, water mixture. (b) The pH of 80% formamide water (●—●); 80% formamide, 5xSSC (□—□); 80% formamide, 0.1 M Tris, aqueous pH 7.8, (○—○). (c) Absorbance at 270 nm of 80% formamide, water (●—●) or 80% formamide, 0.1 M Tris, which is pH 7.8 (○—○).

formamide had been stored for 2 months at 4°C, during which time the conductivity rose from 1.4 μmho to 25 μmho. At 50°C the conductivity of the water: formamide mixture increased from 25 μmho to 70 μmho during the 50 hr incubation (Fig. 1a). The calculated initial and final concentrations of ammonium formate are $8x10^{-4}$ and $2.8x10^{-3}$M. As shown in Fig. 1b, the pH of the unbuffered water:formamide solution decreased from 7.2 to 6.8, suggesting a small amount of volatilization of NH_3. In the presence of a final concentration of 5xSSC the pH remained constant at 8.2. The pH of Tris-OH, adjusted in aqueous solution to pH 7.8 with HCl, was measured as 8.4 in 80% formamide. After 50 hr incubation at 50°C, the measured pH had increased to 9.0. The explanation for this change is not known to us.

The high absorbance of commercial formamide appears to be a contaminant and not a decomposition product since no increase in A_{270} was observed during incubation (Fig. 1c).

Effect of Formamide on the $T_{1/2}$ of Various Duplex Polynucleotides. The effects of formamide concentration on the $T_{1/2i}$ of human and E. coli

DNA:DNA and ribosomal RNA:DNA hybrids are shown in Figs. 2 and 3. The $T_{1/2i}$ for either E. coli or human DNA decrease linearly with increasing concentration of formamide. However, the effect of formamide on the $T_{1/2i}$'s of ribosomal RNA:DNA hybrids is considerably more complex. Initially at low concentration of formamide a large reduction of $T_{1/2}$ is observed. As the formamide concentration is increased the $T_{1/2i}$'s are progressively less affected than the $T_{1/2i}$'s of the corresponding DNAs. The most important point is that at high formamide concentrations the RNA:DNA hybrids are much more stable than the DNA:DNA duplexes.

Representative melting curves of synthetic duplex polymers are given in Figure 4. The results of all the determinations of $T_{1/2d}$'s as a function of formamide concentration are shown in Figure 5. The experimental results indicate that the $T_{1/2d}$'s of all double stranded synthetic polymers respond differently to increasing concentrations of formamide. For example, the $T_{1/2d}$ of poly dA:dT is reduced by 0.75°C per % formamide) whereas the $T_{1/2d}$ of poly dG:dC is reduced by 0.5°C per % formamide). Although the $T_{1/2d}$'s of most synthetic RNA:DNA hybrids decrease linearly with increasing concentration of formamide, the $T_{1/2d}$ of poly rG:dC decreases nonlinearly and thus is similar to RNA:DNA hybrids.

Association Rates. The experiments described in the previous section confirm the hypothesis that RNA:DNA duplexes are more stable than DNA:DNA duplexes in

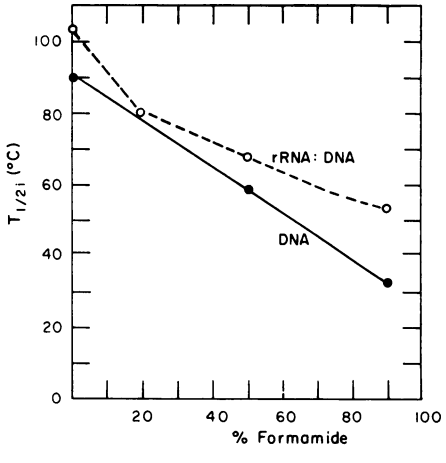

Fig. 2 The $T_{1/2i}$'s of E. coli DNA and rRNA:DNA hybrids are shown as a function of formamide concentration in 0.3 M NaCl, 0.01 M Tris, pH 7.8. Approximately 5μg of E. coli ^{32}P DNA (specific activity 2000 cpm/μg) was used to determine the $T_{1/2i}$ for each melting curve. The $T_{1/2i}$'s of rRNA:DNA hybrids were determined with 2500 cpm for each melting curve.

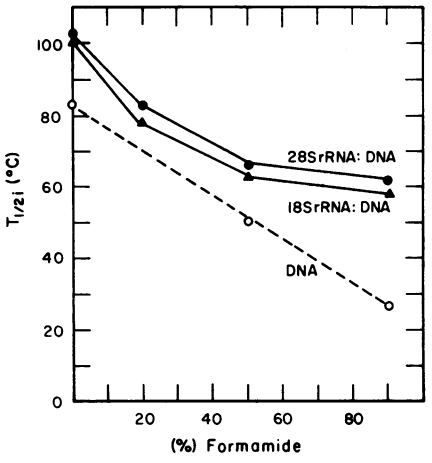

Fig. 3 The effect of formamide concentration (0.3 M NaCl, 0.01 M Tris, pH 7.8) on the $T_{1/2i}$'s of human 18S or 28S rRNA:DNA hybrids and RD-114 DNA. For each DNA melting curve, 10,000 cpm were used. Each melting curve of either 18S or 28S rRNA:DNA hybrids were determined with 2000 cpm.

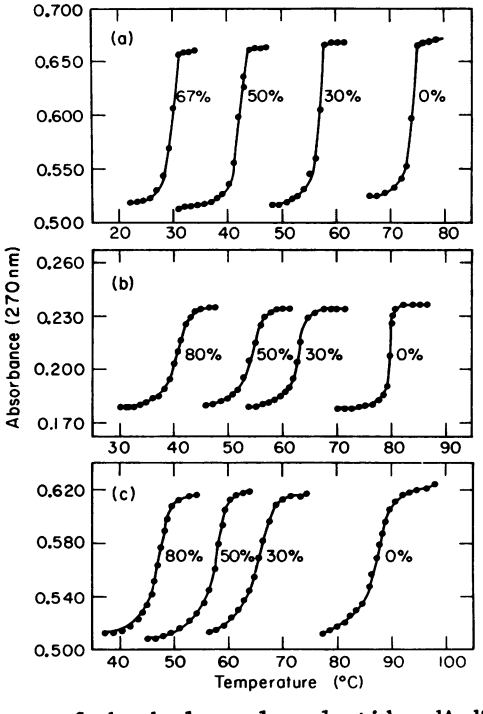

Fig. 4 Melting curves of the duplex polynucleotides dA:dT, dG:dC, and rG:dC in various concentrations of formamide. The buffer and salt concentrations used to generate the melting curves were (a) poly dA:dT, 0.3 M NaCl, 10 mM Tris, pH 7.8, (b) poly dG:dC 10 mM NaCl, 1 mM Tris pH 7.8 and (c) poly rG:dC, 1 mM NaCl 0.1 mM Tris pH 7.8.

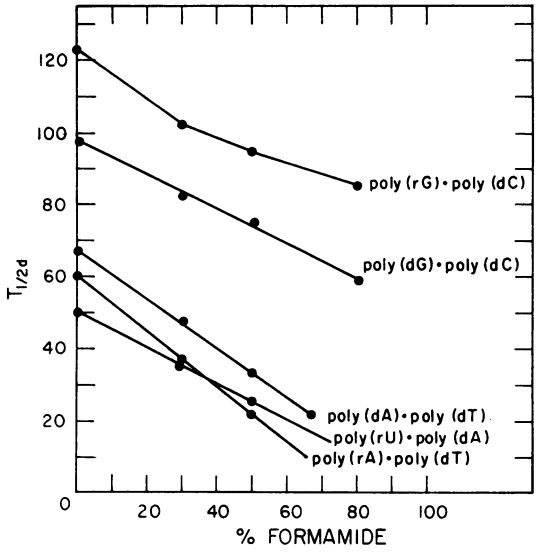

Fig. 5 The effect of increasing concentration of formamide on the $T_{1/2d}$'s of various synthetic duplex polynucleotides. The $T_{1/2d}$ measurements were obtained in 0.3 M NaCl,10 mM Tris pH 7.8 for poly rU:dA and poly dA:dT. Remaining data taken from Fig. 4. All $T_{1/2d}$ measurements are normalized to 0.1 M NaCl (15).

high formamide solvents. We have therefore studied the relative rates and extent of formation of RNA:DNA and DNA:DNA duplexes as a function of temperature using E. coli DNA and ribosomal RNA. These experiments were performed in 80% formamide, 2xSSC. Results of DNA excess experiments, shown in Fig. 6 (a through c), show that as the reaction temperature is raised the DNA association rate is decreased until no reaction occurs. However, RNA:DNA association proceeds with more uniform kinetics and to a greater extent as the reaction temperature is increased (Figure 6a through c). Hybridization is maximal at 50°C, a temperature at which no DNA:DNA association occurs.

The results of RNA excess hybridization experiments are presented in Fig. 7. It can be seen that rate constants do not change appreciably over the 20°C temperature range from 28° to 48°C, however the amount of hybridization is 2 fold greater at 48°C than at 28°C. A reduction in rate of hybridization is not observed until the very stringent hybridization temperature of 56°C is used.

DISCUSSION

Factors affecting thermal stabilities of duplexes. It should first be noted that the assay used for the stability of RNA:DNA hybrids in Figs. 2 and 3 consists of heating the hybrids to a given temperature, quenching, and

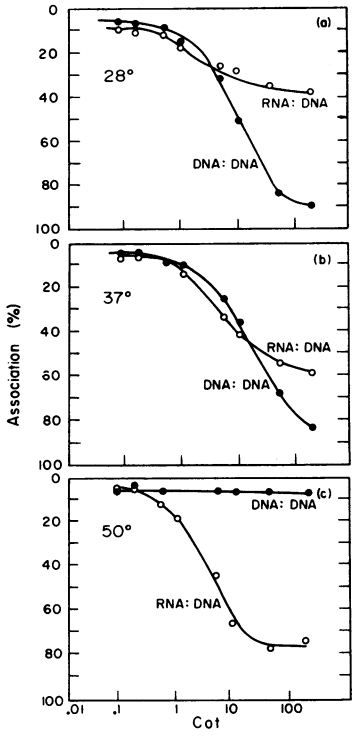

Fig. 6 E. coli DNA excess association with rRNA tracer. Reactions were performed in 80% formamide, 2xSSC, and experimental points determined as given in Materials and Methods.

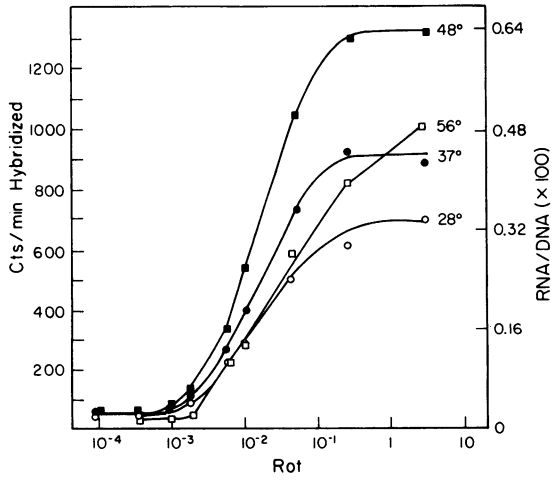

Fig. 7 E. coli rRNA excess association with DNA. Reactions were performed in 80% formamide, 2xSSC. Conditions for association are given in Materials and Methods.

digesting any dissociated RNA with RNAase. RNA that has not dissociated from the complementary DNA strand remains RNAase resistant. Similarly, the stability of DNA duplexes was measured by heating, quenching, and assaying for the amount of single-stranded and double-stranded material by HAP chromatography under conditions in which no further strand dissociation or association occurs.

Thus, these experiments measure the fraction of duplexes which undergo complete strand dissociation at a particular temperature. They are i or irreversibility assays as defined by Hamaguchi and Geiduschek (4). All experiments were conducted with sheared DNA strands of average length 450 nucleotides and with RNA strands that were greater than 900 nucleotides in length. In general, the stability of a duplex molecule is determined by its G+C content. If there is intrastrand base composition heterogeneity, the temperature of dissociation for a given duplex is determined by the base composition of that segment, of length perhaps 50 to 100 nucleotides, which has the highest G+C content within the total duplex. On the other hand, an optical melting curve measures the fraction of base pairs broken at the ambient temperature; these base-pairs will reform on cooling if they are within a duplex molecule held together by some other G+C rich region. These are d or reversible assays. For aqueous solutions, $T_{1/2i}$ is usually 7 to 10° higher than $T_{1/2d}$ (4).

The data in Figs. 2 and 3 confirm the observations by previous investigators that the depression of $T_{1/2i}$ of duplex DNA by formamide is a linear function of the formamide concentration. Our measured value for the slope is -0.63%/(% formamide); McConaughey et al. (2) measured a slope of -0.66%/(% formamide).

The effects of formamide on RNA:DNA hybrids are more complex. The data in Figs. 2 and 3 show that the curves of $T_{1/2i}$ as a function of formamide concentration are concave upwards. There is a relatively large negative slope from 0 to 20% formamide, but a much smaller slope from 50% to 90% formamide. The figures show that at very high formamide concentrations, the difference in stability between RNA:DNA and DNA:DNA duplexes is maximal.

The data in these figures also show that in aqueous solvents, using the $T_{1/2i}$ assays, RNA:DNA duplexes are slightly more stable than DNA:DNA duplexes. For the case of E. coli rRNA:DNA as compared to average E. coli DNA:DNA, the difference in $T_{1/2i}$ is about 6°; of this, about 1° may be attributed to the higher than average (53% vs. 50%) G+C content of rRNA (17). Fig. 3 shows that the $T_{1/2i}$'s of human 18S and 28S rRNA:DNA duplexes are 101° and 103° re-

spectively, whereas that for the average DNA is 82°C. These RNA's have average base compositions of 58% and 67% G+C (16) respectively whereas that for the total DNA is 40%. If we use a value of $\Delta T_{1/2}/\Delta\%(G+C)$ of 41° (19), the $T_{1/2i}$'s of 18S and 28S rDNA duplexes are estimated as 89° and 93°, or 12° and 10° less than that for the RNA:DNA hybrids.

The data of Birnstiel et al. (5) indicate that in aqueous 0.1XSSC, RNA:DNA hybrids are if anything less stable than DNA:DNA duplexes. However, these authors compared the stability of RNA:DNA hybrids pretreated with RNAase and on membrane filters to optical melting curves of DNA:DNA duplexes. Conditions on a membrane filter may be different than conditions in solution. More importantly, we suspect that internal nicks are introduced into RNA:DNA hybrids on filters by RNAase; these nicks will lower the thermal stability.

The determination by Hutton and Wetmur (20) of the optical melting temperature of the cRNA:DNA duplex of ϕX174 DNA indicates less than 1% difference of $T_{1/2d}$ from that for the DNA duplex in aqueous solution. Kallenbach (21) has summarized data which show that there is an increasing positive difference in thermal stability in aqueous solution between RNA:RNA and DNA:DNA duplexes as the G+C content increases. Our results for aqueous solutions indicate that, using $T_{1/2i}$ assays which depend on the G+C rich segments of molecules, RNA:DNA hybrids of high G+C content are slightly more stable than the corresponding DNA:DNA duplexes. Thus it appears that RNA:DNA hybrids are intermediate in thermal stability between RNA:RNA and DNA:DNA duplexes.

It is well known that data on relative thermal stabilities of homopolymer duplexes cannot be used for the accurate prediction of trends in the thermal stabilities of irregular natural polymers. Nevertheless, the overall thrust of the homopolymer data in Fig. 5 supports the two qualitative propositions that: a) with increasing G+C content, RNA:DNA duplexes become more stable relative to DNA:DNA duplexes; b) the effect of formamide in destabilizing duplexes is least for G+C rich RNA:DNA hybrids.

In summary then, the important general conclusion of this section is that RNA:DNA hybrids are more stable than DNA:DNA duplexes in high formamide solvents; this difference is greater for G+C rich polynucleotides.

Factors affecting the rate of association of complementary strands. The data in Fig. 2 show that in 80% formamide, 0.3M NaCl, the $T_{1/2i}$'s of E. coli DNA and of E. coli rRNA:DNA hybrids are 38° and 57° respectively. The data on the rates of DNA:DNA and DNA:RNA duplex formation in a DNA excess reaction presented in Fig 6 were measured in 80% formamide, 2xSSC (Na^+) = 0.39M at

28°, 37°, and 50°C, which we estimate to be 12°, 3°, and ~10° below $T_{1/2i}$ for DNA:DNA duplexes and 31°, 22°, and 9° below $T_{1/2i}$ for RNA:DNA hybrids.

At 28° and 37°, the DNA reassociation reactions go almost to completion. The RNA:DNA reaction proceeds slightly faster than the DNA:DNA reaction but only about 20% and 50% of the RNA form hybrids at the respective temperatures. We attribute this incomplete reaction in part to the removal of DNA strands by DNA:DNA reaction and in part to secondary structure in the RNA as discussed below in connection with Fig. 7. Fig. 6c displays the second major general conclusion of the present paper: in a high formamide solvent at a temperature intermediate between $T_{1/2i}$ for DNA:DNA and RNA:DNA duplexes (50°C in this case), the RNA:DNA reaction occurs almost quantitatively whereas there is no DNA:DNA reaction.

In order to determine DNA:RNA association rates without competition from DNA:DNA reactions, the RNA excess reactions reported in Fig. 7 were performed. The observed reaction rates are about the same at 28°, 37°, and 48°, but the extent of reaction at the plateau is about twice as great at 48° as at 28° and corresponds to reaction with 0.64% of the DNA.

The observation that the extent of reaction is less at 28°C than at 48°C is unexpected. For a reaction between a long RNA and an equally long or longer DNA molecule containing the full complementary sequence, one expects that if base pairing is initiated at one segment of the molecule, a complete duplex will form by a zippering reaction. In the present case, reaction is occurring between moderately long RNA strands and short (450 nucleotide) DNA strands. We interpret the incomplete reaction at 28° and at 37°C to indicate that there are some G+C rich segments in the RNA that form secondary structures that are stable at these temperatures and are not capable of initiating a pairing reaction with a complementary DNA strand.

The plateau level of hybridization of 0.64% observed at 48° corresponds to 10 (16S + 23S) rRNA genes per 2.5×10^9 daltons of DNA. Since the DNA was purchased from a commercial source and was not extracted from cells in stationary phase, we do not believe that our data can be used to calculate the number of rRNA genes per haploid genome (22). The large effect of temperature on the apparent saturation level of hybridization as shown in Fig. 7 may be characteristic of high formamide solvents and may not occur for hybridizations in aqueous solution. Nevertheless, the results in Fig. 7 suggest the possibility of error in RNA excess saturation hybridization experiments conducted at only one temperature.

It is of interest to compare the DNA:DNA and DNA:RNA reaction rates in

80% formamide, 2xSSC (0.39 M Na^+) with those observed at the optimal temperature at the same electrolyte concentration in aqueous solution. From the data of Wetmur and Davidson (3) or equivalently from the data of Britten and Kohne (23), we calculate a $\underline{cot}_{1/2}$ for *E. coli* DNA (complexity, 4.0×10^6 nucleotide pairs, strand length 450 nucleotides) of 2.7 M sec in aqueous 0.39 M Na^+ at T_m -25°. The observed value in 80% formamide at 28° ($T_{1/2}$ - 12°) is 10, or 3.6 fold slower. The DNA rates in Fig. 6a may be at a temperature slightly above the optimal for reassociation, so the rate ratio between aqueous and formamide solutions under optimal conditions is probably about 3.0.

RNA:DNA rates cannot be calculated from the DNA excess data in Fig. 5 without assuming a value for the reiteration frequency of the rRNA genes in the sample. The data in Fig. 7 give comparable values of $\underline{rot}_{1/2}$ of 0.02 M sec at 28°, 37°, and 50°. From the equations of Wetmur and Davidson (3), for a strand length of 450 nucleotides and a complexity of 5.0×10^3 nucleotides, we calculate a $\underline{rot}_{1/2}$ of 0.00172 M sec in aqueous solution, assuming that RNA:DNA reactions have the same rates as DNA:DNA reactions. The observed rate in 80% formamide is 12X slower.

Optimal conditions for RNA:DNA reactions. Our colleague, Dr. Peter Chandler, has examined chain breakage of RNA by several high formamide solvents. He finds that incubation of full length *E. coli* 23S rRNA for 50 hrs at 50°C in 80% formamide, 2xSSC, results in one chain break per 1000 nucleotides. However, there is no detectable degradation of the RNA upon incubation in 80% formamide, 0.4 M NaCl, 0.01 M Pipes buffer (pH 6.4), as judged by methyl mercury gel electrophoresis (14). Thus we currently recommend the latter solvent for hybridization reactions.

Acknowledgments

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†Dedicated to Jerome Vinograd.

*Abbreviations: $T_{1/2i}$, midpoint of the irreversible (HAP or ribonuclease assay) thermal melting curve of a duplex polynucleotide; $T_{1/2d}{}^i$ midpoint of the melting curve measured at ambient temperature (optical assay), both as originally introduced by Hamaguchi and Geiduschek (4). HAP, hydroxyapatite. NaP,

equimolar mixture of NaH_2PO_4 and Na_2HPO_4, pH 6.8. SSC, 0.15 M NaCl, 0.015 M trisodium citrate. NTE buffer, 0.1 M NaCl, 0.01 M Tris, 0.001 EDTA, pH 7.80. rot and cot are initial RNA or DNA concentrations in units of moles/liter of nucleotides times time of incubation in seconds.

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