Use of Formamide in Nucleic Acid Reassociation†

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ABSTRACT: The effect of formamide on DNA–RNA hybridization has been examined by directly comparing the thermal stabilities of the duplexes formed at various criteria. Although increasing formamide concentrations in the reassociation buffers elevate the mean thermal stabilities of the hybrids, there is no simple linear relationship between the mean thermal stabilities of hybrids and the formamide concentrations. Also, there is no simple linear relationship between the formamide concentration necessary to produce hybrids of specified thermal stability and the temperature of hybrid formation. Finally, we have noted a large decrease in the rate of reaction under some conditions.

Molecular hybridization has become a powerful tool in nucleic acid studies; the technique has recently been reviewed by Walker (1969), McCarthy and Church (1970), and Kennell (1971).

For a variety of reasons the utility of this technique would be greatly enhanced by procedures allowing the use of low temperatures (25–45°). These include the absence of thermal degradation of the reactants and enhanced retention of DNA on nitrocellulose filters (Bonner *et al.*, 1967); the use of aminoacyl-tRNA, radioactively labeled in the aminoacyl moiety (Weiss *et al.*, 1968; Nass and Buck, 1970); and the production of very stringent reassociation conditions at salt concentrations and temperatures compatible with hydroxyapatite chromatography (Schmeckpeper, 1972).

When one designs hybridization systems for eukaryotic nucleic acids the criterion of reassociation is a prime consideration. Britten and Kohne (1966; 1968) have demonstrated that some of the eukaryotic genome is represented by families of similar but not necessarily identical DNA sequences of varying repetition frequency. Furthermore, various degrees of sequence relatedness exist in such hybrids as a function of certain reassociation parameters, namely salt concentration, concentration of organic solvents, and temperature, which collectively produce the reassociation criterion (Britten, 1967; McCarthy and McConaughy, 1968; Church and McCarthy, 1968). The degree of sequence relatedness is measured by the extent of base mismatching which can be determined by the T_m[1] or $T_{m,i}$ of the hybrids (Laird *et al.*, 1969). Finally, the reassociation criterion influences the amount of DNA measured as repetitive (Kohne, 1970; K. D. Smith, R. C. Angerer, L. T. Bacheler, B. J. Schmeckpeper, and E. Stavnezer, 1972, manuscript in preparation). Thus for eukaryotic repeated nucleic acid sequences, the percentage of mispaired bases and the portion of the genome studied depend upon the reassociation criterion.

Several investigators have recognized the utility of lowered temperatures for nucleic acid studies and have used a variety of organic solvents as denaturants (Helmkamp and Ts'o, 1961; Helmkamp and Ts'o, 1962; Herskovits, 1962; Levine *et al.*, 1963; Subirana, 1966; Strauss *et al.*, 1968) and in reassociation studies (Bonner *et al.*, 1967; Kourilsky *et al.*, 1970). Recently McConaughy *et al.* (1969) described their efforts to relate formamide concentration to the thermal stability of duplex DNA and to certain reassociation parameters. They found that, from about 45° to 90°, every 1% formamide in the nucleic acid solution reduced the T_m of double-stranded DNA 0.72°. This relationship has been widely applied to eukaryotic nucleic acid reassociation (McCarthy and Church, 1970; Williamson *et al.*, 1970; Pagoulatos and Darnell, 1970; Spelsberg *et al.*, 1971) on the assumption that the effect of formamide on helix disruption is the same as its effect on duplex formation from single strands. None of the preceding studies has directly tested this assumption. Thus an important question is still unanswered. Can formamide replace increased temperature in placing constraints on base mispairing during reassociation as predicted by the relationship relating duplex DNA structure to formamide concentration and temperature (McConaughy *et al.*, 1969)? We felt that this question could be answered directly by comparing the specificity of reassociation at low temperature in formamide with the specificity of reassociation at a standard high-temperature criterion. We have chosen as a standard criterion the reassociation which occurs at 68°, in 2 × SSC in 18 hr; this criterion allows approximately 10–15% mispaired bases (Church and McCarthy, 1968; Laird *et al.*, 1969) and is widely used. We also conducted a survey of reassociation specificity as a function of temperature and formamide concentration and studied the rate of reaction since it was likely that this would change in the formamide solutions (Subirana, 1966; Wetmur and Davidson, 1968; Thrower and Peacocke, 1968).

Materials and Methods

Preparation of [^{32}P]*RNA.* Female Swiss Webster mice from our laboratory colony received intraperitoneally 1 mCi of [^{32}P]P_i (New England Nuclear) in 0.20 ml of 0.25 M Tris-HCl (pH 7.0) at 4:00 p.m.; 16 hr later the mice were killed by cer-

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[1] Abbreviations used are: T_m, mean temperature of denaturation measured optically at ambient temperature; $T_{m,i}$, mean temperature of irreversible strand dissociation; SSC, standard saline citrate, 0.15 M NaCl–0.015 M trisodium citrate; RNase, pancreatic ribonuclease; PB, neutral sodium phosphate buffer, where the indicated molarity designates the anion concentration and the cation concentration is 1.5 times higher; C_0t, parameter used in measuring reassociation and equal to the product of molar concentration of nucleotides and time of incubation in seconds; cRNA, complementary RNA synthesized *in vitro* from a DNA template; SSC–TES, 0.15 M NaCl–0.0075 M trisodium citrate–0.0075 M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

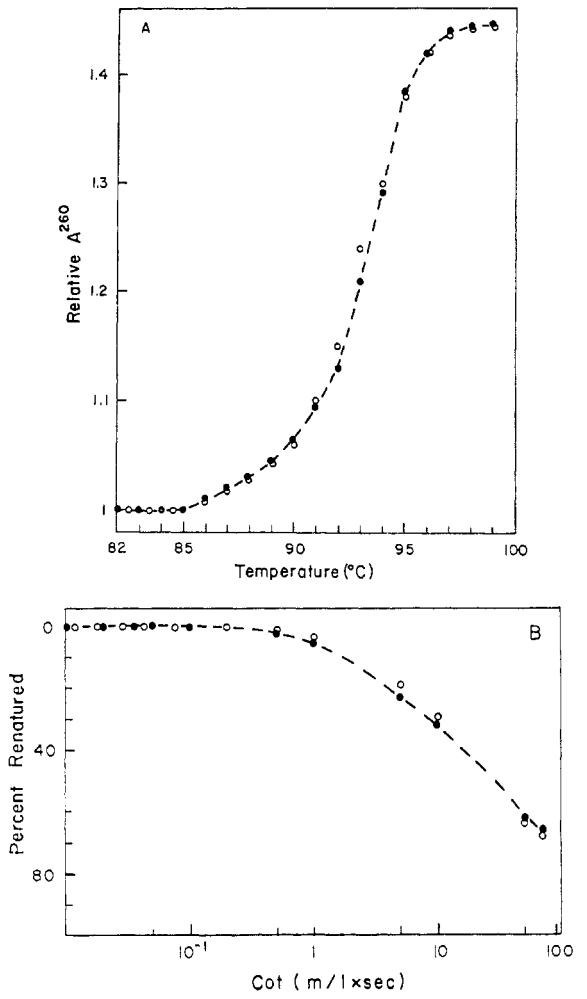

FIGURE 1: (A) Thermal stability profiles of renatured control *E. coli* DNA (●) and renatured formaldehyde-treated and reversed *E. coli* DNA (○). Samples were melted in 0.12 M PB at a concentration of 100 μg/ml. (B) Kinetics of renaturation of control *E. coli* DNA (●) and formaldehyde-treated and reversed *E. coli* DNA (○). Samples were renatured at 60° in 0.12 M PB at a concentration of 100 μg/ml.

vical dislocation. Total cellular RNA was purified from excised livers by the hot phenol–sodium dodecyl sulfate–ethanol precipitation precedure described by Church and McCarthy (1968).

Preparation of Mouse Nuclear DNA. Nuclei were prepared by a procedure similar to that of Chauveau *et al.* (1956) as modified by Wang (1967) with the following exceptions. (1) Excised tissue was homogenized in ice-cold 0.32 M sucrose–0.01 M Tris-HCl (pH 7.6 at room temperature)–0.005 M $MgCl_2$. (2) Only one low-speed (1500*g*) centrifugation was performed. (3) The nuclei were pelleted through heavy sucrose by centrifugation in the SS-34 head of the Servall centrifuge at 16,000 rpm (30,900*g*) for 50 min. The DNA was isolated by a modification of published procedures wherein the nuclear pellets were resuspended in 0.075 M NaCl–0.025 M EDTA, then brought to 50 mM EDTA–1% sodium dodecyl sulfate. Pronase (Calbiochem, B grade) was added to a final concentration of 100 μg/ml and the preparation incubated at 37° for 3–5 hr until the clumps had dispersed and then phenol extracted (Thomas *et al.*, 1966). DNA was further purified by chloroform extraction and ethanol precipitation (Marmur, 1961). Contaminating RNA was removed by room temperature dialysis against 0.3 N NaOH (Bock, 1967; B. J. Schmeckpeper, 1971, unpublished data) or by incubation with RNase (Worthington, electrophoretically pure) and then pronase (preincubated for 3 hr at 37° to destroy nuclease activity), followed by phenol extractions and ethanol precipitation as before. DNA prepared by the second method was always tested for RNase activity and pronase digestion was repeated until RNase activity was undetectable. Contaminating carbohydrates were removed by centrifugation with Norit (Church and McCarthy, 1968).

DNA used for the synthesis of RNA or the preparation of satellite-depleted DNA was sheared by passing native DNA 4–5 times through a 26-gauge needle (Brown and Weber, 1968). Unfractionated DNA used for filters was left at high molecular weight, as isolated.

Preparation of Mouse [^3H]DNA. On days 12 and 13 of pregnancy, Swiss Webster female mice were injected intraperitoneally with 200 μCi of [*methyl*-^{3}H]thymidine (Schwarz Bio-Research). On day 14 of pregnancy the mice were sacrificed and the embryos dissected from the uterus. The nuclei were isolated as described above for nuclear DNA through the low speed centrifugation. The nuclei were resuspended in SSC and lysed by the addition of sodium dodecyl sulfate to 1%. The DNA was isolated by the Marmur (1961) procedure and further purified as described above for nuclear DNA.

Preparation of Mouse Satellite-Depleted DNA. Sheared, denatured mouse nuclear DNA was reassociated in 0.05 M PB to an equivalent C_0t of 0.1 (Britten and Smith, 1970). The reassociation was stopped by the addition of an equal volume of freshly diluted 2% formaldehyde–1 M NaCl–0.05 M PB, and the incubation continued at 60° for 15 min (E. Stavnezer, 1970, personal communication; Grossman, 1968). The DNA was cooled to room temperature and bound to an hydroxyapatite (Bio-Gel HTP, Bio-Rad) column, equilibrated with 0.05 M PB, 1% formaldehyde, at room temperature. Satellite-depleted DNA was eluted by raising the phosphate concentration to 0.15 M. Double-stranded DNA was eluted with 0.30 M PB. The formaldehyde reaction was reversed (see below) by overnight dialysis of the satellite-depleted DNA at room temperature against several hundred volumes of 0.12 M PB (pH 8.5). The DNA was then dialyzed into 0.1 × SSC and used directly or into water and then lyophilized and redissolved in the appropriate buffer.

Preparation of Escherichia coli DNA. DNA was prepared from *Escherichia coli* B (purchased from General Biochemicals, Chagrin Falls, Ohio) by the procedure of Marmur (1961) and further purified as described above for mouse nuclear DNA.

Reversibility of the DNA–Formaldehyde Reaction. Formaldehyde hydroxymethylates free amino groups (Grossman, 1968) and therefore prevents hydrogen bonding in nucleic acids. This hydroxymethylation of nucleic acids can be reversed as judged by ultraviolet spectral data (Grossman *et al.*, 1961) but when radioactive formaldehyde is used, some of the bases are detected as permanently hydroxymethylated (Grossman, 1968). However, the results of two experiments, presented in Figure 1 and described below, indicate that the formaldehyde reaction has no irreversible effects on DNA reassociation. In the first experiment, purified *E. coli* DNA (100 μg/ml) in 0.01 M PB was heat denatured and cooled to 60°. An equal volume of 2% formaldehyde–1 M NaCl–0.01 M PB, at 60°, was added and mixed, and the preparation incubated at 60° for 15 min. It was then cooled to room temperature and the reaction reversed by dialyzing overnight at room temperature against 0.12 M PB (pH 8.5; Grossman *et*

al., 1961). This was followed by dialysis against deionized water and lyophilization. The sample was redissolved and dialyzed, along with a second untreated sample of *E. coli* DNA, against 0.12 M PB. Both samples were heat denatured, incubated at 60° overnight, then placed in a cuvette in a Gilford recording spectrophotometer, equilibrated at 60°. The concentration of DNA in each sample was 100 μg/ml. The melting profiles of the renatured DNA samples were obtained by continuously increasing the temperature (*ca.* 0.5°/min) with a circulating water bath (P. M. Tamson, model T3). As seen in Figure 1A, the T_m's of the samples were identical as were the total hyperchromicities; thus at least 98% of the bases in formaldehyde-treated DNA, after reversal, were able to base pair as well as those in untreated DNA. In the second experiment, a sample of *E. coli* DNA was formaldehyde treated and reversed as above. It and a second untreated sample, both in 0.12 M PB, were heat denatured in the Gilford spectrophotometer. The temperature of the cuvette chamber was adjusted to 60° and the reassociation monitored continuously by hypochromicity at 260 nm. As seen in Figure 1B, the formaldehyde-treated DNA, after reversal, renatured exactly as the control DNA. From these two experiments we conclude that the formaldehyde treatment has no irreversible effects upon properties of DNA strand reassociation.

In Vitro Synthesis of [^{14}C]*cRNA*. RNA complementary to native mouse nuclear DNA was synthesized using *E. coli* DNA dependent RNA polymerase purified by the method of Burgess (1969). The batch synthesis mixture was initially as follows in a total volume of 10–30 ml: 0.04 M Tris-HCl (pH 7.9), 0.02 M $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol (Cleland's Reagent), 0.15 M NH_4Cl, 0.8 mM ATP, 0.8 mM GTP, 0.8 mM CTP, 0.8 mM UTP (ATP from Sigma; other nucleotides from Schwarz BioResearch), 1–2 μCi/ml of [^{14}C]-ATP (uniformly labeled, specific activity approximately 460 μCi/μmole, New England Nuclear), 100–400 μg/ml of DNA, and 10–40 units/ml of polymerase, where one enzyme unit polymerizes 1 nmole of AMP in 15 min at 37° using the reaction conditions of Burgess (1969). In all cases, the ratio of DNA to enzyme was such that the enzyme was just saturated by template. The incubation temperature was 37°. The time of incubation was chosen so that, on a weight basis, the ratio of RNA synthesized to DNA primer was between 1 and 4. With frequent additions of nucleotides the reaction continued for up to 6 hr. To stop the reaction deoxyribonuclease I (bovine pancreas, Worthington, grade DPFF) was added to a final concentration of 25 μg/ml and the incubation at 37° continued for 30 min.

A fine precipitate containing the cRNA appeared after 1 hr of synthesis, but could be completely dissolved by dialyzing the preparation for 1–2 hr against a 40- to 100-fold molar excess of EDTA (pH 7.5) relative to the Mg^{2+} concentration, 1% sodium dodecyl sulfate (to inhibit RNase activity) at room temperature (Millette and Trotter, 1970). The RNA was purified by phenol extraction (pH 5.1); gel filtration over Sephadex G-25 (medium; Pharmacia Fine Chemicals, Inc.) equilbrated in 0.1 × SSC or 1 mM Tris-HCl (pH 7.4)-10^{-4} M $MgCl_2$; a second digestion with deoxyribonuclease (in 0.01 M Tris-HCl-10^{-3} M $MgCl_2$); phenol extraction; and gel filtration over Sephadex G-75 (medium; Pharmacia Fine Chemicals, Inc.) equilibrated in one of the buffers used for the first gel filtration. Virtually all the radioactivity was excluded from the Sephadex G-75 column and well separated from the included peak of DNA oligonucleotides, with the specific activity (cpm/A_{260} unit) predicted for RNA synthesized from nucleotides of the given specific activity. The RNA was judged free from protein by the relative absorbancies at 260 and 230 nm (260/230 = 2.5).

Preparation of the Filters. The DNA was immobilized on membrane filters (0.45 μ pore diameter, type HAWG, Millipore Filter Corporation) by a modification of the procedure of Gillespie and Spiegelman (1965) as described by Church and McCarthy (1968). The filter was rinsed by passing through 900 ml of 4 × SSC, then soaked in Denhardt's preincubation medium (Denhardt, 1966) for 10 min and air-dried at room temperature for at least 2 hr. Small disks containing the desired amount of DNA were then cut from the filter and baked at 60° for 16 hr. Baked filters were stored dry at 4°. Blank filters were made as above, except no DNA was passed through the filter. The amount of DNA on the cut filters was monitored in one or more of three ways: (1) decrease in A_{260} of the DNA solution during filtration, (2) A_{600} after the diphenylamine reaction (Burton, 1968), or (3) radioactivity when [^{3}H]DNA was used.

Reassociation Conditions and Thermal Stability Measurements. High-temperature hybridizations were carried out in a covered water bath at 60° or 68° in 2 × SSC or 4 × SSC (pH of both was 5.5 at room temperature), 10^{-3} M EDTA. Low-temperature hybridizations were carried out in a covered water bath at 25, 33, 37, 45, or 50° in 2 × SSC or 4 × SSC (made from 10 × SSC, pH 4.1 at room temperature), 10^{-3} M EDTA and varying concentrations of formamide; the final pH of the formamide-containing buffers was about 5–6 in all cases. The conditions for individual experiments are indicated in the figure legends and the tables. In a total volume of 100 or 200 μl, each hybridization vial contained one experimental filter, one control filter when appropriate, and RNA in the appropriate solvent; a covering of light mineral oil retarded evaporation. Unless otherwise indicated the hybridizations at 60 or 68° were stopped after 18 hr and the reactions at low temperatures were stopped after 48 hr. After the high-temperature hybridization, each filter was rinsed 3 times, for 10 min each, in 5 ml of hybridization buffer at the temperature of reassociation. After the low-temperature incubations, each filter was rinsed in two 5-ml aliquots of hybridization buffer, for 10 min each at the incubation temperature; this was followed by two 5-ml rinses with the hybridization buffer minus formamide for 5 min, also at the incubation temperature, and a final rinse in hybridization buffer minus formamide at room temperature. The filters were air-dried and placed in a 2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene–toluene liquid scintillation fluor (Amersham/Searle) and counted in a Packard Tri-Carb liquid scintillation spectrophotometer for 40 min each. Each filter was then rinsed 3 times in chloroform to remove fluor and pairs of duplicate filters were placed in test tubes containing 5 ml of 0.5 × SSC or SSC. All samples were placed in a fast-heating, circulating water bath, (P. M. Tamson, model T9) and equilibrated to the starting temperature of the melt. The temperature was increased in intervals, as indicated in the figures, and duplicate filters were removed after 5-min incubation at each temperature. A standard criterion (68°, 2 × SSC) control melting curve was generated with each different low temperature studied; the variation among control curves is a measure of the sensitivity of the assay and differences in $T_{m,i}$'s of less than 1° are not significant. The amount of DNA on the filters after the melt was determined by the diphenylamine reaction (Burton, 1968) after the filters were removed from the scintillation fluor and rinsed in chloroform.

Time Course Measurements. Two reaction mixtures containing identical amounts of [^{14}C]cRNA were set up in 2 ×

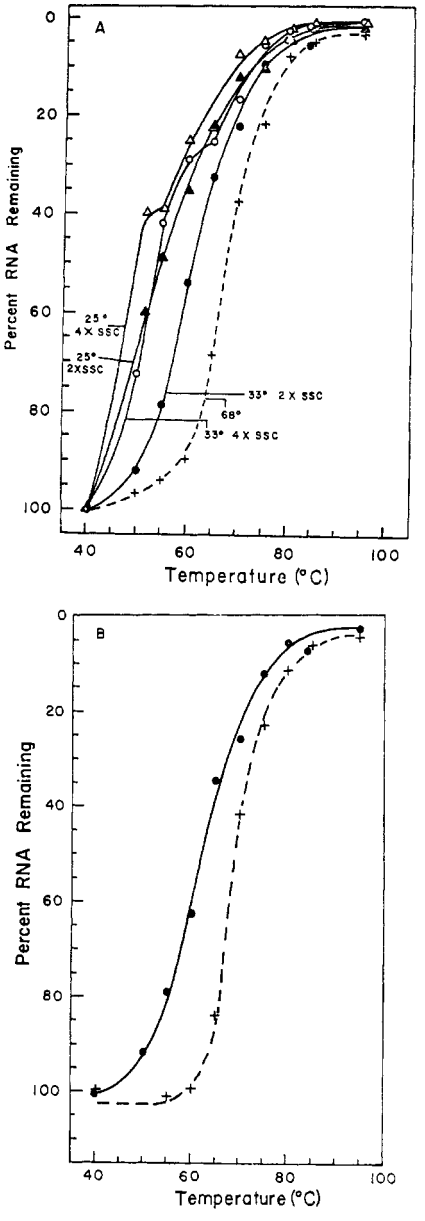

FIGURE 2: Thermal stability profiles of mouse satellite-depleted DNA–cRNA hybrids reassociated at various criteria. Hybrids were formed by incubating filter-bound mouse satellite-depleted DNA and cRNA in 0.10 ml buffer; all buffers contained 10^{-3} M EDTA and varying concentrations of salt and formamide. All thermal stability measurements were made in 0.5 × SSC. Specific activity of the RNA was 1230 cpm/μg. Each point is the average of duplicates. (A) +--+, 4.5 μg of DNA + 64 μg of RNA in 2 × SSC, no formamide, 68°; 180 cpm bound, $T_{m,i}$ = 67.4°. ●—●, 4.5 μg of DNA + 32 μg of RNA in 2 × SSC–50% formamide, 33°; 410 cpm bound, $T_{m,i}$ = 59.1°. ▲—▲, 4.5 μg of DNA + 32 μg of RNA in 2 × SSC–50% formamide, 25°; 794 cpm bound, $T_{m,i}$ = 53.8°. O—O, 4.5 μg of DNA + 21 μg of RNA in 4 × SSC–50% formamide, 33°; 675 cpm bound, $T_{m,i}$ = 53.6°. Δ—Δ, 4.5 μg of DNA + 21 μg of RNA in 4 × SSC–50% formamide, 25°; 931 cpm bound, $T_{m,i}$ = 49.5°. (B) Corrected for DNA remaining on the filter after the melt, +--+, 4.5 μg of DNA + 64 μg of RNA in 2 × SSC, no formamide, 68°; corrected $T_{m,i}$ = 68.4°. ●—● 4.5 μg of DNA + 32 μg of RNA in 2 × SSC–50% formamide, 33°; corrected $T_{m,i}$ = 61.5°.

SSC-TES (pH 7.5 at room temperature)–10^{-3} M EDTA and 2 × SSC–50% formamide–10^{-3} M EDTA, respectively. Equal portions were pipeted into vials, and the reaction initiated by the addition of a mouse [^{3}H]DNA filter and an *E. coli* DNA filter. The samples were incubated for varying lengths of time at either 68° (no formamide) or 33° (with formamide). The reaction was stopped by removing the filters from the vials and rinsing the filters as described above. The filters were dried and then both isotopes counted in 2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene–toluene fluor in the scintillation counter. The ^{3}H cpm were corrected for overlapping ^{14}C cpm; no ^{3}H cpm overlapped the ^{14}C cpm.

Reagents. The formamide used in these experiments was purchased from Eastman Kodak Company and was filtered over a column of chelex-100 resin (Bio-Rad, 100–200 mesh) to remove contaminants which absorb ultraviolet light. Phenol was redistilled shortly before use. Sodium dodecyl sulfate was recrystallized from 70% ethanol. All other reagents were analytical grade. Stock solutions were filtered through HAWG Millipore filters and Chelex-100 resin to remove trace contaminants.

Results

Sequence Specificity of the Low-Temperature Reaction. The first experiments were designed to determine whether or not formamide reassociation criteria can be calculated from the relationship between formamide concentration and thermal stability established for double-stranded DNA (1% formamide lowers the T_m by 0.72°, McConaughy *et al.*, 1969). If this is possible, then 50% formamide in the hybridization buffer should compensate for lowering the reaction temperature 35°. Therefore, the restrictions on base pairing at 33°, 50% formamide–2 × SSC, should be equivalent to those at 68°, 2 × SSC. We have used the parameter of hybrid thermal stability, as measured by $T_{m,i}$ (McCarthy and McConaughy, 1968), to determine the equivalency of reassociation conditions. Two reassociation conditions are considered equivalent if they place the same constraints on base mismatching of the reacting strands as evidenced by identical $T_{m,i}$'s.

The data presented in Figure 2 show that when [^{14}C]cRNA was incubated with mouse satellite-depleted DNA immobilized on filters, the hybrids which formed at 33°, in 50% formamide, 2 × SSC had a $T_{m,i}$ which was about 7° lower than the hybrids which formed at 68°, 2 × SSC. This means that the low-temperature system allows more distantly related sequences to pair, resulting in a higher percentage of mismatched bases. Further relieving the restrictions on sequence recognition by increasing the salt concentration (33°, 4 × SSC–50% formamide, Figure 2A), lowering the temperature (25°, 2 × SSC–50% formamide, Figure 2A), or both (25°, 4 × SSC–formamide, Figure 2A) progressively reduced the $T_{m,i}$'s of the hybrids so formed. Similar results were obtained with liver [^{32}P]RNA and total mouse DNA.

Loss of DNA from Filters. During the reassociation reaction some of the DNA comes off the filters, as evidenced by the loss of diphenylamine-positive material (Burton, 1968) from the filters. This is apparently a function of the temperature of incubation (B. J. Schmeckpeper and R. C. Angerer, 1971, unpublished data). Furthermore, DNA was lost from the filters during the melt. Therefore, unless only nonhybridized DNA were released from the filter, DNA loss would result in some loss of hybridized RNA. This in turn would tend to make the hybrids appear less thermally stable than they were. To assess the effect this would have on our results, we measured the DNA content of each filter by diphenylamine (Burton, 1968) after incubation at its appropriate melting temperature for hybrids formed at 25°, 2 × SSC–50% formamide; for all hybridization conditions at 33°, 2 × SSC, and

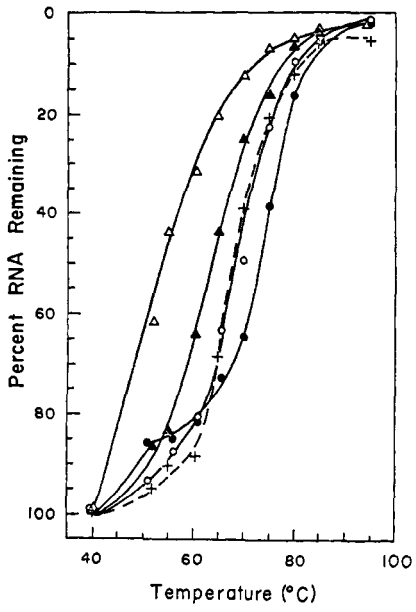

FIGURE 3: Thermal stability profiles of mouse satellite-depleted DNA–cRNA hybrids formed at 37° in formamide. Hybrids were formed by incubating filter-bound mouse satellite-depleted DNA (4.2 μg) with cRNA in 2 × SSC–10^{-3} M EDTA in varying concentrations of formamide in 0.10 ml. All thermal stability measurements were made in 0.5 × SSC. Specific activity of the RNA was 1900 cpm/μg. +- -+, 19.5 μg of RNA, no formamide, 68°; 180 cpm bound, $T_{m,i}$ = 67.2 . ●—●, 17.5 μg of RNA, 70% formamide, 37°; 92 cpm bound, $T_{m,i}$ = 72.5°. ○—○, 19.5 μg of RNA, 50% formamide, 37°; 215 cpm bound, $T_{m,i}$ = 68.4°. ▲—▲, 19.5 μg of RNA, 30% formamide, 37°; 311 cpm bound, $T_{m,i}$ = 63.4°. △—△, 19.5 μg of RNA, 10% formamide, 37°; 643 cpm bound, $T_{m,i}$ = 53.4°. Each point is the average of duplicates.

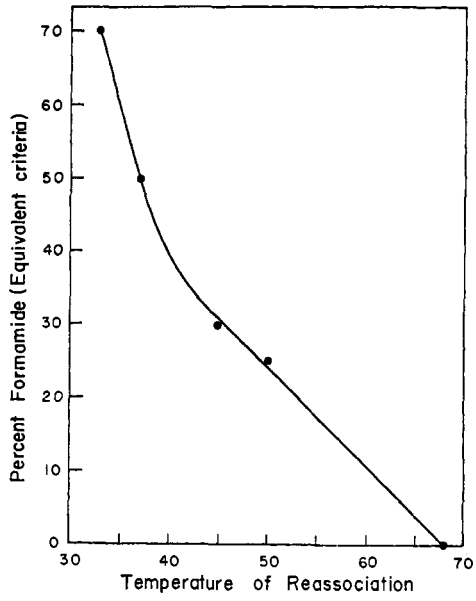

FIGURE 4: Relationship between temperature of reassociation and formamide concentration in the reassociation buffer necessary to produce a criterion equivalent to standard criterion (68°, 2 × SSC). Hybrids were formed in 2 × SSC, 10^{-3} M EDTA in the formamide concentrations and at the temperature indicated.

varying formamide concentration; and for a standard hybrid formed at 68°, 2 × SSC. We made two assumptions: (1) that hybridized DNA was lost from the filter during the melt with the same probability as nonhybridized DNA and (2) that all filters from the same batch and hybridized at the same temperature contained the same amount of DNA at the beginning of the melt. We then calculated the per cent of the RNA remaining at each temperature based on counts per minute of RNA bound microgram of DNA still bound to the filter after the melt to that temperature. As can be seen in Figure 2B, the corrected values changed the $T_{m,i}$'s of the various hybrids slightly but in no case did this change the interpretation of the uncorrected results. Since this is true for the two lowest temperatures surveyed, the remaining data are presented in uncorrected form.

Survey of Formamide-Reassociation Conditions. Since our first experiments showed that the relationship between duplex DNA thermal stability and formamide concentration did not apply to reassociation at low temperatures, we directly examined reassociation specificity as a function of formamide concentration and temperature. The results of a typical experiment are presented in Figure 3. When cRNA–satellite-depleted DNA hybrids were formed in 2 × SSC at 37°, a hybridization buffer that was 50% formamide produced hybrids of the same thermal stability as those produced at the standard criterion. However, the relationship between duplex DNA thermal stability and formamide concentration (McConaughy *et al.*, 1969) predicts that a 37° criterion equivalent to the standard criterion would require 43% formamide in 2 × SSC. Decreases in the formamide concentration reduced the stringency of sequence recognition and hybrids formed with lower $T_{m,i}$'s; but in 70% formamide, hybrids formed which were better paired than those formed at standard criterion.

The results of several such experiments are summarized in Table I and Figures 4 and 5. Examination of Table I and Figure 4 reveals that the relationship derived for the thermal stability of duplex DNA in formamide (McConaughy *et al.*, 1969) correctly predicts equivalent formamide reassociation conditions above 45°; but below that temperature our data indicate that this relationship does not accurately predict equivalent reassociation conditions. Moreover, the lower the temperature below 45°, the greater the deviation between predicted formamide concentration and that determined directly in this study.

Plots of $T_{m,i}$ as a function of formamide concentration in the hybridization buffer for different temperatures (see Fig-

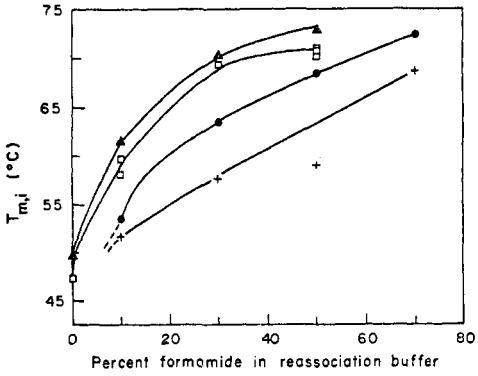

FIGURE 5: Relationship between thermal stability of mouse satellite-depleted DNA–cRNA hybrids and formamide concentration of the reassociation buffer. Temperature of reassociation was 33° (+), 37° (●), 45° (□), or 50° (▲). All thermal stabilities determined in 0.5 × SSC.

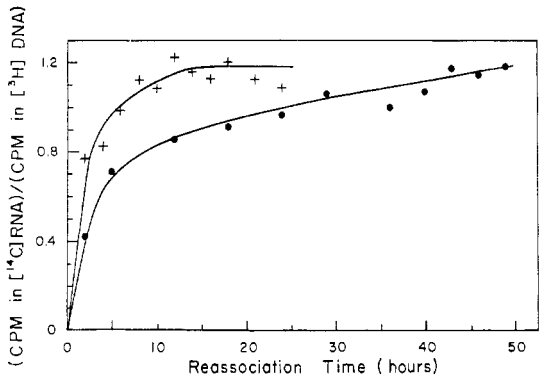

FIGURE 6: Time course of reassociation. Filter-bound mouse [^{3}H]DNA (3 μg) was incubated with 37 μg of [^{14}C]cRNA in 0.20 ml of 2 × SSC–TES–10^{-3} M EDTA at 68° (+—+) or 2 × SSC–10^{-3} M EDTA–50% formamide at 33° (●—●). Specific activity of the RNA was 1200 cpm/μg; specific activity of the DNA was 100 cpm/μg. Cpm bound at the end of the reaction: +—+, 205 cpm of [^{3}H]; 247 cpm of [^{14}C]; ●—●, 306 cpm of [^{3}H], 369 of [^{14}C]. Each point is the average of duplicates.

TABLE I: Deviation from Predicted Value of Formamide Concentration for Criterion Identical with Standard Criterion.

Temperature of Reassociation[a]	% Formamide for Equivalent Reassociation Conditions	
	Predicted[b]	Experimentally Determined
33	50	70
37	43	50
45	32	30
50	25	25
68	0[c]	0[c]

[a] In 2 × SSC–10^{-3} M EDTA and varying concentrations of formamide. [b] From the data of McConaughy *et al.*, 1969. [c] By definition.

ure 5) revealed that there was no simple linear expression relating these parameters although the expected family of curves was generated. Nevertheless, these curves should be useful in guiding the choice of low-temperature-formamide conditions for reassociation at a criterion other than those used in this study.

Rate of Reaction. By directly comparing the extent of reaction as a function of time (Figure 6) it was apparent that the reaction in 50% formamide–2 × SSC at 33° was less than 0.25 as fast as the reaction at 68°, 2 × SSC. The former was still not complete 48 hr after the start of the reaction, whereas the latter was complete by 10–12 hr. Preliminary experiments in 70% formamide at 33° indicated that, as might be expected by the increased viscosity, the reaction rate was further reduced.

Discussion

Use of cRNA and Satellite-Depleted DNA. RNA synthesized *in vitro* from a DNA template has two advantages in these experiments over RNA isolated from whole animals. First, it is easy to prepare milligram quantities of cRNA with higher specific activity than *in vivo* labeled RNA. Second, a larger proportion of DNA sequences, perhaps all of them, will be represented in the *in vitro* RNA; consequently, a larger portion of the DNA will be bound in a hybridization experiment. However, the cRNA will also contain copies of mouse satellite DNA, a fast-reassociating portion of the mouse genome (Waring and Britten, 1966) which does not appear to be transcribed *in vivo* (Flamm *et al.*, 1969). Satellite DNA is 10% of the genome and apparently consists of at least a million identical or near-identical copies of one DNA sequence. Hybrids containing this DNA would be expected to be well-paired and somewhat insensitive to reassociation conditions, as are bacterial nucleic acids (Church and McCarthy, 1968), making thermal stability studies of the repeated portion of the mouse genome more difficult to analyze. Thus the measurements of the thermal stabilities of reassociated cRNA were always made on hybrids of cRNA and satellite-depleted DNA.

Relationship Among Reassociation Temperature, Formamide, and Reassociation Specificity. Based on the previously derived relationship between formamide and duplex DNA thermal stability (McConaughy *et al.*, 1969), reassociation in 2 × SSC–50% formamide at 33° should produce hybrids equivalent to those formed in 2 × SSC at 68°. But the results presented in Figure 2 clearly show that the criterion for reassociation in 2 × SSC at 68° was more restrictive than the low-temperature criterion. These results demonstrate the need to further investigate the effect of formamide and lowered temperature on reassociation specificity in order to establish a low temperature method equivalent to the standard criterion.

An examination of Figures 4 and 5 and Table I leads us to two conclusions. First there is no linear dependence of formamide concentration at equivalent criterion upon the reassociation temperature (Figure 4). Second there is no linear relationship at a given temperature between hybrid thermal stability and formamide concentration in the reassociation buffer (Figure 5). The practical effect of these discoveries is that it is not easy to predict with precision the conditions for DNA–RNA hybridization with formamide which will allow the same degree of base mispairing as that which occurs at 68° in 2 × SSC, or at any other high-temperature criterion. However, there now exist three useful guides for establishing formamide equivalent reassociation conditions: (1) the McConaughy *et al.* (1969) relationship applied above 45°, (2) the relationship presented in Figure 4 (for work equivalent to 68°, 2 × SSC), and (3) the plots of $T_{m,i}$'s (in 0.5 × SSC) as a function of formamide concentration (Figure 5).

Possible Explanations for the Low-Temperature Data. Several arguments might be raised to account for an apparent discrepancy at temperatures below 45° between our work and that previously reported (McConaughy *et al.*, 1969) for the use of formamide during nucleic acid reassociation: (a) hybridization set-up procedure; (b) continuing presence of formamide in the DNA–RNA duplex or in the filter, even after the washing procedure; (c) hydrolysis and subsequent loss of formamide in the hybridization buffer during the 48-hr incubations. The first possibility can be eliminated since the $T_{m,i}$'s of hybrids formed at 45° in 2 × SSC–50% formamide were the same whether the series was prepared and the reaction initiated at room temperature, then incubated at 45°; or prepared at room temperature and initiated after prewarming the reactants to 45°. Also, the $T_{m,i}$ of hybrids formed in 2 × SSC–50% formamide at 33° when the reaction mixture

was warmed to 33° before the addition of the DNA filters was only 2° above the $T_{m,i}$ of hybrids formed under the same conditions but not warmed to 33° before initiating the reaction; the $T_{m,i}$ of the hybrids from the prewarmed series was 5° below the $T_{m,i}$ of the standard criterion hybrids.

The second possibility has not been tested directly. However, even if all the formamide in the hybridization vial had been transferred to the melting buffer, which would then be about 1% formamide, this possibility could not account for the 7° difference in $T_{m,i}$'s. If formamide were hydrogen bonded to the DNA itself, thus interfering with nucleic acid base pairing, we would expect on the basis of model systems (Howard *et al.*, 1966; Huang and Ts'o, 1966) that such single hydrogen bonds would be broken before 40° in 0.5 × SSC.

The last possibility has been tested since aqueous solutions of formamide have been known to undergo slow hydrolysis (Magill, 1934). However, with an Ostwald–Cannon–Fenske viscosimeter (series 200), we were not able to detect any change in viscosity after a 48-hr incubation of 50% or 70% formamide in 2 × SSC–10^{-3} M EDTA, at 37°.

We have already shown (Figure 2B; see Results) that this discrepancy cannot be due to loss of DNA from the filters; nor is it due to incomplete reversal of the formaldehyde reaction with DNA (Figure 1; see Materials and Methods). Finally, since the same phenomenon was noted using *in vivo* RNA and total mouse DNA, the discrepancy cannot be ascribed to a peculiarity of cRNA or satellite-depleted DNA.

Melting Profiles of Hybrids Formed at High Criterion. At criteria higher than the standard criterion we consistently noted a complex melting profile (see Figure 3, 70% formamide curve). In the highest formamide concentrations at 37, 45, and 50°, 15–25% of the RNA was always released by 55°. Control experiments have shown the following: (1) The unstable hybrids did not result from unspecifically bound RNA since they were not detected when *E. coli* DNA or blank filters were incubated with cRNA and then melted; the $T_{m,i}$ of the unspecifically bound RNA was 76°. (2) Preincubation of DNA filters at 68° before hybridization with cRNA in formamide did not reduce the percentage of the RNA in the low-melting fraction; this also argues against the unstable hybrids being the result of DNA loss from the filter during the experiment. (3) Changes in the procedure used for initiating the reactions did not alter the percentage of the RNA that was released from the filter by 55°.

There are several explanations possible for these data. They may be an artifact of formamide hybridization resulting in grossly mispaired hybrids at high criteria. More likely, the unstable hybrids may represent regions in the genome of high AT content and/or short lengths of well-paired sequences.

Rate of Reaction. There are at least three parameters altered in the formamide system, any or all of which might decrease the reaction rate: (1) the increased viscosity of the formamide, (2) the lowered temperature of reaction, (3) the increased dielectric constant of the solvent. We have seen, in fact (Figure 6), that the rate is greatly decreased in 2 × SSC–50% formamide at 33° compared to 2 × SSC at 68°. Furthermore, this fourfold decrease in rate is a minimum estimate since the 33° conditions are not equivalent to the 68° conditions and, therefore, more DNA is available for hybridization at 33° than at 68°.

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