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Nucleic Acid Reassociation in Formamide*

B. L. McConaughy, C. D. Laird, and B. J. McCarthy

ABSTRACT: Conditions are described for effecting deoxyribonucleic acid renaturation and deoxyribonucleic acid–ribonucleic acid hybridization in formamide solutions at low temperatures.

High specificity and rates of reaction can be achieved by appropriate choice of the formamide and salt concentrations and the temperature. Reaction conditions can be deduced from the relationship between formamide concentration and reduction in the thermal stability: 1% formamide reduces the T_m by 0.72°. These methods are valuable for studies of the prolonged reactions occurring between mammalian nucleic acids.

Renaturation of DNA and hybridization of RNA with DNA are normally studied under reaction conditions involving elevated temperatures. The maximum rate of reaction occurs some 25° below the T_m, or mean thermal denaturation temperature (Marmur and Doty, 1961). However, in studies of nucleic acids of complex organisms where reaction times are prolonged, or where biological activity must be preserved, exposure of nucleic acids to high temperatures presents obvious disadvantages such as chain scission and depurination. These difficulties may be avoided by the use of high concentrations of certain salts, such as 6.2 M $NaClO_4$ (Thomas, 1966) or of aqueous solutions of various organic solvents in which the thermal stability of double-stranded polynucleotides is greatly reduced (Herskovits, 1962; Geiduschek, 1962; Levine *et al.*, 1963). A particularly useful organic solvent is formamide in which DNA can be denatured and renatured at room temperature (Helmkamp and Ts'o, 1961; Marmur and Ts'o, 1961; Subirana and Doty, 1966). This principle has been exploited by Bonner *et al.* (1967) to allow the formation of DNA–RNA hybrids with filter-bound DNA at 25° in the presence of formamide.

The present study explores in detail the relationships among the rate and specificity of reaction, the temperature, and the concentrations of formamide and salt. As a result of the incidence of partially related base sequences (Britten and Kohne, 1968), DNA renaturation or DNA–RNA hybridization reactions involving nucleic acids of mammals or other higher organisms may not display locus specificity. In fact, the extent of cross-reaction among related base sequences is dependent upon the reaction conditions such as temperature and ionic strength (McCarthy and McConaughy, 1968; Church and McCarthy, 1968). Thus it is important not only to establish conditions for high rates of reaction and species specificity but also to characterize the nature of the duplexes being formed. Although in aqueous solution cross-reaction among related base sequences is reduced at higher temperatures or lower ionic strengths (McCarthy and McConaughy, 1968), specificity conditions are much more easily adjusted by variation of ionic strength and formamide concentration. High specificity may be obtained at low temperatures, with rates of reaction several times higher than those obtained at 60 or 70° in aqueous solution.

Materials and Methods

Formamide. The formamide used in these studies was purchased from the Eastman Kodak Co. Optical studies in formamide solution require high solvent purity. The criterion applied was that the optical density of 100% formamide at 270 mμ in 1-cm path-length cells not exceed 0.15. All of the experiments described here were carried out with formamide which meets these specifications.

Isolation of Nucleic Acids. Bacillus subtilis DNA was prepared as described by Marmur (1961) and ^{3}H-labeled DNA was extracted from cells grown in minimal medium to which 0.5 μCi/ml of thymidine-methyl-H^3 was added per hour for 4 hr.

DNA was isolated from *Drosophila melanogaster* pupae by a procedure described elsewhere (Laird and McCarthy, 1968).

Mouse and hamster DNA were extracted from liver as previously described by Church and McCarthy (1968). Labeled mouse DNA was prepared from mouse L cells grown in monolayers with 2 μCi/ml of thymidine-methyl-^{3}H added to the medium (McCarthy and McConaughy, 1968).

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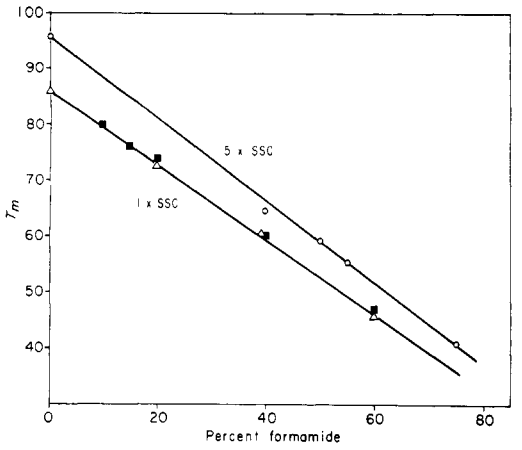

FIGURE 1: Dependence of the T_m of native *B. subtilis* DNA or *B. subtilis* DNA–DNA duplexes upon the concentration of formamide. The melting point of the native DNA in various concentrations of formamide and either 1 × SSC (△) or 5 × SSC (○) was determined optically at 270 mμ. The DNA–DNA duplexes were formed by incubating 1.2 μg of sheared, denatured, ^{3}H-labeled *B. subtilis* DNA with 12 μg of filter-bound *B. subtilis* DNA at 60° in 1 × SSC for 16 hr. These duplexes were then dissociated by heating the filters to increasing temperatures in 2 ml of 1 × SSC containing various concentrations of formamide. (■) Labeled DNA eluted at each temperature was collected by precipitation with trichloroacetic acid and counted.

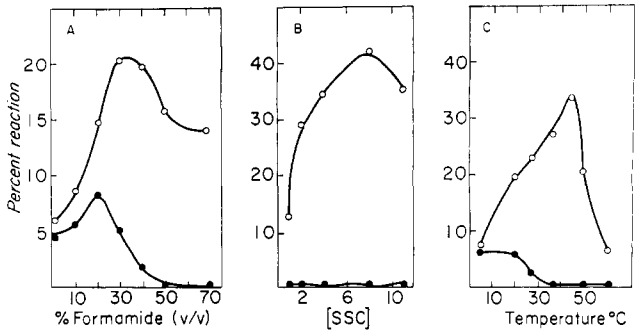

FIGURE 2: Extent of reaction and specificity of DNA–DNA duplex formation in formamide; 1.2 μg of denatured, ^{3}H-labeled *B. subtilis* DNA (9100 cpm) was incubated in 0.2 ml of solvent with 12 μg of filter-bound *B. subtilis* DNA, or 14 μg of filter-bound rabbit DNA, for 22 hr, at conditions described below. At the end of the reaction, the filters were washed for 3 min in 5 ml of the solvent and at the temperature of reaction, and then for 3 min in 5 ml of 2 × SSC to remove the formamide. Per cent of labeled input DNA bound is plotted for the homologous *B. subtilis* DNA (○) and heterologous rabbit DNA (●) filters. (a) Extent of reaction at 20° in 2 × SSC with various concentrations of formamide. (b) Extent of reaction at 37° in 30% formamide at various concentrations of SSC. (c) Extent of reaction in 2 × SSC and 30% formamide at various temperatures.

Pulse-labeled RNA was prepared from L cells as described earlier (Shearer and McCarthy, 1967).

Preparation of DNA Filters. Unlabeled high molecular weight denatured DNA was preadsorbed to nitrocellulose membrane filters according to a method outlined previously (McCarthy and McConaughy, 1968). The filters were saturated with DNA so that a filter with a diameter of 5 mm contained approximately 12 μg of DNA. These filters were incubated for 1 hr at room temperature in the preincubation medium specified by Denhardt (1966) to reduce the background binding in subsequent experiments.

DNA–DNA Duplex Formation and DNA–RNA Hybridization. Radioactively labeled DNA used for duplex reactions was sheared at 12,000 psi in the French pressure cell and denatured by heating to 95° for 10 min followed by fast cooling to 0°. The appropriate amounts of formamide and salt were then added to make up the reaction mixtures. These mixtures were incubated under various conditions with DNA preadsorbed to membrane filters. All reactions were carried out in a total volume of 0.2 ml and at a ratio of DNA on the filter to that in solution of 10:1 so as to minimize the reaction in solution.

Pulse-labeled RNA used for DNA–RNA hybridization was heated to 95° for 10 min and fast cooled. Incubations with filter-bound DNA were carried out at a DNA/RNA ratio of 20:1 in a total volume of 0.2 ml. Other reaction components and conditions are indicated in the figure legends.

At the end of the incubation period the filters were washed in 2 ml of the incubation mixture at the reaction temperature. The filters were then washed twice with 2 ml of SSC (0.15 M NaCl and 0.015 M sodium citrate) to remove the formamide. It is important to rinse away the formamide prior to counting the filters in a toluene-based scintillation fluid so as to avoid quenching.

Thermal Dissociation Profiles. The stability of DNA–DNA or DNA–RNA complexes was assayed by washing the filters at increasing temperatures to obtain thermal dissociation profiles. The duplexes or hybrids were dissociated in 2 ml of 1 × SSC and filters were removed at various temperatures, dried, and counted. The amount of radioactivity remaining on the filter after washing at a particular temperature is a measure of the stability of the complex under those conditions. In some experiments the amount of denatured DNA removed from the filter was determined by precipitation of the eluent with trichloroacetic acid.

The thermal stability of DNA–DNA duplexes formed in solution was characterized by hydroxylapatite chromatography (Bernardi, 1965; Miyazawa and Thomas, 1965). The incubation mixture was diluted with 0.12 M phosphate buffer (pH 6.8) to reduce the formamide concentration below 1% and the sample was then adsorbed to a hydroxylapatite column at 60°. Under these conditions double-stranded DNA binds to the column, whereas single-stranded DNA is eluted. The temperature of the column was then raised and the column washed at various temperatures with the phosphate buffer. The amount of single-stranded DNA eluted at a particular temperature represents the amount of duplex which has been dissociated at that temperature.

Optical Studies of Denaturation and Renaturation. Most optical measurements on nucleic acids are made at 260 mμ. However, since the solvent absorbs strongly at this wavelength, all optical studies described here were monitored at 270 mμ where DNA still has a large absorption (~80% of that at 260 mμ) whereas absorption by the solvent is low. The spectral measurements were carried out in a Beckman DU spectrophotometer fitted with a Gilford automatic recorder. A Haake circulating bath was used to control the temperature which was monitored on the recorder.

The decrease in thermal stability of DNA as a function of formamide concentration was measured optically by denatur-

ing *Bacillus subtilis* DNA in various concentrations of formamide in 1 × SSC or 5 × SSC. Denaturation experiments were carried out at a DNA concentration of 50 μg/ml in cuvets with a 1-cm path length. Only DNA which exhibited a hyperchromic shift of at least 35% was considered pure for spectral measurements. The midpoint, T_m, of the hyperchromic transition was determined as a function of formamide concentration.

The rates of renaturation of mouse liver, *B. subtilis*, and *Drosophila melanogaster* DNA under various conditions of salt, temperature, and formamide concentration were measured in the same apparatus used for the denaturation studies. The DNAs were sheared at 12,000 psi in the French pressure cell before adding other components of the reaction mixture. The renaturation reactions were carried out in glass-stoppered 1-cm path-length cells at a DNA concentration of about 50 μg/ml or in 1-mm path-length cells (at about 1 mg/ml) covered with mineral oil to prevent evaporation during the renaturation. The temperature was increased to 15° above T_m and maintained for 10 min to effect complete strand separation. The temperature was then rapidly cooled to 20° below the T_m and the subsequent decrease in optical density monitored as a function of time.

Results

Reduction of Thermal Stability by Formamide. The effectiveness of formamide in reducing the thermal stability of double-stranded polynucleotides was determined by studying the relationship between formamide concentration and the T_m or mean thermal stability. This relationship was determined in two ways: by optical studies of the denaturation of DNA and by the thermal dissociation of duplexes formed between radiolabeled, sheared, and filter-bound denatured DNA. The first is, of course, a measure of reversible denaturation and the second of irreversible strand dissociation (Geiduschek, 1962). The results presented in Figure 1 show that a linear relationship exists between the T_m and the per cent formamide and that approximately the same slope is obtained for salt concentrations of 1 × or 5 × SSC. The T_m of native DNA or filter-bound duplexes is reduced approximately 0.72° per 1% formamide. This relationship was used in the design of the other experiments to be reported. A similar relationship was found for the thermal dissociation of DNA–RNA hybrids bound to filters.

Formation of Duplexes with Filter-Bound DNA. The effects of variations in the salt and formamide concentrations and temperature on the extent and specificity of DNA–DNA duplex formation were also explored with the DNA-filter method. At room temperature in 2 × SSC labeled *B. subtilis* DNA reacts equally well with filters containing homologous or rabbit DNA (Figure 2A). When formamide is added to the reaction mixture, there is an increase in both the extent and the specificity of the reaction as judged by the relative binding to homologous and heterologous DNA filters. High specificity and extents of reaction are obtained in the presence of 50% formamide. Using the relationship discussed above, the T_m of *B. subtilis* duplexes under these conditions would be approximately 52° [*i.e.*, 87° − (0.7° × 50)]. The reaction also occurs with similar rates in the presence of 70% formamide where the T_m would be approximately 38°. Thus, as in the case of the renaturation of DNA in solution, optimal conditions are found between 10 and 30° below the T_m (Marmur and Doty, 1961). Initial rates of reactions, as well as extents of reaction after long incubations, are increased in higher salt concentrations in formamide solutions (Marmur *et al.*, 1963; Nygaard and Hall, 1964). In 30% formamide at 37°, maximal rates occurred at about 8 × SSC (Figure 2B). The relationship between formamide concentration and temperature is further illustrated in Figure 2C where it is apparent that with a concentration of 30% formamide the maximum extent of reaction in 2 × SSC was obtained at about 45°, again some 20–25° below the T_m of *B. subtilis* DNA in this solvent.

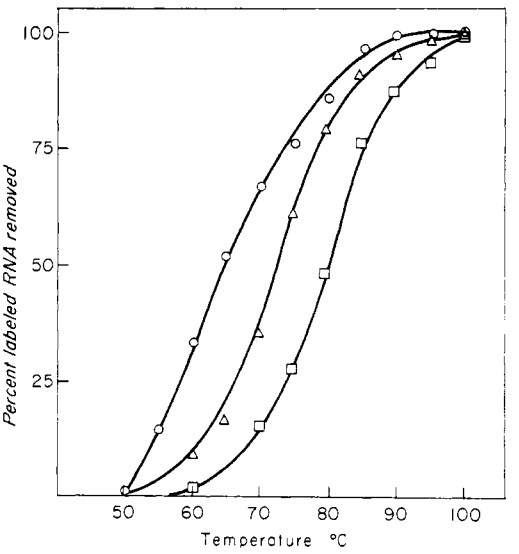

FIGURE 3: Thermal dissociation profiles of mouse DNA–RNA hybrids formed by incubating 1.5 μg of ^{3}H-pulse-labeled nuclear L-cell RNA and 30 μg of mouse DNA bound to filters. The reactions were carried out in 0.2 ml of 2 × SSC and various concentrations of formamide for 16 hr at 50° (○, no formamide; △, 25%, □, 50%). The filters were then washed in 5 ml of the incubation solvent at 50° and then in 5 ml of 1 × SSC at 22° to remove the formamide. The hybrids were dissociated in 1 × SSC as described in the legend to Figure 1.

Thus it appears that the most efficient conditions for the renaturation of *B. subtilis* DNA may be obtained by using fairly high salt concentrations and adjusting the formamide concentration so that the incubation temperature used is about 20 or 25° below the T_m. More extensive optical studies of the renaturation of *B. subtilis* DNA to be described in later paragraphs confirm this point. However, in studies of DNA renaturation or DNA–RNA hybrid formation using nucleic acids of higher organisms, the optimum reaction rate and the species specificity are not the only important considerations in designing experimental conditions. The DNA of most complex organisms contains many partially related base sequences presumably resulting from gene duplication during earlier evolutionary history (Britten and Kohne, 1968). Many of these are sufficiently similar in base sequence to cross-react unless the conditions of specificity are extremely stringent. The extent of cross-reaction among related base sequences is a continuous function of the incubation temperatures, as illustrated by the thermal dissociation profiles of DNA–DNA or DNA–RNA duplexes formed at various temperatures (McCarthy and McConaughy, 1968; Church and McCarthy,

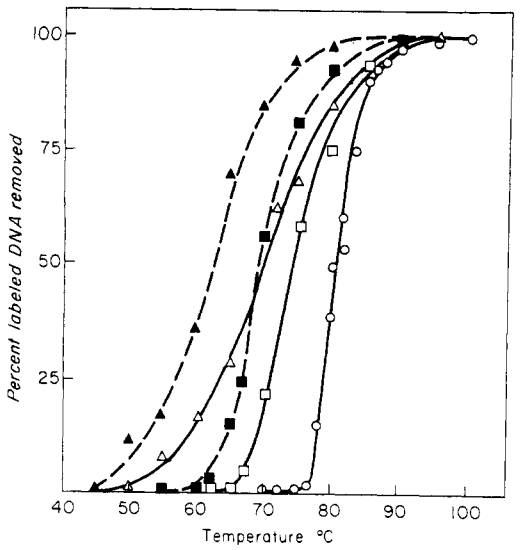

FIGURE 4: Thermal dissociation profiles of duplexes formed by incubating 1.2 μg of ^{3}H-labeled, sheared, denatured L-cell DNA with 12 μg of mouse DNA or hamster DNA bound to filters. The reactions were carried out at 45° for 16 hr in 0.2 ml of 1 × SSC containing various concentrations of formamide. The amount of formamide used and the extent of reaction are as follows. (a) Mouse DNA: △, 8% formamide, 10.3% reaction; □, 25% formamide, 6.4% reaction; ○, 50% formamide, 2.0% reaction. (b) Hamster DNA: ▲, 8% formamide, 3.7% cross-reaction; ■, 25% formamide, 0.88% cross-reaction. There was no measurable cross-reaction with hamster DNA in 50% formamide. Filters were washed in 5 ml of the incubation medium at 45° and then in 5 ml of 1 × SSC. Dissociation of the duplex was accomplished by heating the filters to increasing temperatures. A filter was removed and counted at each indicated temperature.

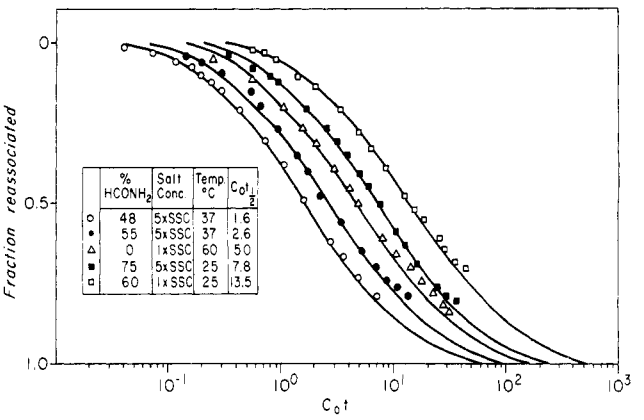

FIGURE 5: The rate of renaturation of sheared denatured *B. subtilis* DNA as a function of temperature and the concentration of salt and formamide. The DNA was denatured at a concentration of 50 μg/ml in a recording spectrophotometer and the rate of renaturation under the conditions shown in the inset was followed by the hypochromicity at 270 mμ. The fraction of DNA reassociated, plotted as a function of the initial concentration (moles of P per liter) multiplied by the time (seconds). The data obtained are plotted on curves representative of theoretical second-order reactions.

1968). Thus the conditions of maximal reaction rate are not necessarily those of choice even though cross-reactions with the DNA of unrelated organisms may be minimal (McCarthy and McConaughy, 1968; Church and McCarthy, 1968).

In view of these complications it is desirable to characterize the DNA–DNA or DNA–RNA duplexes formed under each reaction condition and to compare them with products formed in aqueous solution. Figures 3 and 4 illustrate two such experiments designed to assess the specificity of conditions in which various concentrations of formamide were used. Figure 3 displays the thermal dissociation profiles of DNA–RNA hybrids formed between ^{3}H-pulse-labeled nuclear L cell RNA and mouse DNA at 50° in solutions containing various amounts of formamide. As the conditions of stringency are increased through the addition of formamide, the mean dissociation temperature, measured in the absence of formamide, increases in accordance with the formation of more closely matched DNA–RNA hybrids. Comparison of these profiles with those obtained after incubation in aqueous solution at various temperatures (Church and McCarthy, 1968) allows the specificity of the conditions to be assessed. For example, hybrids as well paired as those obtained in the presence of 50% formamide are obtainable in aqueous environments only by incubating at 75° (Church and McCarthy, 1968).

A parallel experiment illustrates the thermal stabilities of DNA–DNA duplexes formed at higher formamide concentrations (Figure 4). Labeled mouse DNA was incubated with filters containing mouse or hamster DNA at 45° in the presence of increasing concentrations of formamide. The T_m of the duplexes formed increases with formamide concentration although those formed with hamster DNA are always less stable than those with mouse DNA under corresponding conditions. This illustrates the utility of the formamide method for attaining high specificity without subjecting the nucleic acids to high temperature.

Optimal Rates of Reaction in Formamide. A more detailed examination of the dependence of rate of reaction upon the temperature and concentrations of salt and formamide was made through a study of the time course of renaturation of *B. subtilis* DNA. The reassociation of sheared denatured *B. subtilis* DNA was followed by measurements of hypochromicity. The results are expressed in terms of the fraction of DNA reassociated as a function of the concentration multiplied by the time (Britten and Kohne, 1968). The results of several experiments are assembled in Figure 5 in which the curves represent theoretical functions for second-order reactions. Rates of renaturation in formamide are compared with the standard condition of 60° in SSC, where the reaction is half-completed by a C_0t of about 5. This value is close to that obtained for *Escherichia coli* DNA (Britten and Kohne, 1968) in agreement with the similarity of the total amount of DNA per cell in these two organisms.

The compositions of the other incubation mixtures were chosen in accordance with the known relationships between the T_m and the formamide concentration (Figure 1) and the T_m and the ionic strength (Marmur *et al.*, 1963). In addition it is clear that optimal rates of renaturation occur some 20–25° below the T_m (Marmur and Doty, 1961). One example will suffice to illustrate the principles with which the reaction conditions are designed. At 25°, optimal rates will be expected under conditions where the T_m is about 45–50°. In standard SSC, the T_m of *B. subtilis* DNA is approximately 88°. Thus to reduce the T_m to 45° approximately 60% formamide must be

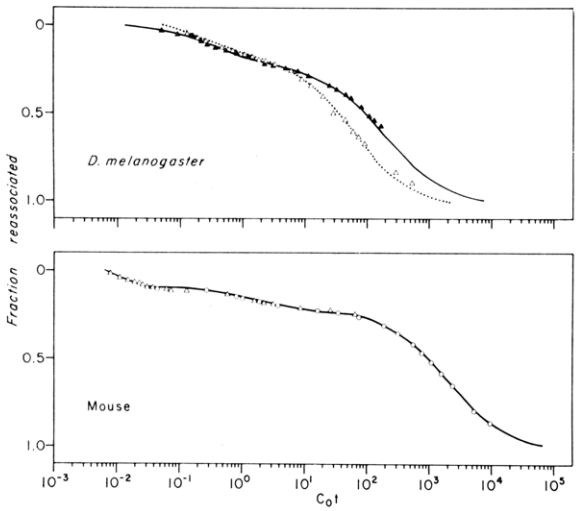

FIGURE 6: The renaturation kinetics of *D. melanogaster* DNA and mouse DNA. The rate of renaturation of denatured *D. melanogaster* DNA in SSC at 60° (▲) or 5 × SSC containing 48% formamide at 37° (△) was followed as described in Figure 5. The rate of reassociation of denatured mouse DNA was measured in 5 × SSC containing 48% formamide at 37° at two concentrations in cuvets with different light paths: 50 μg/ml, 1-cm light path (△) and 500 μg/ml, 1-mm light path. The initial 10% which represents the mouse satellite, reassociated before the temperatue was equilibrated at 37°. The rate of renaturation for the remaining 90% is plotted on curves which represent ideal second-order kinetics for a 15% component (redundant sequences) and a 75% component (unique sequences).

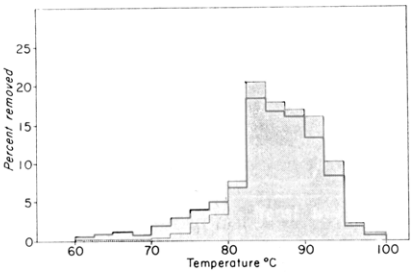

FIGURE 7: Thermal dissociation profiles of *B. subtilis* or *D. melanogaster* complexes by hydroxylapatite chromatography. The incubation mixture contained 30 μg/ml of ^{14}C-labeled *B. subtilis* DNA (120 cpm/μg) and 600 μg/ml of ^{3}H-labeled *D. melanogaster* DNA (10 cpm/μg). The DNA was sheared and denatured prior to incubation in 60% formamide–0.30 M phosphate buffer at room temperature for 5000 hr. This corresponds to a C_0t value of 32,000 for *D. melanogaster* DNA and 1600 for *B. subtilis* DNA. The sample was then diluted 60-fold in 0.12 M phosphate buffer (pH 6.8) and adsorbed to a 3-ml hydroxylapatite column. The column was washed twice with 5-ml aliquots of the phosphate buffer. The temperature of the column was raised in 2.5° increments and washed twice at each temperature. The DNA eluted at each temperature was collected by precipitation with trichloroacetic acid and counted. The shaded area represents the profile for *B. subtilis* DNA.

added according to the relationship established in Figure 1. Alternatively, if higher ionic strength is used to increase the reaction rate, more formamide must be added to reduce the thermal stability of the renatured DNA.

The time course of renaturation at 25° is illustrated for two different concentrations of salt and formamide. In both cases the rates of reaction are slower than at 60° in SSC although at higher ionic strength the reaction rate is increased. By raising the temperature to 37° a considerable increase in reaction rate may be obtained particularly when the ionic strength is high. Two such conditions are illustrated in Figure 5, corresponding to about 20° or about 25° below the T_m.

Since the concentrations of DNA and reaction times for the renaturation of virus or bacterial DNA are relatively short, the use of formamide adds very little convenience to experiments of this type. However, for complex DNA, the method is of obviously greater value since the much more prolonged incubations may be carried out at temperatures which do not damage the nucleic acids. The utility of this approach is illustrated in Figure 6 by the renaturation kinetics of *D. melanogaster* and mouse DNA. The renaturation of *D. melanogaster* DNA is shown both at 60° in SSC and at 37° in 5 × SSC containing 48% formamide. It should be noted that both *Drosophila* and mouse DNA and in fact most higher plants and animals have DNA of approximately 40% G + C as does *B. subtilis*. Thus the optimization of reaction conditions for *B. subtilis* DNA (Figure 5) may also be applied to such DNAs. As in the case of the bacterial DNA, the reaction in formamide at 37° occurs at some three times the rate of that at 60°. Under both sets of conditions *D. melanogaster* DNA shows some 15% redundant sequences confirming earlier observations (C. D. Laird and B. J. McCarthy, submitted for publication). The remainder renatures as a single component of unique sequences. By comparison with the data for *B. subtilis* DNA this rate may be used to calculate the genome size of *D. melanogaster* (C. D. Laird and B. J. McCarthy, submitted for publication). Likewise, the renaturation kinetics of mouse DNA reveals three distinct components: the rapidly renaturing mouse satellite DNA, partially redundant sequences, and unique sequences. The overall profile is very similar to that determined at 60° by Britten (1968).

Renaturation Studies using Hydroxylapatite. As an alternative to measurements of hypochromicity the renaturation of DNA may be followed by chromatography on hydroxylapatite (Bernardi, 1965; Miyazawa and Thomas, 1965). This technique depends upon the adsorption of double-stranded DNA under conditions where single-stranded DNA remains in solution (Britten and Kohne, 1968). Reactions occurring in formamide solutions may also be analyzed in this way providing that the sample is diluted with 0.12 M phosphate buffer so that the formamide concentration is below 1% before application to the column. In these cases reactions should be carried out in the absence of sodium citrate since this adversely affects the hydroxylapatite separation. When these precautions are observed kinetic experiments or isolations of differentiately renaturing components may be performed as described by Britten and Kohne (1968).

Hydroxylapatite chromatography can also be used to characterize the thermal stability of renatured duplexes. By gradually raising the temperature of the column, duplexes are dissociated and removed at a characteristic temperature reflecting their base composition and the degree of base pairing (Miyazawa and Thomas, 1965). For example, with the DNA of eucaryotic organisms, the thermal elution profile of DNA renatured to completion provides information as to the fraction of redundant sequences which form mismatched duplexes.

This principle is illustrated in Figure 7, which shows the thermal elution profile of a mixture of *B. subtilis* DNA and *D. melanogaster* DNA renatured for an extended period at 25°. Since *B. subtilis* DNA contains few redundant base sequences other than the five or so ribosomal cistrons (Kohne, 1968; McCarthy and McConaughy, 1968) and is approximately the same base composition as *D. melanogaster*, the deviation between the two thermal elution profiles may be used as an estimate of the fraction of base sequences forming mismatched complexes. Approximately 10% or so of *D. melanogaster* DNA is distinguishable in this way corresponding well to the estimate made from the kinetics of renaturation (Figure 6) (Laird and McCarthy, 1968, and submitted for publication, 1969).

Conclusions

Some obvious advantages deriving from the use of formamide solutions for nucleic acid annealing or hybridization reactions have been pointed out by Bonner *et al.* (1967) and Weiss *et al.* (1968). These include the decreased degradation of nucleic acids at the lower temperatures used and increased retention of DNA bound to membrane filters. To these we would add the attainment of higher reaction rates and specificity without high temperatures and the absence of microbial growth in formamide solutions even during extended incubation at 37°. Most important, however, is the flexibility introduced into the design of reaction conditions suitable for a given experiment. With nucleic acids of complex organisms this flexibility is extremely important since a multitude of DNA–DNA or DNA–RNA complexes of different degrees of base pairing may be formed depending upon the reaction conditions (McCarthy and McConaughy, 1968; Church and McCarthy, 1968). It is more convenient, as well as less damaging to nucleic acid molecules, to control this specificity with formamide rather than through adjustment of the incubation temperature.

The above paragraphs have illustrated the principles which underlie the choice of formamide concentration, salt concentration, and temperature. At a given temperature this choice depends upon the T_m of the duplexes and the established linear relationship between formamide concentration and the depression of T_m (Figure 1). We wish to emphasize the importance of the correct choice of reaction conditions to control specificity and to point out that this imposes some restriction upon the range of useful conditions. For example, below room temperature, the amount of formamide necessary to depress the T_m is prohibitively high and introduces difficulties in assembling the various components of the reaction mixture. In addition rates of reaction with simple bacterial DNA are low. The same decrease in reaction rate may not, however, be observed with complex DNA because of the existence of high levels of distantly related nucleotide sequences revealed only with low reaction specificity (McCarthy and McConaughy, 1968). According to the principles established in this paper, it appears that the reaction conditions proposed by Bonner *et al.* (1967) for the hybridization of pea DNA and RNA in 30% formamide at 0 or 24° are of quite low specificity. The theoretical T_m of well-paired complexes under these conditions is some 40–60° above the incubation temperatures. Thus it appears that these conditions do not meet the requirements for specificity necessary for meaningful hybridization experiments with nucleic acids of eucaryotic cells.

A good compromise between low reaction rate and nucleic acid degradation is provided by a temperature of 37°. This has the added advantage of availability of temperature control at 37° in most laboratories. At this temperature reaction rates may be augmented by increase in ionic strength without calling for excessively high concentrations of formamide. No doubt rates may be further increased through the addition of more salt but the improvement is likely to be small and practical difficulties are encountered in the preparation of the reaction solution. Alternatively it is possible that other organic solvents which are more effective in reducing the thermal stability of double-stranded nucleic acids may be substituted for formamide (Legault-Demaré *et al.*, 1967).

The obvious utility of the formamide method for studying the renaturation of complex DNAs over extended periods applies equally to the formation of DNA–RNA hybrids from mammals or other higher organisms. By entirely analogous methods extensive hybridization of mouse RNA with the unique sequences of mouse DNA has been obtained. These reactions were carried out in formamide for extended periods (Shearer, 1969). This possibility adds a new dimension to the analysis of gene expression in mammalian cells. These experiments will be described in detail elsewhere.

Acknowledgments

We are indebted to Richard Hull for his excellent technical assistance.

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Purification and Properties of Dog Pancreas Ribosomes and Subunits*

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ABSTRACT: Dog pancreas ribosomes can be purified by chromatography over DEAE-cellulose. The particles are eluted by a gradient of KCl and are successively removed from the column as subunits: (1) 38 S, (2) 57 S, contaminated by some 38 S.

This distribution has been demonstrated by centrifugation of the peak fractions through a 10–30% sucrose gradient of similar composition as the solution in which they were eluted; *i.e.*, 0.01 M Tris, pH 7.3 (25°), 3 mM Mg^{2+}, 0.5 M KCl, and 15 mM mercaptoethanol. The optimum conditions for phenylalanine incorporation have been determined. On incubation in an amino acid incorporation system, the subunits reassemble to monomers. The 38S and the 57S particles have been further purified by preparative-scale sucrose gradient centrifugation. Their phenylalanine incorporation activity was tested. In the absence of polyuridylic acid, neither subunit separately, nor their mixture, demonstrated significant activity. Similarly, incorporation of isoleucine, valine, or lysine was essentially zero. Phenylalanine incorporation in the presence of polyuridylic acid was zero with the 38S particle, 40 $\mu\mu$moles/mg with the 57S particle, and 350 $\mu\mu$moles per mg with the mixture.

The relative ease of purification of bacterial ribosomes, their separation from endogenous mRNA by preincubation, and the stability exhibited by both monomers and subunits have all contributed to the rapid advances which utilize these nucleoprotein particles. In contrast, comparable studies with mammalian ribosomes have been less rapid, largely because of the difficulties in purifying active ribosomes. Since extraneous protein may decrease the functional activity as well as the stability of ribosomes, its removal is essential. Similarly, the presence of endogenous mRNA may compete or hybridize with that added, thus complicating the interpretation of the data. Our first objective in the study of ribosomes from a mammalian source has thus been to purify them according to these two criteria.

Most samples of mammalian ribosomes have consisted of RNA and protein in approximately equal amounts (Petermann, 1964). Beeley *et al.* (1968) obtained dog pancreas ribosomes which analyzed 60% RNA, but their polypeptide-synthesizing activity was not tested. We have found that ribosomes from dog pancreas can be purified by chromatography over DEAE-cellulose to yield biologically active particles of this composition. A KCl gradient is used for elution from the column. The ribosomes dissociate into subunits in the process and a preliminary separation can be made. The eluate is intermediate in composition between the conditions for dissociation established by Martin and Wool (1968) for muscle ribosomes (high ionic strength, pH, and Mg concentration) and that of T'so and Vinograd (1961) for reticulocyte ribosomes (moderate to low ionic strength and zero concentration of Mg). Crude subunits can be further purified by centrifugation through a sucrose gradient of high ionic strength. Under these conditions the endogenous mRNA is largely removed.

The work to be reported includes a description of the purification procedures, gross analyses, and sedimentation studies of the ribosomes and their subunits. Phenylalanine incorporation with and without poly U has been utilized as a measure of polypeptide synthesis capability and of endogenous mRNA content.

Experimental Procedure

Materials. The tissue for most of this work was obtained by excision of fresh pancreas from dogs. This was immediately chilled and stored at −20° or was purchased from Pel-Freez Biologicals, Inc., Rogers, Ark. PMSF[1] was obtained from

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[1] The abbreviations used are: DOC, sodium deoxycholate; ME, mercaptoethanol; PMSF, phenylmethylsulfonyl fluoride; RNP, ribonucleoprotein; TES, tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.