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City University of New York PH.D. 1982

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TECHNIQUES FOR ACCELERATING DNA RENATURATION AND THEIR PRELIMINARY APPLICATION TO GENE ISOLATION METHODS

by

ROBERT WIEDER

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1982

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This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirements for the degree of Doctor of Philosophy.

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ABSTRACT

TECHNIQUES FOR ACCELERATING DNA RENATURATION AND
THEIR PRELIMINARY APPLICATION TO GENE ISOLATION METHODS

by Robert Wieder

Adviser: James G. Wetmur, Ph.D.

Optimum methods for the acceleration of DNA renaturation were investigated. Renaturation rates in solution are effectively accelerated a maximum of ten fold by the addition of NaCl, LiCl, dextran sulfate, NaCl and dextran sulfate or LiCl and dextran sulfate to 2.4 M tetraethylammonium chloride. Acceleration of 100-fold may be achieved by volume exclusion with 35-40% dextran sulfate in 1 M NaCl at 70°C. Renaturation kinetics remain second order and temperature dependent, but become independent of dextran sulfate concentration if sufficient dextran sulfate is used. Dextran sulfate may be selectively precipitated by use of 1 M CsCl.

Reassociation kinetics of DNA at high concentrations on the surface of a phenol emulsion were also investigated. Apparent second order rate constants fall on two intersecting

straight lines when presented as a function of DNA concentration on a log-log plot. The intersection occurs when the available catalytic surface is saturated. The slopes of these lines -0.3 and -1.3 for the low and high concentration ranges respectively are the same in different solvents and at different temperatures. At high DNA concentration, high complexity heterologous denatured DNA apparently competes 2-4 times better for the surface than homologous DNA because it does not participate in the reassociation reaction. Native and partially native DNA molecules cannot compete with single-stranded DNA for a saturated surface. At high DNA concentrations, rates become inversely dependent on the single-strand DNA length.

Both methods of accelerating DNA reassociation rates were employed in experiments carried out in attempts to isolate sequences specific for the Y chromosome of the rat. Three potential batch methods for the isolation of any insertion were attempted. They were deamination of female rat DNA and endonuclease S1 cleavage of the male/female heteroduplexes, BND chromatography of sonicated female DNA/restricted male DNA heteroduplexes and CsCl density gradient centrifugation of the products of renaturation of density-labeled female rat DNA and unmodified male rat DNA. Extensive damage was suffered by the DNA during long renaturation steps with either rate acceleration technique. This prevented significant enrichment of Y-specific sequences. The CsCl density gradient method was found to be useful for separating less complex DNA fragments.

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CHAPTER I. KINETICS OF DNA RENATURATION

A. HISTORICAL PERSPECTIVES

1. Early Studies

When homologous single-strands of DNA are incubated under the appropriate conditions, they renature to a helical conformation. The earliest observations of DNA renaturation were made by genetic (Marmur and Lane, 1960), physico-chemical (Doty, et al., 1960) and immunologic (Levine, et al., 1960) methods. Many factors affecting DNA renaturation were studied by Marmur and Doty (1961) and reviewed by Marmur, Rownd and Schildkraut (1963).

A bell-shaped temperature dependence of renaturation was demonstrated by Ross and Sturtevant (1960) and Marmur and Doty (1961), and confirmed by Britten and Kohne (1966). The temperature dependence was the subject of several mathematical studies (Saunders and Ross, 1960; Flory, 1961; Kallenbach, et al., 1963). An ionic strength effect on renaturation rates was also found (Marmur and Lane, 1960; Marmur and Doty, 1961; Britten and Kohne, 1966; Wetmur and Davidson, 1968).

An inverse relationship was observed between the extent of renaturation and the amount of DNA in a bacteriophage, bacterium or cell (Marmur and Doty, 1961). This phenomenon was attributed to the heterogeneity of the DNA, later to be termed complexity. Cells of eukaryotic organisms were found to have at least three complexity classes of DNA differing by as much as a factor of 10^6 in renaturation rates (Britten and Kohne, 1966; 1968).

A dependence of renaturation rates on the size of the DNA molecules was observed (Marmur and Doty, 1961) and determined to be proportional to the square root of the length of the segments (Wetmur and Davidson, 1968). The renaturation rate of molecules of different lengths is proportional to the square root of the length of the shorter of the two strands (Wetmur, 1971). DNA renaturation was found to be concentration dependent (Doty, et al., 1960) and to follow second order kinetics (Subirana and Doty, 1966). A factor of two drop in the rate constant over a 100-fold increase in DNA concentration (Subirana and Doty, 1966) is a discrepancy which will be discussed later in this chapter.

Renaturation rates were found to be inversely proportional to the microscopic viscosity of the solution (Subirana and Doty, 1966; Thrower and Peacocke, 1966) but relatively independent of the macroscopic viscosity. It was postulated that the rate-determining factor was the rate of coming together of the strands and not their rate of rewinding once in contact (Thrower and Peacocke, 1966). Wetmur and

Davidson (1968) calculated that the reaction was not rapid enough to be subject to diffusion control, leaving the alternative possibility of an excluded volume effect. This point will be discussed later in this chapter.

Wetmur and Davidson (1968) set forth a systematic mathematical interpretation of the factors affecting DNA renaturation. The following is a list of these factors:

a. DNA renaturation in solution is a second order reaction which proceeds by a slow, rate determining nucleation event followed by a rapid zippering.
b. The reaction rate constant is temperature dependent, with a broad, flat maximum at about 20-30°C below the melting temperature (T_m). There is a decrease in rate with either elevation or diminution of the temperature from this range.
c. The reaction rate is inversely proportional to the microscopic viscosity of the solution.
d. The reaction is highly dependent on Na^+ concentration at low ionic strength. The rate changes very little, however, with the increase of Na^+ concentration above 0.4 M.
e. At 0.4 M Na^+, the rate is essentially independent of pH in the 5 to 9 range.
f. The rate constant is inversely proportional to the complexity of the DNA, (N), in nucleotide units.
g. The rate constant for DNA degraded to single-stranded molecular length L expressed in nucleotides is proportional to $L^{0.5}$. Wetmur (1971) later showed that, in fact, the rate constant is proportional to the square root of the

length of the shorter of the two reacting strands.

h. For a given N and L, the rate constant increases slightly with the G+C content of the DNA.

2. Methods of Plotting Renaturation Rate Data

Wetmur and Davidson (1968) described the rate of disappearance of single-stranded DNA as follows. If [A] and [A'] are the single-stranded nucleotide concentrations of two homologous strands, and [A] = [A'], then

$$\frac{-d[A]}{dt} = k_2[A]^2 = \frac{k_2 C_o^2}{4} \quad (1)$$

where k_2 is the second order rate constant. If C_o is the total nucleotide concentration and $C_o = 2[A]$, then

$$\frac{C_o}{C} = \frac{1}{f_{ss}} = \frac{k_2 C_o t}{2} + 1 \quad (2)$$

where f_{ss} is the fraction of unreacted nucleotides remaining. Rate constants can be determined by plotting $1/f_{ss}$ as the ordinate against time. The slope of the line through the data points is $k_2C_o/2$.

Britten and Kohne (1966; 1968) plotted their rate data

from an inverted form of equation (2):

$$f_{ss} = \frac{1}{(k_2/2)C_ot+1} \quad (3)$$

This form of plotting data with f_{ss} as the ordinate against $\log_{10}C_ot$ is known as the C_ot plot. The curves through the data points are inverted sigmoids with a linear range around $t_{1/2}$, the half time of reaction. The rate constant is equal to $2/C_ot_{1/2}$. Rates which diverge by as much as a factor of 10^9 can be plotted together in this manner, where second order reactions with different half times will be parallel. DNAs with more than one complexity, where the reaction is not second order, can also be analyzed when plotted this way (Britten and Kohne, 1966; 1968). Various computer programs have been written to determine rate constants from C_ot plots (Britten, et al., 1979; Kells and Straus, 1977; Pearson, et al., 1977; Murphy, et al., 1979).

Wetmur (1971) developed a method for plotting renaturation data which do not remain linear after the first half-time. The method involved determining an empirical limit past which renaturation does not proceed, and setting it equal to unity. If all data are expressed as a fraction of that number, the curve straightens out for the entire extent of the reaction. Morrow (1974) found that reactions assayed with endonuclease S1 which slowed down after the first half-time would appear linear for the entire extent of the reaction if

plotted as

$$1/f_{ss} = [(k_2/2)C_ot + 1]^{0.44} \quad (4)$$

Neither curve fitting procedure needs to be used, however, if only data from the first half-time of renaturation are used, where the points remain fairly linear.

Smith, et al. (1975) plotted some of their data with $\log_{10}(C_ot/C_ot_{1/2})$ as the abcissa. $C_ot_{1/2}$ is the C_ot at which 50% of the DNA was found in fragments bound to a hydroxylapatite column. In this manner, the data were normalized to a $C_ot_{1/2}$ equal to 1 so that the second order rate constant for the hydroxylapatite reaction was also equal to 1. This permits pooling of data for reactions carried out with different fragment lengths.

B. FACTORS AFFECTING DNA RENATURATION RATES IN SOLUTION

The dependence of k_2 on the eight factors cited previously was formalized by Wetmur and Davidson (1968) into the equation

$$k_2 = \frac{k_N' L_s^{0.5}}{N} \qquad (5)$$

where k_N' is the specific nucleation rate constant, L_s is the length of the shorter strand in nucleotides and N is the complexity of the DNA, also expressed in nucleotides. Below follows a detailed discussion of these factors which contribute to k_2.

1. The Nucleation Event

DNA renaturation is a second order reaction in which the initial nucleation event between two homologous strands determines the rate. The nucleation event has been the subject of many studies. Crothers, et al. (1968), using the data of Blake, et al. (1968), mathematically derived the fact

that more than a single base pair is necessary for establishing a stable nucleus. Other studies showed that the formation of the second (Lee and Wetmur, 1972a; 1972b) or third (Eigen, 1967; Porschke and Eigen, 1971) base pair must occur in order for a stable nucleation site to exist. Once the second base pair is formed between two molecules in register, zippering proceeds at about 0.5 to 10 x 10^7 base pairs per second (Craig, et al., 1971; Porschke and Eigen, 1971). In circumstances where homologous segments are tandemly repeated, a second nucleation occurs downstream from the first one before zippering reaches that site (Bertani and Chattoraj, 1980).

The renaturation rate of a population of heterogeneously sized molecules begins to lose the appearance of second order kinetics past the first half-time when plotted on a reciprocal plot against time. The rates appear to slow because 40 to 60 percent of the length of each molecule renatures with the first nucleation (Miller and Wetmur, 1975) and what remains to be observed is the renaturation of the tails of molecules. The latter step is slower because of the decreased length of the remaining single-stranded entities, and because of inhibition of nucleation in a single-stranded tail by the double-stranded region of the molecule (Smith, et al., 1975; Britten and Davidson, 1976). The latter phenomenon was termed "particle inhibition" and accounts for about a half of the observed decrease in rate.

The reaction, however, remains close to second order.

This fact can be verified by changing the DNA concentration and observing an equivalent change in rate. The reaction, however, is only approximately second order. Over a one hundred fold increase in DNA concentration the rate increases by only a factor of fifty (Subirana and Doty, 1966). This discrepancy has not been adequately explained to date. Rau and Klotz (1975) suggested that this phenomenon is observed in less complex DNAs at higher concentrations because the nucleation rate is comparable to the rate of zippering, especially under conditions of extensive secondary structure in the single-stranded region. This theory, however, neglects to account for the slowing of rates due to size heterogeneity as previously discussed.

In the course of formalizing the parameters affecting DNA renaturation kinetics, Wetmur and Davidson (1968) defined the term β as the number of nucleation sites per nucleotide. If C_o is the total single-stranded nucleotide concentration and N is the complexity of the DNA, then $\beta C_o/2N$ is the concentration of any one nucleation site. The rate of nucleation at any one site then, is $k_N(\beta C_o/2N)^2$ where k_N is the rate constant for nucleation at such a site. The total number of distinct loci able to act as nucleation sites is βN. The rate of nucleation at all sites, therefore, is $k_N \beta^3 C_o^2/4N$. β is probably equal to 1. For simplicity, the nucleation rate constant, k_N, can be redefined to include the β^3 term. If two reacting molecules are in register and are both of length L, the rate of base pair formation would be

$$\frac{-dC_o/2}{dt} = \frac{k_N L C_o^2}{4N} \qquad (6)$$

Comparing this equation with equation (1) derived from empirical findings, the following relationship between k_2, the experimental rate constant and k_N, the elementary nucleation rate constant is established:

$$k_2 = k_N L / N \qquad (7)$$

k_N is independent of concentration and complexity, but is dependent on temperature, ionic strength, microscopic viscosity, length, and weakly on pH and G+C content. Below follows a discussion of these factors. The relationship between k_N' and k_N (equations 5 and 7) is given in equation 9 below.

a. Effect of Temperature on Nucleation

The bell-shaped temperature dependence of renaturation kinetics was demonstrated by Saunders and Ross (1960), Ross and Sturtevant (1962), Marmur, et al. (1963) and many others. The same mathematical studies which demonstrated that renaturation is a two step process also showed that a temperature optimum exists where this process is favorable.

Crothers, et al. (1968) modified the steady-state model of Saunders and Ross (1960) for double helix formation of homopolymers of riboadenylic and uridylic acid to yield a model which encompasses the following: the difference in the rate of formation and equilibrium constants of the first few base pairs and those present in a long helix, and the definition of a kinetic barrier between the formation of the $(AU)_{\alpha}$ and $(AU)_{\alpha+1}$ base pair where $\alpha+1$ is the number of base pairs required for successful nucleation. As stated previously, $\alpha+1$ is 2 (Lee and Wetmur, 1972a; 1972b) or 3 (Eigen, 1967). The rate determining step for helix formation, $\alpha+1$, is determined using a stepwise base pairing model, the temperature dependence of k_2, and the requirement that the activation energy for forming a base pair be small and positive (Crothers, et al., 1968).

The T_m, and therefore the optimum temperature of reassociation, T_r, varies with the solvent used. The T_m also varies with the G+C content of the DNA and with the percent mismatching. These factors will be discussed in the following sections.

b. Effect of Ionic Strength on Nucleation

Single-stranded DNA molecules are polyanions. In order to renature the molecules, the electrostatic repulsion of the

sugar-phosphate skeleton must be overcome or the charges must be shielded. The strong dependence of k_2 on the ionic strength was first attributed to shielding of the polyanionic repulsion by Ross and Sturtevant (1960).

The effects of ionic strength on renaturation in low salt concentration was described by Studier (1969) and at high salt concentration by Wetmur and Davidson (1968). The renaturation rate was found to be proportional to the third power of the sodium ion concentration below 0.4 M NaCl (Studier, 1969). The rate is less dependent on Na^+ concentration above 0.4 M NaCl, but continues to increase approximately linearly until 3 M where it levels off (Orosz and Wetmur, 1977). Renaturation rates in KCl and CsCl increase linearly by a factor of 3 between 0.4 and 3 M. Rates in LiCl increase more rapidly, such that 0.9 M LiCl yields an equal rate to that achieved with 3 M NaCl. Using the data of Wetmur and Davidson (1968), Britten (1969) and Britten and Smith (1970) developed an empirical formula by which they could calculate the second order rate constant given the Na^+ concentration.

Manning (1976) developed a theory using published data and previous calculations (Manning, 1969) to correctly predict a power dependence of k_2 on the ionic strength. Using polyelectrolyte theory and the previous assumption of a preequilibrium between two separated, closely aligned but unbonded strands (Wetmur and Davidson, 1968), Manning determined that the nonbonded precursor to the nucleated species consists of an aligned pair of complementary segments

about 13-16 nucleotides. This length, considerably greater than the nucleus, correlates well with the statistical segment length of single-stranded DNA calculated by Wetmur (1971) from the radius of gyration of single-stranded DNA measured by Krasna, et al. (1970).

The theory and previous measurements (Record,1975; Dove and Davidson, 1962) show that the T_m becomes a decreasing function of ionic strength when divalent cations are added to the monovalent ionic solution. This is due to the preferential binding of the divalent metals to the phosphates and their displacement of the monovalent cations. Manning's theory predicts an inverse variation of k_2 with the monovalent cation concentration when the divalent cation concentration is at least equal to the nucleotide monophosphate concentration. The theory also predicts an increased dependence of the rate on the ionic strength with longer oligomers, with a leveling off of the effect at 15 base pairs. This agrees with the empirical data of Porschke, et al. (1973) who found that k_2 increases as the square root of the ionic strength with a hexamer, as the 0.7 power of the ionic strength with a decamer, and as the square of the ionic strength with a 14-mer.

Other solvent systems have been used to achieve renaturation conditions. Formamide containing solutions have been used when lower renaturation temperatures were required (McConaughy, et al., 1969). In 0.035 to 0.9 M NaCl solutions, 1 to 50% formamide lowers T_m by 0.6°C per percent formamide

(Hutton, 1977). The effect is somewhat greater at higher NaCl concentrations. The rate of renaturation also decreases in aqueous formamide solutions. At T_m-(25$\pm$5)^oC, the rate decreases linearly by 1.1^oC for every percent formamide from 1 to 50. In more than 50% formamide, the rate effectively loses its dependence on the NaCl concentration above 0.8 M NaCl.

Urea lowers the T_m by 2.25^oC for every mole per liter from 1 to 8 M, while decreasing the rate by about 8% for every molar at the optimum T_r (Hutton, 1977). The T_m of DNA in 40% dimethylsulfoxide (DMSO) and 0.2 M NaCl is 27^oC lower than the T_m of DNA in 0.2 M NaCl (Escara and Hutton, 1980). The drop in T_m is not linear. The renaturation rate, however, is double under these conditions over that in salt alone. Escara and Hutton (1980) attributed this effect to the decreased dielectric constant of the DMSO solution.

Other solvent systems studied were solutions of ethylene glycol (Wetmur and Davidson, 1968), sodium perchlorate (Wetmur and Davidson, 1968; Chang, et al., 1974), tetramethyl and tetraethylammonium chloride (Me_4NCl and Et_4NCl) (Chang, et al., 1974), and a variety of tetraalkylammonium salt solutions (Orosz and Wetmur, 1977). Et_4NCl at 2.4 M and Me_4NCl at 3.2 M turned out to be of special utility. In addition to lowering the T_m to 63 and 93^oC respectively, yielding a sharp hyperchromicity, they also had the effect of negating any nucleotide composition effect on T_m or T_r (Melchior and VonHippel, 1973). Klump (1977) measured enthalpies for melting DNA in various alkylammonium salt solutions. The

effect of tetraalkylammonium salts of negating T_m differences between A+T and G+C base pairs was found to be due to preferential binding of the tetraalkylammonium salts to the A+T pairs (Shapiro, et al., 1969) in the minor groove of DNA (Golas, et al., 1980), and partially to modification of the solvent structure (Golas, et al., 1980).

c. Effect of pH on Nucleation

Wetmur and Davidson (1968) found that DNA renaturation rates are essentially independent of pH in the range from 5 to 9. As the pH rises above 9, however, the thymine and guanine acidic protons of single-stranded DNA get partially titrated and the rate of renaturation decreases even at constant T_m-T_r. The T_m remains essentially unchanged in high salt in the pH range from 5 to 9. On either side of this range, it drops precipitously. In acid, the helix can be titrated to over 50% of maximum before transition. In base, the nucleotide residues of double-stranded DNA do not titrate until the onset of denaturation because their pk values are shifted by the free energy of helix stabilization (Record, 1967).

d. Effect of Viscosity on Nucleation

Early studies (Subirana and Doty, 1966; Thrower and Peacocke, 1966) found that increasing the viscosity of the microenvironment around the DNA by the addition of sucrose diminished the rate of renaturation. Addition of sucrose and glycerol at high concentrations of NaCl, and of $NaClO_4$ to DNA solutions also lowers the T_m and the T_r slightly (Wetmur and Davidson, 1968). At the T_r, the rate of renaturation is inversely proportional to viscosity in concentrated NaCl. The inverse proportionality of renaturation rates to the microscopic viscosity was confirmed in studies with ethylene glycol, Me_4NCl and Et_4NCl (Chang, et al., 1974).

A theory of a simple translational diffusion control of renaturation (Schmitz and Schurr, 1972) is contradicted by two arguments raised by Wetmur and Davidson (1968). The overall rate is too slow to be diffusion controlled, especially if segmental diffusion is permitted. Elevating the temperature above T_r would increase the rate of a diffusion controlled reaction, not lower it. An alternate theory which has gained both theoretical and experimental support was proposed by Wetmur and Davidson (1968). The mechanism involves the previously discussed preequilibrium step, followed by the viscosity-limited, rate-determining formation of the second base pair (Lee and Wetmur, 1972a; 1972b).

The latter theory also predicts that polymers which raise

the macroscopic viscosity of the solution but have no effect on the microenvironment do not decrease the renaturation rate. Empirically, addition of native heterologous DNA (Subirana and Doty, 1966; Walker and McCallum, 1966) or polyacrylate (Subirana and Doty, 1966) did not lower the renaturation rate of tracer molecules. Larger concentrations of inert polymers such a dextran or sodium dextran sulfate increase the rate of DNA renaturation (Chang, et al., 1974; Wetmur, 1975; Wieder and Wetmur, 1981; this thesis, chapter 2).

Wetmur (1975) explained the acceleration of renaturation (R) by an inert polymer as the simple excluded volume effect:

$$R = e^{0.4\beta |\eta| c} \qquad (8)$$

β is the proportionality constant between the excluded volume of a polymer and the volume of the polymer, $|\eta|$ is the intrinsic viscosity of the polymer and c is the weight concentration of the polymer. The theory predicts that volume exclusion by the polymer effectively increases the DNA concentration in the rest of the solution. The theory does not address itself to the probability of a phase transition which might occur if high enough concentrations of polymer were used (Wieder and Wetmur, 1981; this thesis, ch. 2). Rates as high as 100 fold those without dextran sulfate have been achieved (Wieder and Wetmur, 1981; this thesis, ch. 2).

A second method of rate acceleration called the Phenol Emulsion Reassociation Technique (PERT) was discovered by

Kohne, et al. (1977) and further investigated by Wieder and Wetmur (1982). With this technique, DNA renatures on the surface of phenol bubbles in the presence of a chaotropic salt at room temperature. At low DNA concentrations, thousands fold rate accelerations have been achieved. This and the volume exclusion rate acceleration method will be discussed in chapters 3 and 2.

e. Effect of Length on Nucleation

DNA renaturation is most often carried out with DNA fragments that are considerably less than the size of the genome which is being studied or the size of the fragments isolated from cells. Fragmentation can be achieved in several ways as described by Britten, Graham and Neufeld (1974). DNA can be sheared with a syringe or by blending, by a high pressure press or by sonication. The methods yield a normally distributed sized population of molecules. Single strands can also be quantitatively degraded by DNase or heat treatment and alkaline hydrolysis (Lindahl and Nyberg, 1972; Lindahl and Andersson, 1972). This procedure yields a random distribution of molecule sizes. Another, more preferable method of achieving a random size distribution of molecules is by restriction enzyme cleavage of the DNA. Renaturation of restriction fragments results in double strand formation along

the entire length of the fragment from a single nucleation event since the fragments are not circularly permuted. Hence, renaturation of restriction fragments proceeds to completion and does not slow down after the first half-time.

Wetmur and Davidson (1968) experimentally determined that the rate constant, k_2, is proportional to the square root of the length of the reacting molecules when both strands are of the same size (eq. 5). They found this relationship to hold true over a one thousand fold variation in length. The theoretical equation derived from the assumption of a rate limiting nucleation step followed by rapid zippering (equation 7) predicts the rate constant to be dependent on the length of the strands. These two equations imply that k_N, the elementary nucleation rate constant is inversely proportional to the square root of the length of the reacting molecules. Thus the specific nucleation rate constant is given by

$$k_N' = k_N L^{0.5} \qquad (9)$$

The decreased availability of nucleation sites with increased length was explained as being due to an excluded volume effect when reacting molecules fail to penetrate each other completely. All of the factors predicted by this theory, the temperature profile of renaturation, the length effect in the preequilibrium step, the lack of an effect of circular permutations on renaturation rates, and a decreased reaction rate of circles versus linear molecules were verified

experimentally by Kinberg-Calhoun and Wetmur (1981).

In 1971, Wetmur determined that when homologous strands of two different length distributions renature, the rate depends on the square root of the shorter strand. Other studies confirmed this relationship (Lee and Wetmur, 1972; Hutton and Wetmur, 1973c; Hinnebusch, Clark and Klotz, 1978; Chamberlin, et al., 1978). Apparent renaturation rates of tracers which are shorter than the drivers can be underestimates for two reasons. The driver-driver self reaction is faster than the driver-tracer reaction due to the length effect. The displacement of the shorter tracer from driver-tracer hybrids by branch migration of homologous driver molecules also contributes (Lee, Davis and Davidson, 1970; Green and Tibbets, 1981).

f. Effect of Nucleotide Composition on Nucleation

Studies with DNAs from different organisms of differing G+C content and with homopolymers have uncovered a trend pointing to a weak dependence of the rate of renaturation on G+C content. Data are tenuous, at best, and contradictory findings do exist. Wetmur and Davidson's (1968) as well as Seidler and Mandel's (1971) data with various DNA's suggest a trend of increasing k_N' with increasing G+C content. Gillis, et al. (1970), while measuring DNA content with a

diphenylamine colorimetric reaction and Lee and Wetmur (1972), while studying homopolymers, found the opposite effect of faster rates with increased adenine content. Wetmur (1976) suggests, after considering all of the available data and the errors in measurements as well as in the assumptions in all of the studies that the renaturation rate has a weak positive dependence on the G+C content of the DNA.

g. Effect of Base Mismatching on Nucleation

Base mismatches cause about a 1.1 to 1.5°C drop in T_m for every percent of the bases that are mismatched, depending on whether the mismatch mimics a transition or a transversion (Gralla and Crothers, 1973; Hutton and Wetmur, 1973b; Chang, et al., 1974; Kallenbach and Dorst, 1972; Lee and Wetmur, 1973). The result is a loss of about 2.8 kilocalories of stabilization free energy per base pair (Lee and Wetmur, 1973). Chang, et al. (1974) developed a theory predicting a 1.5°C upper limit in the T_m drop per percent mismatching.

The effect of mismatching on renaturation rates has been studied by means of various modifications of DNA bases. The effects studied include deamination (Hutton and Wetmur, 1973b; Marsh and McCarthy, 1974; Bonner, et al., 1973), glyoxylation (Hutton and Wetmur, 1973b), modification by chloroacetaldehyde (Lee and Wetmur, 1973), and modification by N-acetoxy-N-2-

acetylaminofluorene (Chang, et al., 1974). The consensus of the studies indicates that rates decrease by about a factor of two for a 10-15°C reduction in melting temperature, which is due to a 10% level of mismatching. The differences in the results are due to the differences in T_m-T_r among the different data sets. It is necessary, therefore, to choose the renaturation temperature carefully to favor the renaturation of the desired sequences in a system with both perfectly matched as well as mismatched sequences. Hutton and Wetmur (1973b) derived a theory which relates renaturation rate to melting temperature changes.

2. Length Effect

From equation 7, derived by assuming renaturation to consist of a slow nucleation step followed by a rapid zippering, it is expected that the rate of renaturation would depend on the length of the molecules. The fact that it depends on the square root of the length has been explained in the previous section as being due to the inverse dependence of nucleation on the square root of the length due to an excluded volume effect.

3. DNA Complexity

Marmur and Doty (1961) first observed that renaturation was inversely proportional to the amount of DNA in a bacteriophage or cell. Britten and Kohne (1966) found that DNA from different organisms sheared to the same length reacted with rate constants inversely proportional to complexity. This inverse dependence was found to hold true for six orders of magnitude of complexities of DNAs from various sources.

The complexity of DNA in a genome is defined as the number of bases that are present in a unique order without repetition. For bacteriophages, viruses and bacteria, the entire DNA of the organism is a unique sequence represented only once. Hence, the length of the genome is also the complexity. Britten and Kohne (1966) discovered that the DNA of higher organisms, that is most eukaryotes, has in it three classes of DNA complexity. Their study showed the presence of a class of low complexity sequences, represented about 10^6 times per genome, a more heterogenous class of higher complexity sequences, represented from 10^3-10^5 times, and a class of very high complexity, representing about 70% of the DNA, present in only one copy per haploid genome. Sequences present in multiple copies renature at a faster rate than unique sequences when fragmented DNA is being renatured simply because they are present at a higher concentration than unique

sequences. Particular care must be employed when measuring the complexity by renaturation because repetitive sequences are often interspersed among unique segments. It is imperative that the fragment size to which the DNA is sheared be smaller than the average repeat length of the sequence whose complexity is being determined (Hutton and Wetmur, 1973a), or an erroneous and inconsistent answer will be obtained.

Before the inverse relationship of renaturation rate and complexity was determined, the only way to measure the complexity of lower organisms was by assuming that the length was equal to complexity, and determining the length by physical means. This was accomplished for viruses and phage by measuring the length of the intact genome electron microscopically by the Kleinschmidt technique (Davis, Simon and Davidson, 1971) with internal standards of known base pair lengths, and for bacteria by measuring the DNA content of the cell by the diphenylamine colorimetric technique (Gillis, et al., 1970). Because of the various complexity classes, the complexity of unique sequence DNA from higher organisms can only be determined by following its renaturation kinetics in a system where k_N' has been determined with DNA of known complexity.

C. METHODS OF ASSAYING RENATURATION KINETICS

Renaturation rates can be determined by measuring the rate of formation of base pairs or the rate of formation of molecules with at least one double-stranded region. The latter measurement yields a rate approximately double that of the former. Methods which measure base pair formation include spectrophotometric hypochromicity, optical rotation, endonuclease S1 digestion of homogeneously labeled molecules, CsCl density centrifugation, and differential binding of intercalating dyes (Hutton and Wetmur, 1972). Methods which measure hybrid molecule formation include hydroxylapatite chromatography and filter hybridization. Below follows a discussion of several of these methods.

1. Measurement of Base Pair Formation

a. Optical Determination of DNA Renaturation Rates.

The initial studies of renaturation kinetics were carried out on synthetic homopolymers using the temperature-jump (T-jump) technique (Eigen and DeMaeyer, 1963) and the stopped-flow technique (Ross and Sturtevant, 1960; 1962). T-jump experiments measure the rate of return to equilibrium of a system which is artificially perturbed, whereas stopped-flow experiments measure the rate of reaction of two species which are rapidly mixed. These two methods will not be considered any further since they are not used for the measurement of reassociation of DNA from biological sources.

The optical method which is commonly employed to measure renaturation kinetics is spectrophotometry described by Wetmur and Davidson (1968). DNA can be denatured in boiling water or by addition of enough NaOH to make the solution 0.1 N. It is then ice quenched in the case of heat denaturation, or brought to pH 7 with NaH_2PO_4. The temperature in the cuvette is then suddenly brought to T_r by a burst of voltage across two leads in a sealed cuvette and maintained at that temperature during renaturation. Alternatively, a water jacketed cuvette can be employed in a recording spectrophotometer connected through a series of stopcocks to two recirculating constant temperature

baths, one at T_m+10°C and the other at T_r+10°C (Wetmur, 1971). Melting, renaturation, and remelting can be achieved by switching between baths. To determine the rate from renaturation curves, the absorbance at 260 nm (A_{260}) is monitored. A_{260} of single-stranded DNA, which is the absorbance at zero time (A_o), is assumed to be 1.36 times A_{260} of fully native DNA, or the absorbance after an infinite time of renaturation (A_∞). Absorbance data points at various time points (A) are recorded and plotted in one of the ways previously discussed. f_{ss} is the equivalent of $A-A_\infty / A_o-A_\infty$.

b. Enzymatic Determination of Renaturation Rates

Endonuclease S1 is a nonspecific endonuclease which cleaves single-stranded but not double-stranded regions of DNA (Ando, 1966). If the DNA is homogeneously labeled, renaturation rates can be assayed by endonuclease S1 digestion. Homogeneous labeling can be achieved by growing the organism in 3H-thymidine or α-^{32}P-nucleotide triphosphate. In vitro iodination with $TlCl_3$ as the catalyst (Commerford, 1971; Orosz and Wetmur, 1974), or nick translation with DNase I and DNA polymerase I with ^{3}H or ^{125}I nucleotide triphosphates, or α-^{32}P-nucleotide triphosphate (Kelly, et al., 1970) also achieves random labeling. Samples at zero time and at various time points thereafter are diluted into

endonuclease S1 buffer which has 0.01 M sodium acetate pH 4.2 and 0.001 M $ZnCl_2$, and incubated with endonuclease S1 at 37°C. Several methods can be used to separate cleaved nucleotides from intact DNA, including trichloroacetic acid precipitation (Ando, 1966), binding of hybrids to DEAE cellulose filters (Maxwell, et al., 1978), or thin layer chromatography in 1 M HCl (Wieder and Wetmur, 1981; 1982; this thesis, chapters 2 and 3). Rates can again be determined by plotting the data in any of the ways discussed before. f_{ss} will be the counts which are acid soluble and migrate with the solvent front, divided by the total number of counts.

2. Measurement of Renaturation of Molecules by Hydroxylapatite Chromatography

Hydroxylapatite differentially binds single and double-stranded DNA when the temperature and phosphate concentration are varied (Britten, et al., 1974). The advantages of using hydroxylapatite chromatography for renaturation rate determination are many. Both renatured and single-stranded fractions can be separated in this manner preparatively. Reactions appear to go to completion, since any molecule with a double-stranded region appears fully renatured. Hence, rate plots continue to remain linear even after the half-time of reaction. Specific conditions can be

found for selecting hybrid molecules with any degree of mismatch desired (Fox, et al., 1980). Generally, the conditions in the range of 0.12 M phosphate buffer at 60°C or 0.08 M phosphate buffer at 70°C will only elute single strands and leave double strands stuck to the colums. A disadvantage which exists with hydroxylapatite columns is their slow flow rates.

D. REASSOCIATION OF DNA MOLECULES IN SOLUTION TO DNA BOUND TO SOLID SUPPORTS

It is often necessary to carry out renaturation experiments between tracer molecules in solution and DNA strands immobilized on a solid support. There have been several solid supports used to date to which nucleic acids have been coupled. Nygaard and Hall (1964), Gillespie and Spiegelman (1965) and Westphal and Dulbeco (1968) had all used nitrocellulose filters to bind DNA. Litman (1968) physically immobilized DNA on cellulose, whereas Gilham (1968) covalently linked DNA to cellulose via the terminal phosphate by the carbodiimide method. Wagner, et al. (1971) linked the 5' terminal phosphates of polyinosinic acid, while Poonian, et al. (1971) linked the single-stranded ends of DNA molecules to cyanogen bromide activated sepharose. Robberson and Davidson (1972) used an ε-aminocaproic acid methyl ester converted to a hydrazide, coupled to CNBr activated agarose to bind periodate oxidized 3' termini of RNA. Noyes and Stark (1975) have linked DNA to diazotized m-aminobenzyl oxymethyl (DMB) cellulose primarily through guanine and thymine residues of single strands.

Several methods have also been developed to transfer DNA or RNA fragments separated on agarose gels onto solid supports of nitrocellulose paper or activated filter paper, and either

physically or chemically link the fragments to the support. Once on the support, tracer molecules can be hybridized to the immobilized fragments to detect homologous sequences. The procedure of transferring DNA fragments to nitrocellulose paper from agarose gels and hybridizing probes to them was first described by Southern (1975). Transfer of RNA fragments to nitrocellulose paper and efficient retention thereof was worked out by Thomas (1980). Transfer and covalent linkage of agarose gel separated RNA fragments to DMB filter paper was first described by Alwine, et al. (1977). Hybridization of probes to gel separated DNA or RNA fragments linked to filter paper or DMB paper has become a very popular and useful method of detecting sequence homology.

In order to localize specific nucleic acid sequences in chromosomes, Gall and Pardue (1969) applied the technology of nitrocellulose filter hybridization to chromosome preparations and developed the technique of in situ hybridization. The technique is useful for large, heterogeneous sequences, or for sequences present in multiple copies. The difficulty with identifying single-copy sequences in chromosomes, however, is the lack of sufficient counts incorporated to unambiguously visualize the position of incorporation on an X-ray film. Recently, Gerhard, et al. (1981) were able to localize a unique gene by direct in situ hybridization. In order to get enough counts incorporated for autoradiographic visualization, they fused the desired cDNA sequences to a plasmid which they labeled to high specific activity by nick translation with

^{125}I-dCTP (Kelly, et al., 1970). Hybridization of a probe with complementary strands yielded the further advantage of forming networks, leading to further incorporation of radioactivity. Specific unique sequences have also been localized by in situ hybridization with an RNA probe by localization with anti-RNA-DNA fluorescent monoclonal antibodies (Stuart, et al., 1981).

The kinetics of hybridization of tracer nucleic acid molecules to DNA on solid supports has been studied by several investigators (McCarthy and McConaughy, 1968; Spiegelman, et al., 1973; Flavell, et al., 1974). The concentration of DNA bound to the solid support is in excess to that in solution, in order to minimize tracer-tracer renaturation (McCarthy and McConoughy, 1968). If the concentration of the tracer in solution is A and the concentration of DNA bound to the support is B, then the disappearance of the tracer from solution follows pseudo-first order kinetics, dependent only on the unchanging DNA concentration on the support (Flavell, et al., 1974). The equations describing the rate are:

$$-dA / dt = kAB \qquad (10)$$

$$1/f_{ss} = e^{kBt} \qquad (11)$$

The data points at various times should be linear if plotted with $-\ln f_{ss}$ as the ordinate versus time. The slope of the line will be kB where k is the reaction rate constant.

Flavell, et al. (1974) found however that the rate of DNA reassociation to DNA immobilized on nitrocellulose filters did not follow ideal kinetic behavior. The initial rates of reassociation were proportional to B only at low B values, but the rate did not increase linearly at high B values. They postulated that filter hybridiztion essentially involved two processes: 1) translational diffusion to the filter, and 2) hybridization with the filter bound DNA. At low B values, the reaction is limited by 2), whereas at high B values, hybridization around the filter is so fast that the solution surrounding the filter is depleted of A and the reaction becomes limited by diffusion of tracer to the filter. This implied that at high B values, equation (10) should be replaced by

$$-dA / dt = JA + kAB \tag{12}$$

where J is the diffusion term and is defined as $0 < JA < kAB$. Thus, the reaction is governed by two different factors, each more important than the other under different circumstances. Under conditions where renaturation is fast, the rate is limited by the diffusion term. This contention is supported under conditions of high B by an increased rate when the reaction is shaken, by the inverse dependence of the rate on the molecular weight of the DNA in solution, and the relative independence of the rate on the complexity of the DNA.

Under slow reaction conditions, the hybridization term is

rate limiting. The rate is independent of shaking, it is directly proportional to the concentration of DNA on the filter, and is inversely proportional to the complexity of the DNA. The rate is independent of the molecular weight of the DNA, however, as opposed to the square root of the length dependence in solution, and the calculated rate constant is about ten times too slow. Flavell, et al. (1974) explain these anomalies as being due to inadequate accessibility of the tracer to the DNA fixed to the filter. The rate may also only depend on the length of the bound strands. The multiply bound strands could be the short strands and the rate governed by these L_s. The work described in chapter 3, below, using the PERT procedures for accelerating DNA renaturation reactions also involves novel forms of DNA strand length dependence. Wahl, et al. (1979) were able to accelerate renaturation rates of tracer to DNA bound to DMB paper by addition of 10% dextran sulfate.

Szabo, et al. (1975) worked out the kinetics of in situ hybridization. The rate of renaturation in this case is also pseudo first order, but the species in excess is the tracer concentration, since the sequences in the chromosome are only present once. If B is the concentration of the sequence in the chromosome and A is the concentration of the probe, then the rate of saturation of the hybridizable sequence in the chromosome is

$$-dB / dt = kAB \qquad (13)$$

$$B = B_o e^{-kAt} \quad (14)$$

The fraction of sites which have reacted with complementary DNA at any one time is

$$B_o - B \,/\, B_o = 1 - e^{-kAt} \quad (15)$$

Szabo, et al. (1975) found that the rate of renaturation was very similar to that predicted by the first order rate equations, and that rates were also similar to analogous filter disc hybridization rates. The rate constant was again found to be inversely proportional to the complexity of the RNA probe.

CHAPTER II. ONE HUNDRED FOLD ACCELERATION OF DNA RENATURATION IN SOLUTION

A. INTRODUCTION

The half-time for renaturation of single-copy sequences to a mammalian DNA at a concentration of 1 mg/ml (all sequences) is about 2-3 days in concentrated NaCl solutions at the optimum temperature for renaturation. Two methods exist for significantly accelerating DNA renaturation. The first method employs volume excluding inert polymers such as dextran sulfate (Wetmur, 1975). The reactions occur in homogeneous solution, follow second order kinetics, and may be described by equation 5 with increased k_N'. A simple excluded volume theory predicts that the acceleration, R, for DNA renaturation due to the addition of an inert polymer with intrinsic viscosity $[\eta]$ to a weight concentration c should take the form

$$R = e^{0.4\beta[\eta]c} \tag{8}$$

β is a proportionality constant between the excluded volume of the inert polymer and the physical volume of the inert polymer. Using dextran sulfate volume exclusion in 2.4 M Et_4NCl, Wetmur (1975) achieved DNA renaturation rates up to

20 times those found in 0.4 M NaCl. The acceleration of DNA renaturation using dextran sulfate has recently been extended to reactions between DNA in solution and complementary DNA on solid supports. Leiden, et al. (1980), Wahl, et al. (1979) and P. Szabo (personal communication) have achieved accelerations of reactions involving DNA bound to nitrocellulose filters, DNA covalently attached to diazobenzyloxymethyl paper and DNA fixed in Drosophila melanogaster chromosomes respectively. Any reported accelerations beyond 10-20 fold have been the result of detection of aggregated DNA probe molecules renatured with DNA on the solid support. The solubility of dextran sulfate in the solvent systems employed in these studies has to a great extent established the limits for the acceleration process. One of the aims of this work is to quantitatively explore the limits of acceleration of DNA renaturation rates using the volume exclusion technique.

The second method for accelerating DNA renaturation involves the use of a phenol-water emulsion. (Kohne, et al., 1977). The phenol emulsion reassociation technique (PERT) requires continuous agitation to maintain a large surface area between phenol and water. Renaturation apparently occurs near this interface. The use of a chaotropic salt enhances this acceleration of DNA renaturation at room temperature. The mechanism of the additional enhancement cannot simply be attributed to a decreased DNA melting temperature because the rate of renaturation of the DNA with the PERT system is very

weakly dependent upon the temperature. Because of the heterogeneity of the system, second order kinetics are not obeyed at all DNA concentrations. The observations of Kohne, et al. (1977) that DNA at greater than or equal to 1 mg/ml renatures less than 20 times faster in 2 M NaSCN at room temperature using PERT than at 65°C in 0.4 M NaCl using no acceleration method have been verified (see chapter 3). On the other hand, accelerations of many thousand fold are possible if the DNA is diluted, although the half time for renaturation is essentially the same or greater than the value found at 1 mg/ml DNA. The PERT technique has been the subject of further study and will be discussed later in this thesis.

Orosz and Wetmur (1977) showed that DNA renaturation is fastest when the cation present is Li^+ and found that addition of NaCl to DNA in 2.4 M Et_4NCl increases DNA renaturation rates. These salt effects, if extended or combined, might constitute a third method for accelerating DNA renaturation. The other aim of this work, in addition to exploration of the limits of DNA renaturation acceleration using volume excluding polymers, is to explore various salt effects which might substitute for or complement volume excluding polymers. A significant acceleration of DNA renaturation using salt effects has been achieved, but often with the loss of second order kinetic behavior when approaching the level of acceleration which would be competitive with the other two methods. The greatest rate accelerations are achieved using the PERT method with dilute DNA or the volume exclusion method with concentrated DNA.

B. EXPERIMENTAL

1 Materials

Dextran sulfate, M_r = 500,000, was obtained from Pharmacia. Standard curves were prepared to relate dextran sulfate concentration in solutions containing various salts to refractive index measured at 25°C with a Bausch and Lomb Abbe 3-L refractometer. Unknown dextran sulfate concentrations in solutions of known salt concentration were determined using refractive index measurements and the appropriate standard curve. Tetraethylammonium chloride (Et_4NCl) was purchased from Eastman Organic Chemicals and purified as described by Chang, et al. (1974). $Na^{125}I$, carrier free was purchased from Amersham. All other chemicals were reagent grade. Polyethyleneimine impregnated Polygram Cel 300 PEI thin layer chromatography (tlc) plates from Macherey Nagel were purchased from Brinkman. Endonuclease S1 was obtained from Sigma and assayed as by Hutton and Wetmur (1973a). E. coli B DNA type VIII and calf thymus DNA were purchased from Sigma. The DNAs were sonicated for many applications using eight thirty-second pulses at 0°C with a Heat Systems Sonifier Cell Disruptor.

2. Single-Copy DNA

Single-copy calf thymus DNA was prepared by hydroxylapatite chromatography on Biogel HTP (Bio-Rad) in 0.15M potassium phosphate buffer, pH 7.0, of sonicated denatured calf thymus DNA which had been renatured to C_ot 15 (Britten, et al., 1974). C_o is the initial molar DNA nucleotide concentration and t is time in seconds.

3. ^{125}I-Labeled Probe DNA

DNA was labeled _in vitro_ with ^{125}I according to the procedure of Chan, et al. (1976), which maximizes ^{125}I incorporation while minimizing chain scission. Prior to iodination, DNA was extracted with phenol-chloroform to remove trace levels of associated proteins. ^{125}I-labeled probe DNAs were used with bulk DNAs of the same molecular weight.

4. Measurement of Renaturation Rates

E. coli DNA renaturation rates were determined using a Beckman Spec 25 connected to two recirculating Lauda baths (Wetmur, 1971). Reactions which were too rapid to follow with this apparatus or which could not be measured because of light scattering artifacts were determined using a radioactive tracer and the more complex calf thymus DNA. ^{125}I-labeled single-copy DNA was used to follow the renaturation of single-copy sequences in bulk unfractionated calf thymus DNA. The reaction products at various time intervals were determined by digesting a DNA sample with endonuclease S1, spotting the sample on a tlc strip and developing with 1 M HCl. The tlc strips were then cut in half and counted in a Beckman Biogamma counter. After correcting for any counts which run without digestion with endonuclease S1 or which do not run when a boiled and ice quenched sample is digested with endonuclease S1, the ratio of counts which run to counts which do not run is plotted as a function of time. The slope of the best fit line through the first half time of reaction is taken to be $k_2C_o/2$. k_2 values thus obtained are self-consistent estimates of the non-second order renaturation rate constant.

C. RESULTS AND DISCUSSION

1. Sodium Chloride and Tetraethylammonium Chloride

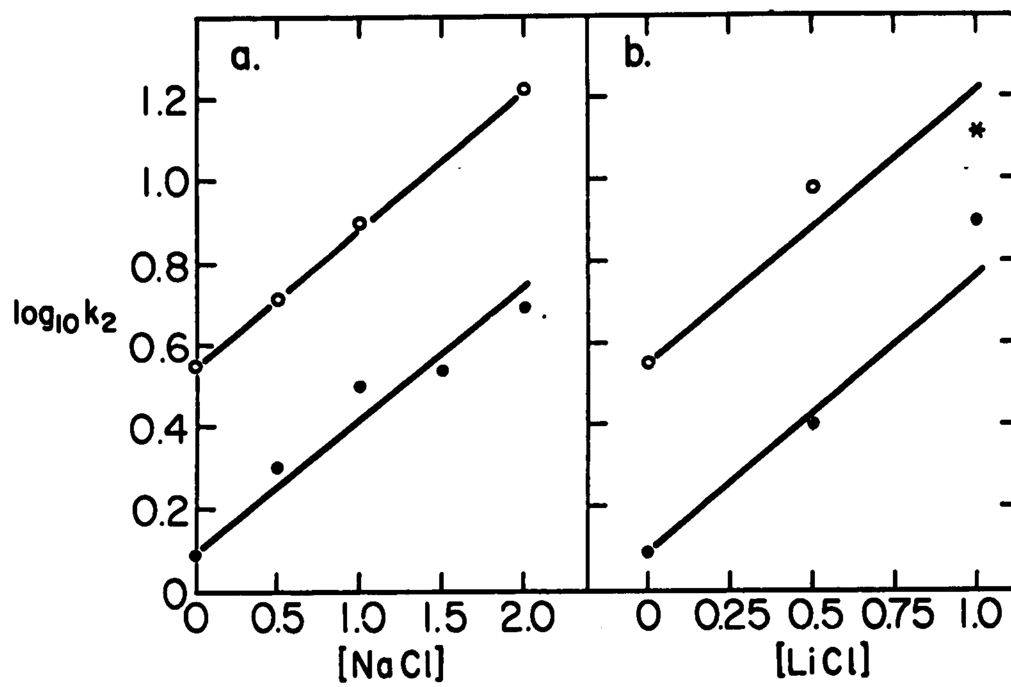
The effect of NaCl on DNA renaturation in 2.4 M Et_4NCl at 45°C was investigated by Orosz and Wetmur (1977). Similar but more extensive results are shown in figure 1a. k_N' for DNA in 2.4 M Et_4NCl at 45°C is 2.25×10^5. Addition of NaCl to DNA in 2.4 M Et_4NCl has little effect on the melting temperature but does increase the rate of renaturation of DNA by as much as five-fold. Ordinarily, dilution of DNA by 100-fold leads to no more than a two-fold increase in the second order rate constant. This same approximately second order dependence of renaturation rates is found in 2.4 M Et_4NCl plus NaCl up to 1.5 M. At the highest NaCl concentrations, the renaturation rates appear to increase more than expected with DNA dilution. Also, renaturation reactions involving longer DNAs appear to terminate prematurely when concentrated ($\geq$ 0.5 mg/ml) DNA solutions are used. These findings are interpreted to indicate a competition between DNA aggregation and renaturation in solution containing 2 M NaCl and 2.4 M Et_4NCl.

Addition of NaCl to DNA in Et_4NCl might be viewed as being addition of Et_4NCl to DNA in NaCl. If so, the net

Fig. 1. The effect of addition of salts to 0.5 mg/ml DNA solutions in 2.4 M Et_4NCl on the rate constant for renaturation (k_2) at 45°C. DNAs of two different molecular weights were investigated: (o) long; (•) sonicated; (*) long - this reaction terminated before 50% completion.

(a) NaCl addition

(b) LiCl addition



a.
b.
1.2
1.0
0.8
0.6
0.4
0.2
0
$\log_{10} k_2$
0
0.5
1.0
1.5
2.0
[NaCl]
0
0.25
0.5
0.75
1.0
[LiCl]
*

result of adding Et_4NCl is to lower the melting temperature without affecting the rate of renaturation. Anderson, et al. (1978) have shown that Na^+ binds four times more strongly to native DNA than does Et_4N^+. Substitution of Na^+ for Et_4N^+ in the denatured DNA counterion atmosphere, as well as increasing the ionic strength, apparently increases the renaturation rate by an amount comparable to the difference in microscopic viscosity at the renaturation temperatures in NaCl and Et_4NCl. The mixed solvent system is convenient because the low renaturation temperature and relatively rapid renaturation rate with concentrated DNA. The phenomenon which leads to increased renaturation rates for dilute DNA in concentrated NaCl, 2.4 M Et_4NCl solutions might be of practical value, but there seems little advantage over other acceleration systems described in the introduction and below.

2. Lithium Chloride and Tetraethylammonium Chloride

The effect of LiCl on DNA renaturation rates in 2.4 M Et_4NCl at 45°C is shown in figure 1b. The figure shows the results for DNAs of two different lengths. Addition of LiCl was found to have little effect on the melting temperature and was also found to have little effect on the base composition dependence of DNA melting. LiCl appears to be about twice as effective as NaCl of the same concentration in accelerating

DNA renaturation rates with 0.5 mg/ml DNA. Significant deviations from the normal concentration dependence of k_2 begin to be observed in 0.5 M LiCl and are marked in 1 M LiCl. In addition, with long DNA and 1 M LiCl, premature terminations of renaturation occured prior to 50% completion (see figure 1b). These results are again interpreted to indicate aggregation of single-stranded DNA. 0.5 M LiCl or 1 M NaCl in 2.4 M Et_4NCl may be used to obtain a reproducible 2-fold increase in renaturation rate. Use of high concentrations of NaCl or LiCl in 2.4 M Et_4NCl or of LiCl alone might be useful as an alternative to the PERT procedure with dilute DNA solutions. LiCl solutions, especially, deserve further study. In terms of obtaining the shortest possible renaturation half-time for a given DNA, however, dextran sulfate volume exclusion remains the most effective method and is the subject of study below.

3. Dextran Sulfate and Tetraethylammonium Chloride

The effect of 0-12 percent dextran sulfate 500 (intrinsic viscosity 0.9) on DNA renaturation rates in 2.4 M Et_4NCl was investigated by Wetmur (1975). Accelerations of 10 fold were achieved under conditions where second order kinetics are obeyed. It has been subsequently determined that acceleration of DNA renaturation rates does not change the relationship

between k_2 and L_s (eq.5). It was also found that higher concentrations of dextran sulfate may be achieved at elevated temperatures. DNA renaturation rate accelerations are not increased by increasing the dextran sulfate concentration to 15-20 percent (table 1). If 2.4 M Et_4NCl is to be used as a solvent for DNA renaturation addition of 10-15 percent dextran sulfate is the best means of providing a reproducible 10-fold rate acceleration. The acceleration is at least 3-fold more effective than that due to NaCl or LiCl addition with 0.5 mg/ml DNA. A method for subsequent removal of dextran sulfate from a DNA renaturation solution is described below.

4. Dextran Sulfate and Tetraethylammonium Chloride with Added Sodium or Lithium Chloride

Renaturation rate constants (k_2) and rate acceleration (R) for 0.5 mg/ml E. coli DNA in 2.4 M Et_4NCl plus added NaCl or LiCl are listed in table 1. The plateau value for rate acceleration is about 10-fold in 2.4 M Et_4NCl. Further addition of NaCl to solutions containing concentrated dextran sulfate fails to lead to a homogeneous solution. 0.5 M LiCl fails to improve upon rate accelerations achievable without added salt. 1.0 M LiCl and 5 percent dextran sulfate does lead to about a 2-fold additional acceleration. However, the

TABLE 1.

Volume Exclusion-Induced Acceleration of DNA Renaturation in 2.4*M* Et_4NCl[a]

Dextran Sulfate (%)	Added Salt	k_2	R[b]
0	0	1.2	1
5	0	4.7	4
10	0	11	9
15	0	12	10
0	1*M* NaCl	2.5	2
10	1*M* NaCl	12	10
15	1*M* NaCl	11	9
0	0.5*M* LiCl	2.4	2
5	0.5*M* LiCl	10	8
10	0.5*M* LiCl	Precipitation	
0	1*M* LiCl[c]	8	7
5	1*M* LiCl[c]	25	21

[a] *E. coli* DNA, 0.5 mg/ml.
[b] *R* is the rate acceleration compared to rates in 2.4*M* Et_4NCl.
[c] Reactions proceeded over 50% to completion but terminated prematurely.

form of the renaturation reaction is altered, indicating aggregation of the DNA (sonicated) competing with renaturation. It is concluded that no additional advantage is gained by adding NaCl or LiCl to DNA solutions in 2.4 M Et_4NCl containing the volume excluding polymer dextran sulfate.

5. Dextran Sulfate and Sodium Chloride

In order to attempt to find a method for further accelerating DNA renaturation DNA in 1 M NaCl plus dextran sulfate was studied. 2 M NaCl was also investigated but no advantage over 1 M NaCl was found. Renaturation rates are known to be higher in NaCl than in Et_4NCl by about a factor of two, presumably due to a difference in microscopic viscosity of the solvents at the renaturation temperatures (Orosz and Wetmur, 1977).

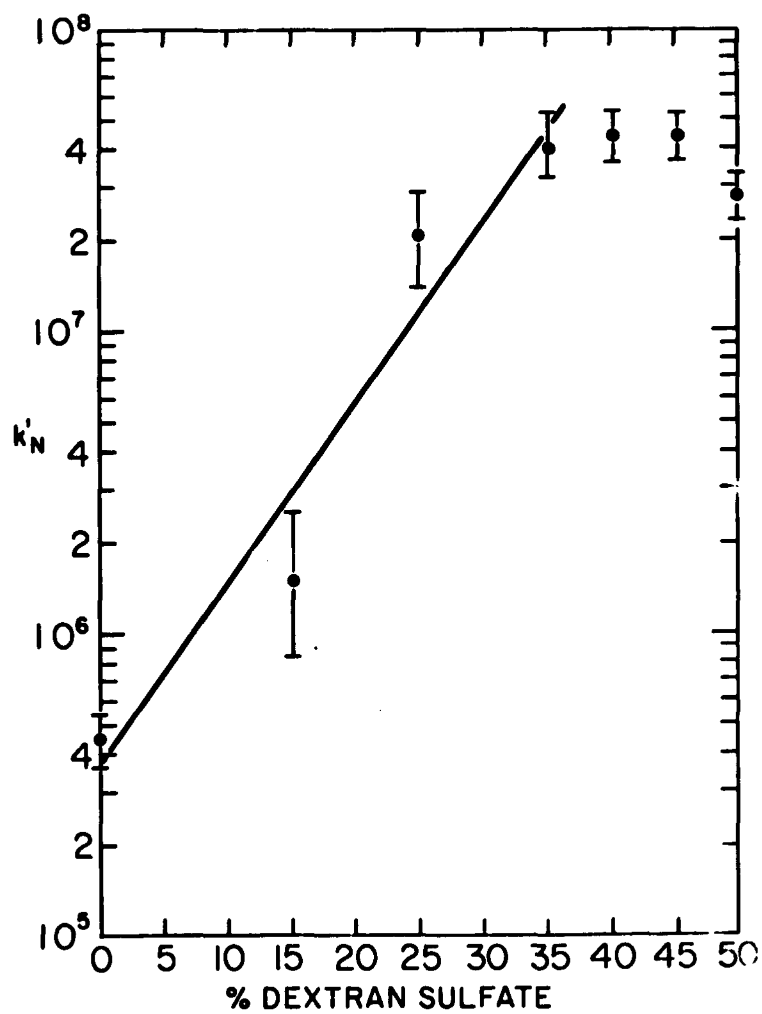
Dextran sulfate is more soluble in 1 M NaCl than in 2.4 M Et_4NCl. When greater than 25 percent dextran sulfate in 1 M NaCl was used as a solvent for E. coli DNA renaturation optical methods of following renaturation became unsatisfactory for three reasons. First, a refractive index gradient developed in the cuvette when the sample was heated or cooled leading to artifactual absorbance changes. Second, the DNA could not be denatured at 100°C thus obviating simple measurement of a reference absorbance for denatured DNA.

Finally, the reaction rates became extremely fast - faster than might have been anticipated by the simple replacement of Et_4NCl with NaCl. For these reasons, studies in 1 M NaCl with dextran sulfate were carried out with calf thymus DNA at 1.0 mg/ml using a tracer ^{125}I-labeled single-copy calf thymus DNA. The solutions were prepared by addition of solid dextran sulfate and NaCl to denatured calf thymus DNA plus tracer. The use of NaCl instead of Et_4NCl is quite compatible with tracer experiments because NaCl at moderate concentration inhibits endonuclease S1 less than does Et_4NCl. Dextran sulfate does not affect endonuclease S1 digestion following dilution from either 1 M NaCl or 2.4 M Et_4NCl.

The results of renaturation rate measurements in 1 M NaCl with various concentrations of dextran sulfate are shown in figure 2. The 0 and 15 percent dextran sulfate data were confirmed using spectrophotometric measurements. Because of the difficulty in measuring DNA melting in solutions containing 25 percent or more dextran sulfate, the conditions for optimum renaturation were investigated. Maximum renaturation rates were found to occur at 70°C for dextran sulfate containing solutions just as would be found with only 1 M NaCl. k_N' values may be converted to R values relative to 2.4 M Et_4NCl (or 0.7 M NaCl) by division by 2.25×10^5. Many of the R values in figure 2 exceed 100, or 10 fold better than the plateau value for acceleration of DNA renaturation rates by dextran sulfate in 2.4 M Et_4NCl.

Because of heterogeneity of DNA sizes and sequence

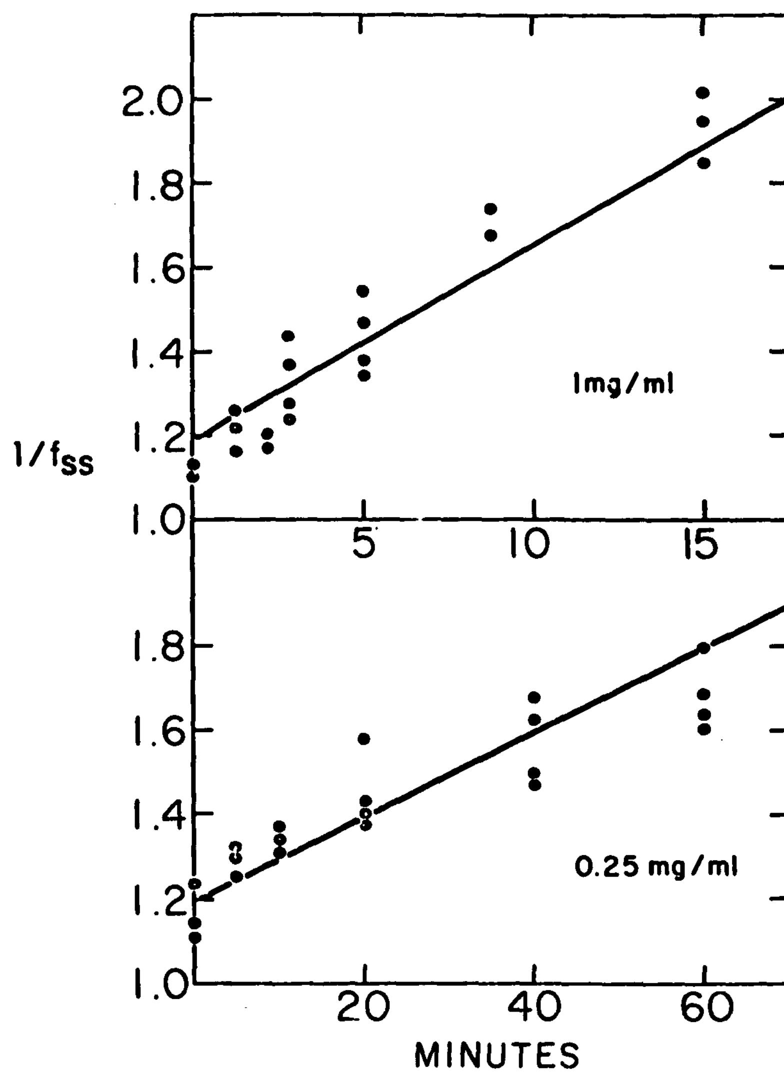
Fig. 2. The nucleation rate constant k_N' at 70^oC is shown as a function of added dextran sulfate in 1 M NaCl. The DNA used for determination of k_N' was sonicated calf thymus DNA at 1.0 mg/ml.



10^8
4
2
10^7
k'_N
4
2
10^6
4
2
10^5
0 5 10 15 20 25 30 35 40 45 50
% DEXTRAN SULFATE

phases, renaturation reactions slow down more in the later stages than would be the case for a simple second order reaction. For this reason, renaturation rate constants are usually derived from the behavior of the DNA during the first half of the reaction. Determination of reaction order is based on the effect of changing the initial DNA concentration. The effect of a four-fold change in DNA concentration on the renaturation kinetics of single-copy calf thymus DNA in 1 M NaCl plus 45 percent dextran sulfate is shown in figure 3. The reciprocal of the fraction of single-stranded DNA, f_{ss}, is shown as a function of time on two different scales. The results are in agreement with second order renaturation of single-copy calf thymus DNA. The half time of reaction is less than 20 minutes at 1 mg/ml calf thymus DNA. The half time for renaturation of E. coli DNA under comparable conditions is of the order of a second, which explains our failure to measure such a rate. Beginning with denatured E. coli DNA, the reaction is completed before it is possible to heat and cool a sample to and from the renaturation temperature.

Fig. 3. The reciprocal of the fraction of a single-stranded single-copy calf thymus DNA is shown as a function of time for two concentrations of calf thymus DNA. The solvent is 1 M NaCl, 45 percent dextran sulfate at 70°C. The experiments were followed using the endonuclease S1 assay.



2.0
1.8
1.6
1.4
1.2
1.0
1/fss
1mg/ml
5
10
15
1.8
1.6
1.4
1.2
1.0
0.25 mg/ml
20
40
60
MINUTES

6. Dextran Sulfate and Sodium Chloride with Organic Solvents

Table 2 lists nucleation rate constants for DNA renaturation in 2 M NaCl plus 15 percent dextran sulfate with added Et_4NCl or organic solvents. Addition of organic solvents which raise or lower the dielectric constant might be expected to affect both DNA-DNA interactions and the excluded volume due to the dextran sulfate. Small amounts of Et_4NCl lowered DNA melting temperatures without significantly affecting k_N'. This result is in agreement with studies with 2.4 M Et_4NCl, dextran sulfate and lower concentrations of NaCl. Adition of formamide, N,N-dimethylformamide and dimethylsulfoxide, solvents with variable and opposite effects on the dielectric constant, lower DNA melting temperatures but also decrease k_N' in each case. The compatibility of dextran sulfate and formamide or other denaturing solvents in addition to Et_4NCl may be of importance for accelerating various types of DNA renaturation experiments, especially those involving DNA attached to a support.

TABLE 2.

Effect Of Adding Organic Solvents On Renaturation of DNA in 2*M* NaCl[a]

Dextran Sulfate (%)	Organic Solvent	$k'_N \times 10^{-6}$
0	0	0.6
15	0	1.8
15	10% Formamide	1.5
15	20% Formamide	1.2
15	30% Formamide	0.9
15	10% *N,N*-Dimethylformamide	1.3
15	10% Dimethyl sulfoxide	0.9
15	0.5*M* Et_4NCl	1.9

[a] Measurements were carried out at 25°C below the melting temperature.

7. Removal of Dextran Sulfate

Dextran sulfate may be selectively precipitated from a DNA solution of CsCl to a concentration of 1 M. In 1 M NaCl, the refractive index was found to behave according to a straight line form, with percent dextran sulfate, having intercept 1.343 and a slope 0.00105. After precipitation of dextran sulfate from solutions of 1-40 percent dextran sulfate and dialysis into 1 M NaCl, no dextran sulfate could be detected in solution by refractive index measurements capable of detecting much less than one percent. The DNA concentration of the supernatant fractions was determined using ^{125}I-labeled DNA in the presence of 0.5 mg/ml DNA. The DNAs were partly native and partly denatured. Greater than 99 percent of the initial ^{125}I activity per unit volume could be accounted for in the supernatant after precipitation when the effect of CsCl on ^{125}I detection was taken into account.

D. FURTHER DISCUSSION

The most rapid rates of DNA renaturation appear to occur in concentrated NaCl solutions with large quantities of dextran sulfate. Acceleration can be achieved in solvents which lower DNA melting temperatures, such as Et_4NCl, but not to the same level as in NaCl at 70^oC. A straight line is drawn in Figure 2, which relates $\log_{10}k_N'$ to dextran sulfate concentration. This line does not fit the data as well as would a sigmoid curve indicative of a phase transition type of phenomenon. On the other hand, a straight line fit was obtained in 2.4 M Et_4NCl (Wetmur, 1975), indicating a simpler type of excluded volume mechanism. Polymers with the same charges would be expected to be incompatible (Flory, 1953). If so, a concentrated DNA phase could form. Simply equating the chemical potential of water in the two phases would lead to a predicted volume concentration of DNA in this phase which would be similar to the dextran sulfate concentration in the larger dilute DNA phase. Thus a 500-fold concentration of DNA from 1.0 mg/ml could occur in 25 percent dextran sulfate with a similar acceleration of DNA renaturation. If not all of the DNA were found in the concentrated DNA phase, the rate of DNA renaturation would be accelerated less and still would be concentration dependent. These predictions might be altered, at least quantitatively, if electrostatic effects on excluded

volume were taken into account. The precise way of dealing with phase equilibria involving concentrated polyelectrolytes has yet to be determined. Recent calculations involving the parallel phenomenon of native DNA condensation induced by polymers (Naghizadeh and Massih, 1978) or salts (Post and Zimm, 1979) may lead the way to studies involving polymer incompatibility with denatured DNA. Nevertheless, whether or not a phase transition is involved with dextran sulfate and DNA in NaCl, the DNA renaturation reaction is extremely fast, quite reproducible, and responds to the same kinetic variables as does renaturation in simple salt solutions.

CHAPTER III. FACTORS AFFECTING THE KINETICS OF DNA REASSOCIATION IN PHENOL-WATER EMULSION AT HIGH DNA CONCENTRATIONS

A. INTRODUCTION

The Phenol Emulsion Reassociation Technique (PERT) was described in an elegant study by Kohne, Levison and Byers (1977). The method permits rapid reassociation (renaturation) of homologous single strands of DNA in a phenol-water emulsion at room temperature. Many factors were found to affect the reassociation rate constant. Among these factors were the complexity of the DNA, the concentration and chaotropicity of the salt present, the phenol concentration, the pH, the temperature, and the vigorousness of agitation of the reaction vessel. The complexity of the DNA affected the rate constant in the same way as is found with reassociation reactions in homogeneous solutions. In contrast, the rate constant with the PERT was found to be independent of DNA strand length. In addition, the rate constant with the PERT was found to be concentration dependent, indicating that DNA reassociation in a phenol-water emulsion is not a second order reaction at all DNA concentrations. The authors also pointed out the

importance of adding the phenol to a reaction mixture last as well as the importance of diluting an emulsion only into a concentrated salt solution. When all of these factors are controlled, the PERT is an extremely useful method for accelerating DNA reassociation. The PERT is more effective than the dextran sulfate system (Wieder and Wetmur, 1981; chapter 2 of this thesis) for accelerating DNA reassociation at low to moderate DNA concentrations. Many groups have employed the PERT since its discovery (Byers, et al, 1979; Dutko and Oldstone, 1979; Drlica, et al., 1978; Henny and Smith, 1979; Shih, et al., 1977; Stanfield and Lengyel, 1979; Wetmur, et al., 1979; Dvorak and Riman, 1980).

Kohne, Levison and Byers (1977) also found that the reassociation rate constant was affected by the presence of nonhomologous DNA species. Single stranded DNA was found to interfere with the reaction whereas native DNA had no effect or enhanced the rate. They suggested that certain areas of the phenol-water emulsion were about 20-30°C below the DNA melting temperature, thus producing optimum conditions for DNA reassociation. They also suggested that single-stranded DNA aggregated at the phenol-water interface where reassociation occurs. This model may account for the effect of DNA concentration and competing DNAs on reassociation rate constants. This study concentrated on factors affecting DNA reassociation at high DNA concentrations using the PERT system. In addition to verifying and extending the data concerning the effect of DNA concentration and the effects of

various solvent parameters on the rate of reassociation of DNA, it was determined that the reactions become length dependent at high DNA concentrations. The effects of competing DNAs, including partially reassociated reaction intermediates have also been examined. These studies of the reaction mechanism for the PERT were carried out using an endonuclease S1 assay for base pair formation.

B. EXPERIMENTAL

1. Chemicals and Biochemicals

All chemicals used were reagent grade. Liquefied phenol was obtained from Fisher. Polyethyleneimine impregnated CEL 300 PEI thin layer chromatography (tlc) plates from Macherey Nagel were obtained from Brinkman and pre coated tlc PEI-cellulose F plates from MC/B Manufacturing were purchased from VWR. Endonuclease S1 was obtained from Sigma and assayed as previously described (Hutton and Wetmur, 1973a). *E. coli* B DNA type VIII was purchased from Sigma. The DNA was sonicated using eight thirty-second pulses at $0^{o}C$ with a Heat Systems Ultrasonics Sonifier Cell Disruptor.

2. Single-Copy DNA

Single copy calf thymus DNA was prepared by hydroxylapatite chromatography on Biogel HTP (Bio-Rad) in 0.15M potassium phosphate buffer, pH 7.0, of sonicated denatured calf thymus DNA which had been renatured to C_ot 15 (Britten, et al., 1974). C_o is the initial molar DNA nucleotide concentration and t is time in seconds.

3. ^{125}I-Labeled Probe DNA

DNA was labeled *in vitro* with ^{125}I (Chan, et al., 1976) under conditions which maximize ^{125}I incorporation while minimizing chain scission. Prior to iodination, the DNA was extracted with phenol-chloroform to remove trace levels of associated proteins. ^{125}I-labeled probe DNAs were used with bulk DNAs of the same molecular weight.

4. PERT Reactions

All reactions were carried out in a final volume of 0.4 ml in a 1/2 dram screwcap glass vial with a teflon lid in the cap. Reaction mixtures were made up with phenol added last. Zero time points were taken prior to addition of phenol. The reactions were shaken in a polyallomer tube clamped to a Sybron Thermolyne shaker from Fisher.

5. Measurement of Renaturation Rates

^{125}I labeled E. coli DNA was used to follow renaturation The reaction products were assayed by diluting 25ul aliquots at various time points into ice cold 2 M NaCl. The dilutions were four and eight fold for thiocyanate and phosphate containing experiments respectively. The dilution resulted in clearing of the emulsion removing the surface on which renaturation had taken place. These samples were subsequently diluted ten fold into endonuclease S1 assay buffer (0.2 M NaCl 0.1 M NaAc pH 4.17 and 0.001 M $ZnCl_2$) and assayed as in chapter 2. After the DNA samples were digested with endonuclease S1, they were spotted on tlc strips and developed with 1 M HCl. The tlc strips were then cut in half and counted in a Beckman Biogamma counter. After correcting for any counts which comigrate with the solvent front without digestion with endonuclease S1, generally fewer than 5% of the total counts, or which do not move from the origin when a boiled and ice quenched sample is digested with endonuclease S1, about 10% of the total counts, the ratio of counts which migrate to counts which do not is plotted as a function of time. The slopes of the curves past the first half-time as well as the extent of renaturation varied with the concentration of the DNA, as will be shown later. For these reasons, the rate constant was determined from the slope of

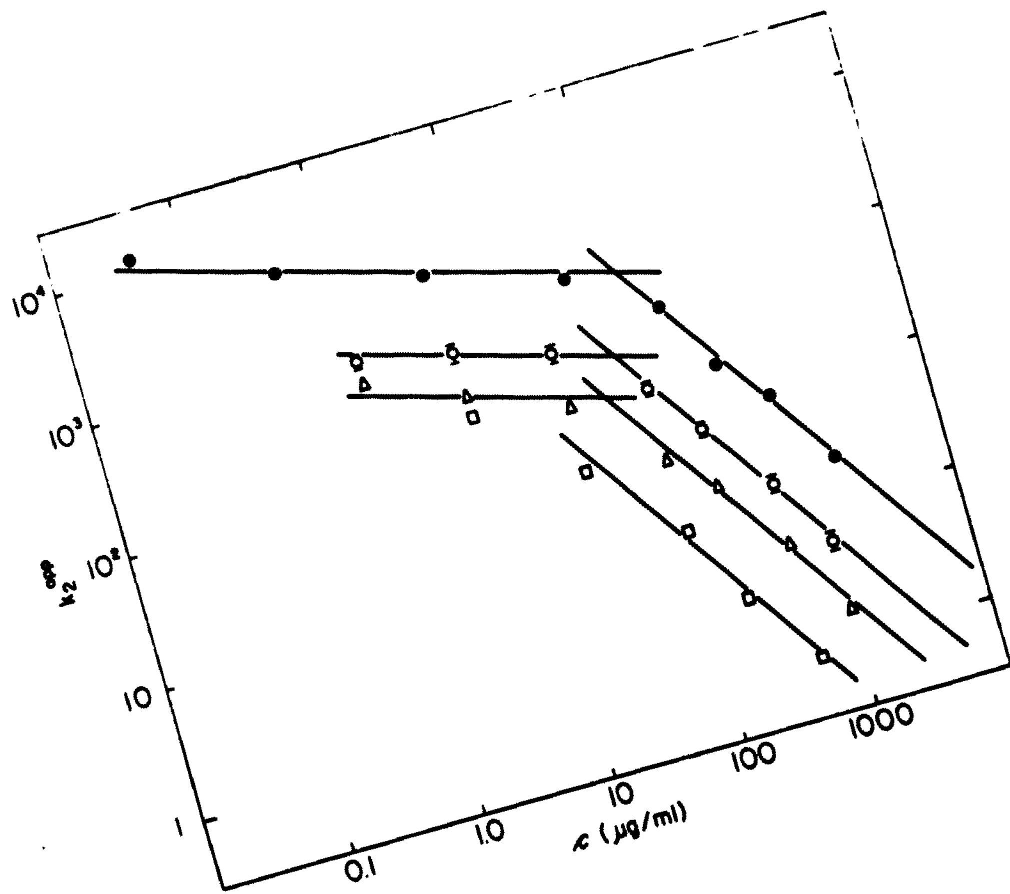
the best-fit line through the first half-time of reaction, or $1/f_{ss}$ between 1 and 2, which was taken to be $k_2^{app}C_o/2$.

C. RESULTS

1. Definition of Two DNA Concentration Ranges for PERT at Various Temperatures

Figure 4 shows the apparent second order rate constant for DNA reassociation, k_2^{app}, as a function of DNA concentration for _E. coli_ DNA in 2 M NaSCN under four conditions. The rate constants of reactions in phenol emulsions fall along two intersecting straight lines. In the low DNA concentration range, the rate constant drops by about a factor of 10 when the DNA concentration is increased 1000-fold. In the high DNA concentration range, the rate constant drops more than a factor of 10 when the DNA concentration is increased 10-fold. In order of increasing values, the open symbols in figure 4 show k_2^{app} in 9% phenol at 4°, 37° and 25°C (room temperature). In 2 M NaSCN, a chaotropic salt, reassociation rates appear to be optimal at room temperature. The closed symbols show k_2^{app} values in 12.9% phenol obtained by Kohne, et al. (1977) at 25°C. The low DNA concentration lines in figure 4 are drawn to be parallel to the extensive data of Kohne, et al. (1977). The new data is in fair agreement with this assumption. In the high DNA concentration range, the parallel lines are a best

Fig. 4. The effect of the DNA concentration (c) on the apparent reassociation rate constant (k_2^{app}) in 2 M NaSCN and 9% phenol as measured by endonuclease S1 digestion is shown at various temperatures: (o) room temperature; (Δ) 37°C; (□) 4°C. Data from Kohne, et al. (1977) at room temperature in in 2 M NaSCN and 12.9% phenol as measured by hydroxylapatite chromatography is also shown, (•).



10^4
10^3
10^2
10
1
k_2^{app}
0.1
1.0
10
100
1000
c (μg/ml)

fit through all four of the data sets. There is no indication that temperature affects the slope of the k_2^{app}-DNA concentration plots in this DNA concentration range.

The data of Kohne, et al. (1977) at 25°C are 4 to 8 fold higher than the new data at 25°C. A factor of 2 difference may be accounted for by the difference between hydroxylapatite chromatographic determination of reaction progress and the slower apparent rates detected using endonuclease S1 digestion. The remaining difference may be due to a combination of different phenol concentrations, differences in agitation procedures and differences in DNA single-stranded molecular weights. The latter factor is described in subsequent sections.

Figure 5 shows the apparent second order rate constant for DNA reassociation, k_2^{app}, as a function of DNA concentration for E. coli DNA in 0.48 M phosphate buffer and 9% phenol. In order of increasing values, the symbols in figure 5 show k_2^{app} at 4°, 25° and 37°C. The straight lines were drawn with the same slopes as obtained from figure 4. There is no indication that substitution of phosphate buffer for NaSCN affects the slope of the k_2^{app}-DNA concentration plots. Again, there is no indication that temperature affects the slope in the high DNA concentration range. With the nonchaotropic salt, 0.48 M phosphate buffer, DNA reassociates faster at 37°C than at 25°C. The k_2^{app} values at high DNA concentrations are approximately the same in 0.48 M phosphate buffer at 37°C as in 2 M NaSCN at 25°C.

Fig. 5. The effect of the DNA concentration (c) on the apparent rate constant (k_2^{app}) in 0.48 M sodium phosphate buffer and 9% phenol as measured by endonuclease S1 digestion is shown at various temperatures: (o) room temperature; (△) 37°C; (□) 4°C.

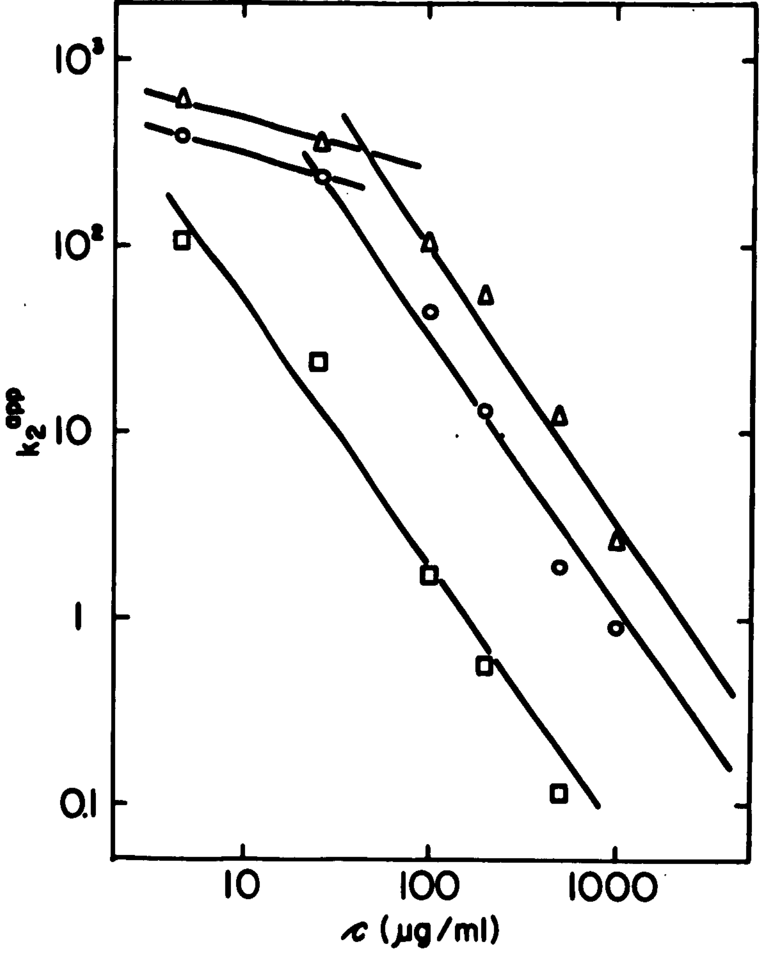
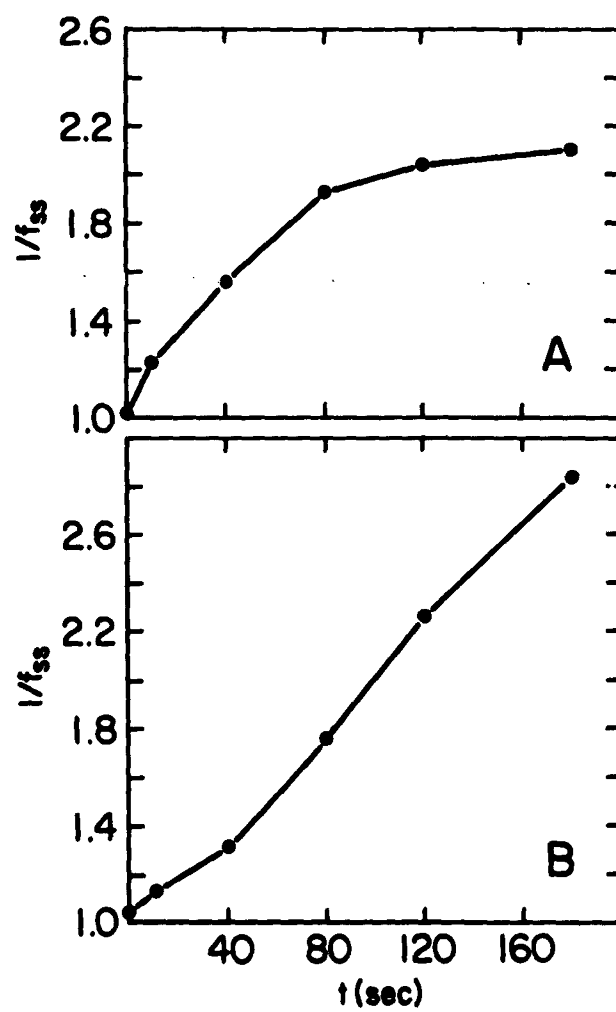

10³
10²
10
1
0.1
k_2^{app}
10
100
1000
c (μg/ml)

Figure 6 shows examples of actual rate plots for E. coli DNA in the low and high DNA concentration ranges, both done with the same tracer and carrier. As may be seen in panel A, which shows an experiment in the low concentration range, the rate plot curves downward substantially. The downward curvature with increasing time is even greater than seen for reassociation of DNA in solution when endonuclease S1 digestion is used as the assay for reaction progress. The additional downward curvature occurs because only ends of partially native molecules are left to react and these molecules are less associated with the reaction surface. In panel B, which shows an experiment in the high concentration range, the rate plot curves upward or is straight over a substantial fraction of the reassociation reaction. Late in the reaction, the rates are greater than in panel A because the DNA concentration is higher. Earlier in the reaction, the rates are much lower than would be expected for such a high DNA concentration because the emulsion surface is saturated. The forms of these rate plots reflect differences in the competition reactions of native, denatured and partially renatured DNA for the phenol-water interface. Studies of these competition reactions are described in the next section.

Fig. 6. Reassociation rate plots for *E. coli* DNA in 2 M NaSCN and 9% phenol at room temperature. (A) 25 ug/ml; (B) 500 ug/ml.


2.6
2.2
1.8
1.4
1.0
1/f$_{ss}$
A
2.6
2.2
1.8
1.4
1.0
1/f$_{ss}$
B
40
80
120
160
t (sec)

2. Competition Between Various Forms of DNA

Figure 7 shows the effects of added DNAs on the apparent second order rate constant, k_2^{app}, for reassociation of 5 ug/ml E. coli DNA. The concentration, C_o, used to calculate k_2^{app} is that of the E. coli single-stranded DNA only. Panel A shows competition experiments in 2 M NaSCN, and panel B shows identical experiments in 0.48 M phosphate buffer, both at room temperature. The total DNA concentration, c, includes both 5 ug/ml ^{125}I labeled E. coli DNA and the added DNA, c_{added}. For the experimental points,

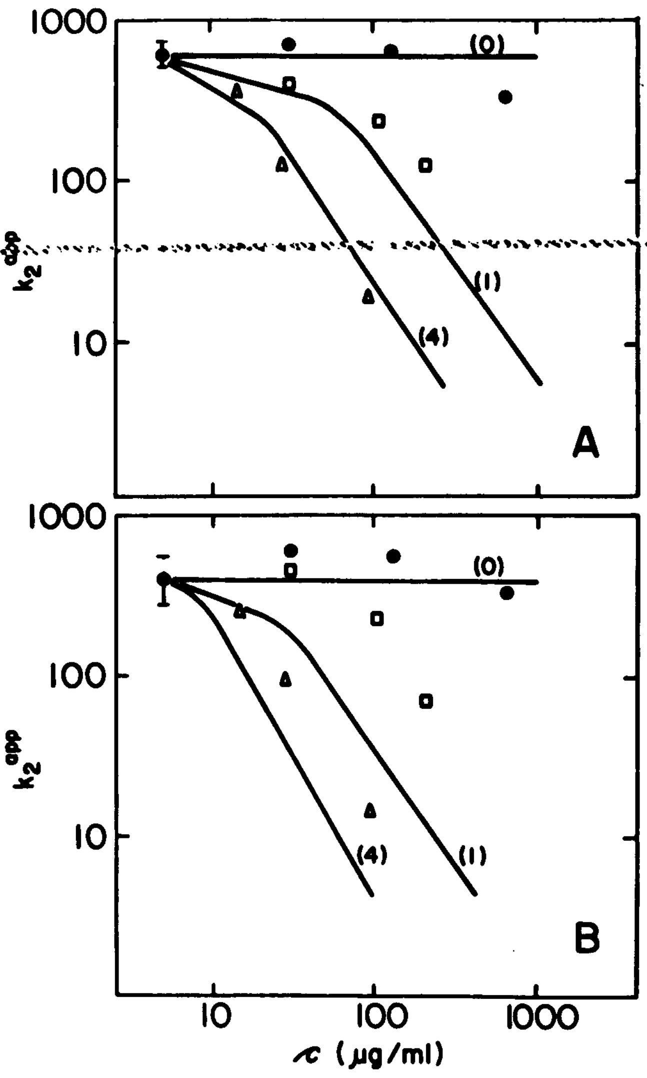
$$c = 5 + c_{added} \tag{16}$$

The solid curves give expected k_2^{app} values for

$$c = 5 + Nc_{added} \tag{17}$$

where N = 0, 1 or 4. The (0) curve thus describes added DNA having no effect on k_2^{app}. If any other added single-stranded DNA behaved in the same way as added single-stranded E. coli DNA, except for participation in the reassociation reaction, this DNA would affect k_2^{app} in the manner depicted in curves (1). Curve (4) thus refers to added DNA having an effect 4 times that of single-stranded E. coli DNA. The data for these

Fig. 7. The effect of various concentrations (c) of different competitors on the rate of reassociation of 5 ug/ml E. coli ^{125}I DNA probe in 2 M NaSCN (A) and 0.48 M sodium phosphate buffer (B). The competitors used were: native calf thymus DNA (o), single-stranded single-copy calf thymus DNA (△), and single-copy calf thymus DNA renatured to approximately one half-time (□). Lines labeled (0), (1) and (4) represent theoretical curves of 0:1, 1:1 and 4:1 ratios of homologous single-stranded DNA to probe concentration respectively.



1000
100
10
k_2^{app}
(0)
(1)
(4)
A
B
10
100
1000
c (μg/ml)

curves were obtained from figures 4 and 5.

The solid symbols show experimental results with added native DNA. Addition of native DNA has little or no effect on the reassociation reactions at concentrations up to 625 ug/ml. In fact, at some native DNA concentrations, native DNA appears to increase k_2^{app}. The open triangles show results with added single-stranded single-copy calf thymus DNA. Addition of this DNA greatly reduces k_2^{app}. The same experiment was performed in 2 M NaSCN and 0.48 M phosphate buffer at 37°C. The same type of rate reduction was observed (data not shown). In fact, single-copy calf thymus DNA has an effect 2-4 times that of single-stranded E. coli DNA.

The open squares show results of competition with renatured DNA. Single copy calf thymus DNA was renatured one half-time as determined spectrophotometrically. The length of the double-stranded regions in partially renatured sonicated DNA averages 60% of the length of the original double-stranded molecules (Miller and Wetmur, 1975). About 15-20% of the mass of the DNA in this sample of competitor is completely denatured and 80-85% is composed of molecules which contain both native and denatured regions. The observed competition may be accounted for if only the fully denatured DNA molecules compete.

3. Physical Association of Single-Stranded DNA with the Emulsion

Centrifugation experiments were performed with ^{125}I *E. coli* DNA in a 9% phenol emulsion with 2 M NaSCN at room temperature in a Damon IEC clinical centrifuge at a setting of 5/7 for 5 minutes. Increasing quantities of unlabeled competing DNAs were added and the specific activity ratio of the solution after centrifugation without competitor to that with competitor was determined. Table 3 presents the data which were obtained with 5 ug/ml ^{125}I *E. coli* DNA alone and with native and single-stranded competitor. Only about half of the *E. coli* DNA appears to be associated with the emulsion at 5 ug/ml *E. coli* DNA and with the centrifugation conditions employed. The aliquots were taken at approximately equal times after addition of phenol and vortexing. The fraction of counts associated with the emulsion is time dependent because small droplets coalesce to produce larger droplets with lower total surface area. Therefore, the numbers reported in table 3 are only used to provide trends. The concentrations of the competitors given in table 3 correspond to those of the competitors used to obtain figure 7.

Single-stranded calf thymus DNA, when added to the reaction, caused a decreasing association of the single-stranded *E. coli* DNA with the emulsion. As little as 10 ug/ml of single-stranded calf thymus DNA seemed to have a

TABLE 3.

EFFECT OF COMPETITORS ON THE ASSOCIATION OF ^{125}I-LABELED *E. coli* SINGLE-STRANDED DNA WITH PHENOL

	Concentrations (ug/ml)			
^{125}I DNA	Native DNA[a]	Denatured DNA[a]	Renatured DNA[b]	Ratio[c]
5				1.0
5	25			1.4
5	125			1.2
5	625			1.1
5		10		0.8
5		25		0.7
5		100		0.7
5		500		0.3
5			25	0.9
5			100	1.0
5			200	0.9

[a] calf thymus DNA

[b] single copy calf thymus DNA renatured to one half time (spectrophotometric assay)

[c] cpm removed with competitor/ cpm removed without competitor (approximately 50%)

substantial effect on the association. When added, at moderate concentrations, native calf thymus DNA has the opposite effect. In fact, at 25 ug/ml added native DNA, the stabilization of the single-stranded _E. coli_ DNA-phenol emulsion complex is even greater than at 625 ug/ml. Finally, renatured single-copy calf thymus DNA seems to have little effect on the association of _E. coli_ single-stranded DNA with the emulsion.

4. Length Effect

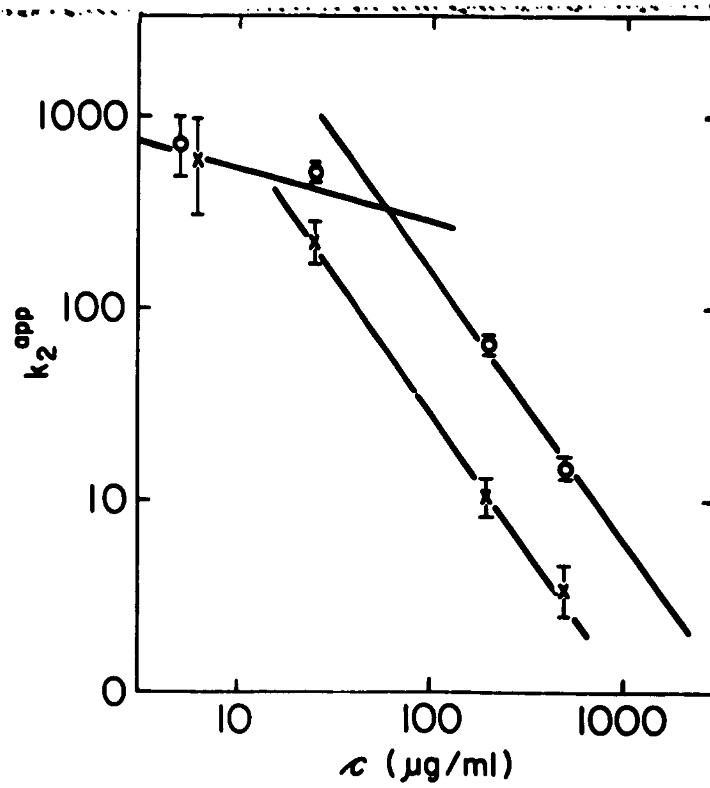
^{125}I labeled _E. coli_ DNA of two different lengths was prepared. Sonicated and commercially available intact _E. coli_ DNA were iodinated _in vitro_ according to the procedure of Chan, et al. (1976). A 50 to 1 deoxycytidine to iodide ratio was used at pH 4.6 to label the intact molecules, whereas only a 15 to 1 deoxycytidine to iodide ratio was present at pH 4.4 to label the sonicated molecules. The lengths of the two DNAs were determined by following their renaturation kinetics in the spectrophotometer in 1 M NaCl (Wetmur and Davidson, 1968). A factor of two in the rates of reassociation in 1 M NaCl at 70°C was observed, signifying a factor of four in their lengths. In contrast to previous experiments where only tracer amounts of ^{125}I DNA were used in experiments of greater than 5 ug/ml concentrations, here the entire population of

molecules was labeled. The results are presented in figure 8.

At 5 ug/ml, the rates obtained with PERT in 9% phenol and 2 M NaSCN appear the same. At higher concentrations, however, there appears to be a length effect. At 25 ug/ml the shorter molecules renature about 2.3 times faster than the longer ones. At 200 ug/ml and 500 ug/ml, the difference in rates is about a factor of 4 to 6. Thus it appears that in the low DNA concentration range, length has little or no effect on the rate of DNA renaturation, as Kohne, et al. (1977) had found, but in the high DNA concentration range, increasing single strand DNA length reduces the rate of reassociation.

Fig. 8. The effect of the DNA concentration (c) on the apparent rate constant (k_2^{app}) in 2 M NaSCN and 9% phenol at room temperature as measured by endonuclease S1 digestion with ^{125}I labeled E. coli DNAs of different lengths.

(o) Sonicated DNA; (x) unsonicated DNA.


1000
100
10
0
10
100
1000
k_2^{app}
c (µg/ml)

D. DISCUSSION

At all the temperatures studied and in both phosphate buffer and NaSCN, DNA reassociation reactions using PERT appear to have the same concentration dependence within either the low DNA concentration range of the high DNA concentration range (figures 4 and 5). Consider first the low DNA concentration range extensively studied by Kohne, et al. (1977). The reaction order appears to be 1.7. If the reactions occured at the surfaces of phenol droplets and the surface area remained constant, the reaction order would be expected to be 2 if all the DNA were associated with the surface. At 5 ug/ml single-stranded DNA, a concentration nearing the upper limit of the low DNA concentration range, about 50% of the DNA is associated with the emulsion. Because no more than twice 50% of the DNA can be associated with the surface, the deviation from second order cannot entirely be attributed to a change in the fraction of DNA associated with the emulsion surfaces. DNA reassociation in solution deviates slightly from second order behavior (Wetmur, 1976). The same phenomenon may contribute to the -0.3 deviation. A third possibility is an increase in the emulsion surface area with increasing DNA concentration. If the surface area increases, the DNA is diluted and the rate constant will decrease. The latter explanation is suggested here because the stabilization of the phenol emulsion by added DNA was observed. If the

phenol droplets are associating slower and dispersing at the same rate, an increased surface area might be associated with stabilization of the phenol emulsion by DNA.

The form of the rate curves shown in figure 6 changes when moving from the region of low DNA concentration to the region of high DNA concentration. In the high DNA concentration region, the k_2^{app} values are slight overestimates of the initial rates just as the k_2^{app} values at low DNA concentrations are underestimates. Since the k_2^{app} values were determined from the initial portion of the curves during the first half of the reaction, the errors introduced were minor, probably of the order of 10%. However, the errors were consistent within each DNA concentration range and the relative values of k_2^{app} and initial rate constants remain the same within either DNA concentration range. Thus, the interpretation of the rate curves affects the absolute value of k_2^{app} but not the slopes of the lines in figures 4 and 5.

In the region of high DNA concentration, the slope of the lines in figures 4 and 5 is -1.3. The slope of -1.3 implies a 0.7 order reaction. One explanation of this reaction order would be a continued expansion of phenol-water surface area with increasing DNA even after the surface appears to be filled. If the surface were proportional to the DNA concentration in the region of high DNA concentration, the reaction order would be 1. The deviation toward zero order could be due to incomplete association of DNA with phenol to create new surface. The centrifugation experiments reported

in table 3 include results from the region of high DNA concentration. A significant fraction of the single-stranded DNA remains associated with the emulsion at 500 ug/ml DNA. These results are consistent with a level of expansion of the surface area with added DNA in the region of high DNA concentration leading to an apparent 0.7 order reaction.

Competition experiments were performed with added denatured, native or renatured DNA. The results in figure 7 are compared with theoretical curves where added DNA has no effect (0), the expected effect if the ^{125}I labeled DNA behaves as a tracer in a more complex DNA reassociation (1), and where the added DNA has an effect equivalent to that expected for a four-fold higher concentration of added DNA (4). The native DNA has little effect on the reassociation rate of the 5 ug/ml ^{125}I-labeled E. coli DNA, implying that it does not compete for or alter the properties of the emulsion surface. Denatured DNA has an effect 2-4 times that expected. Part of this effect is due to the continuous presence of all of the added more complex DNA rather than a continuously decreasing amount of single-stranded DNA as could be the case if all the DNA were E. coli DNA. The curvature of the rate is intermediate between that of the 1.7 and 0.7 order reaction rate plots seen in figure 6 and thus k_2^{app} appears to be lower with competing complex DNA. Another effect of the failure of the added complex DNA to renature may be the continued presence of those lengths of DNA which compete best for the emulsion surface. The effect of length

on DNA reassociation using PERT is discussed below. Finally, the partially renatured DNA competes in a manner which is intermediate between the native and denatured DNA. The competition may primarily be attributed to the fully denatured DNA remaining in the competing DNA sample, although some effect of partially native molecules cannot be excluded.

In the region of low DNA concentration, most or all of the single-stranded DNA is associated with the emulsion surface and the surface is not full. Kohne, et al. (1977) observed little or no length dependence for DNA reassociation using PERT in the low DNA concentration region. The data in figure 8 is consistent with this result. DNA reassociation is described by (Wetmur, 1976)

$$k_2^{app} = k_N \; L \; / \; N \qquad (18)$$

where L is the length (nucleotides) of the DNA, N is the complexity (base pairs) of the DNA and k_N is the nucleation rate constant. In solution, a length-independent nucleation rate constant may be defined by

$$k_N' = k_N \; L^{0.5} \qquad (9)$$

because k_N is found to be inversely proportional to the square root of the length of the DNA molecules. Using PERT, a length-independent nucleation rate constant would take the form

$$k_N'' = k_N L \tag{19}$$

because k_N is inversely proportional to L in the region of low DNA concentration.

Two possible explanations could account for the L^{-1} dependence of k_N in the region of low DNA concentration. Either the base pair formation steps involved in forming a nucleation site depend on L^{-1} or there is an effect which limits available nucleation sites in a manner proportional to L^{-1}. The argument against the former mechanism (Wetmur and Davidson, 1968) seems likely to hold as well on a phenol-water interface. The energy involved in deforming single-stranded DNA chains to form a nucleation site of 2-3 base pairs should be less than thermal noise levels and independent of L. There are two ways of limiting available nucleation sites. First, reactions could occur only at the ends of DNA molecules. Such a mechanism would lead to the predicted dependence of k_2^{app} on L. There is no experimental evidence to support such a unique mechanism. Second, nucleation sites could be limited by an excluded volume effect. Such an effect has been postulated to explain the k_N dependence on $L^{-0.5}$ in solution (Wetmur and Davidson, 1968; Wetmur, 1976). An excluded volume calculation has been used to predict the correct functional dependence of k_N on lengths of single-stranded DNA molecules when the molecules have different lengths, when molecules are circularly permuted and when the molecules are linear and

circular (Kinberg-Calhoun and Wetmur, 1981). Substitution of a random-walk two-dimensional radial distribution function for the three-dimensional one used in this theory leads to the correct predicted dependence of k_N on L^{-1}. In fact, a simple excluded volume model for reaction of DNA molecules limited to a circle on the phenol-water emulsion surface will lead to the same prediction. Deviation from L^{-1} dependence of k_N will occur if the distribution functions are non-random due to intramolecular excluded volume effects. However, the range of L values tested experimentally has been too limited to detect such nonideal behavior.

At high DNA concentrations, k_2^{app} for long DNA depends on DNA concentration in the same way (0.7 order) as is the case for shorter DNAs. However, the absolute value of the rate constants is significantly reduced. The longer DNA apparently saturates the phenol-water emulsion surface at a lower DNA concentration than the shorter DNA. The reduced k_2^{app} with longer DNA is seen not only in the data in figure 8 but also in the enhanced competition by heterogeneous (length) complex single-stranded DNA at high concentrations (figure 7 and table 3). The magnitude of the effect of L on k_2^{app} in the region of high DNA concentration is larger than may be explained solely on the basis of a nonrandom radial distribution function for the DNA on the emulsion surface. An effect of DNA length on surface area available on phenol-water emulsion surfaces could be proposed, but such an explanation cannot be verified because no measurements of available surface area have

been performed.

DNA reassociation measurements using PERT in the region of high DNA concentration may be quantitative if solvent composition, temperature and pH are controlled and a reproducible agitation system is employed. The phenol must be added last, and the samples for assay must be diluted into a high salt buffer to solubilize the phenol. Finally, the length of the DNA must be controlled because the length effect of k_2^{app} in the region of high DNA concentration using the PERT is greater than the length effect on k_2^{app} in solution reassociation experiments.

CHAPTER IV. SEQUENCE ORGANIZATION OF THE EUKARYOTIC GENOME

A. INTRODUCTION

One of the aims of this project is to devise a general method of isolating specific sequences from a eukaryotic genome. In order to undertake such a study, a knowledge of the organization of the DNA in the organism under scrutiny is necessary. The experiments reported in the next chapter describe attempts at isolating sequences specific for the Y chromosome of the rat. It is for the above reasons that this chapter undertakes a discussion of the organization of eukaryotic genomic DNA and concludes with a discussion of the Y chromosome.

B. SEQUENCE ORGANIZATION

Britten and Kohne (1966) found that many eukaryotes contain in their nuclei several classes of DNA sequences which are distinguished by their copy number. In particular, they found mammalian nuclear DNA to consist of three classes of DNA

sequences: highly repeated sequences at about 10^6 copies per cell, middle repeated sequences at anywhere from 10^2 to 10^5 copies per cell, and single-copy sequences, making up about 10%, 15-20% and 70% respectively of the genome. Most organisms in the animal kingdom have about 70% of their genomes as unique sequences, regardless of the size or complexity of the genome (Davidson, et al., 1975).

1. Highly Repeated Sequences

The highly repeated sequences, in actuality, belong to three categories. A part of the highly reiterated sequences is found in satellite DNA representing 3-5% of the genome. These are sequences which band separately on either side of the main genomic band in isopycnic centrifugation experiments. They are arranged in long, uninterrupted tandem repeats (Walker, 1971) near the centromeres of some of the chromosomes (Pardue and Gall, 1970). The four human satellite DNAs have since been assigned to locations on quinacrine banded chromosomes (Gosden, et al., 1975). Satellite DNA sequences vary in their homogeneity from an exact repeat of a short oligonucleotide sequence (Skinner, et al., 1974) to longer repeats of more complex and divergent sequences (Southern, 1970). Southern (1975) found a major repeat unit of about 240 base pairs when he analyzed mouse satellite DNA with several

restriction endonucleases. Cooke and Hindley (1979) showed with cloned fragments that human satellite III DNA consists of a number of non-cross-reacting sequences which nevertheless share the same restriction site. They found that certain classes of satellite DNA are unique for certain chromosomes, and postulated satellite DNA to have a possible role in the identification of chromosomes for cellular processes.

The second category of highly repeated DNA is about an order of magnitude more complex than satellite DNA (Cech, et al., 1973). It is part of main band DNA and is interspersed with the less repetitive or non-repetitive sequences. It is estimated that at least 5% of the mouse genome is composed of segments of these rapidly renaturing sequences about 1500 base pairs long, alternating with sequences of more complex DNA about 2200 base pairs long. These sequences are about two times as mismatched as satellite DNA.

The third category of highly repetitive DNA, termed "foldback", "snapback", or "zero-time" DNA, contains inverted repeated sequences (Wilson and Thomas, 1974) which renature with first order kinetics and comprise 6% of the total human genome (Dott, et al.,1976). They appear to be heterogeneous in length with an overall average of 190 base pairs. The inverted repeats are present in almost all families of repetitive frequencies, and are interspersed in the chromosomes. There are approximately 2×10^6 inverted repeats per haploid human genome (Dott, et al., 1976). It was suggested, even before their existence was confirmed, that

inverted repeats might correspond to acceptor sites which could interact with regulatory proteins (Jelinek and Darnell, 1972). The inverted repeat regions are transcribed into heterogeneous nuclear RNA but are no longer present in mRNA (Jelinek, et al., 1974). Shorter inverted repeats have been consistently found in the nontranscribed 5' region of eukaryotic genes (Nishioka and Leder, 1979; Efstratiadis, et al., 1977).

2. Middle Repetitive Sequences

The arrangement of middle repetitive sequences in the genome of eukaryotes has been studied extensively. It became apparent that two basic categories of sequence organization exist within eukaryotic genomes. The genomes of eukaryotes are divided into a category in which repetitive DNA is interspersed with single-copy DNA at fairly short intervals in at least part of the genome, and a category where this type of interspersion pattern is not observed. At first it seemed that the former was the general rule, with exceptions existing only in some insects, but as more organisms were analyzed, the latter category began to include members of most unlikely phyla. Below follow descriptions of the two categories with a cross-sectional survey of their members.

a. The "*Xenopus* Pattern" of Interspersed Repeats

Davidson, et al. (1973b) analyzed the genome organization of the amphibian *Xenopus laevis* by renaturation and hydroxylapatite chromatography of different sized fragments. They found that approximately 50-55% of the genome consisted of an interspersion of middle repetitive sequences about 0.3 ± 0.1 kilobases (kb) among nonrepetitive sequences about 0.8 ± 0.2 kb pairs. The short element repeats represent about 60-75% of the repetitive sequences in *Xenopus* (Davidson, et al., 1973a). About 25% of the repetitive sequences were found to be organized in longer stretches of greater than 2000 nucleotides, referred to as "clustered repetitive regions". Their distribution in the genome was not ascertained. Approximately 25% of the genome was found to consist of mainly nonrepetitive regions, though most of it contained rare interspersed repetitive elements spaced 4000 or more bases apart (Davidson, et al., 1973b).

Davidson, et al. (1973a) found a positive correlation between the lengths of the repeated segments and the heat stability of duplex molecules. The short interspersed repeats had up to 15% mismatched sequences. When fractionated on a size exclusion gel filtration column, the shortest molecules manifested the greatest degree of mismatch, with a gradual increase in thermal stability with the length of the repeat.

This was still true after correcting for the T_m difference due to duplex length. Of the repeated DNA in the genome, the 25% fraction in the "clustered repeat" pattern exhibited the lowest degree of mismatch and, hence, the least amount of evolutionary divergence of all the repeats.

A wide cross-section of the animal kingdom exhibits the "*Xenopus* pattern" of genome organization, although the fraction of the genome arranged in this pattern is variable. Generally, the lengths of the interspersed repeats are around 300 base pairs and the lengths of the interspersed single-copy regions are between 0.8 and 3 kilobase pairs. Occasionally, these values vary substantially.

Davidson, et al. (1975) suggested that the "*Xenopus* pattern" of sequence organization originates from a remote evolutionary stage predating the divergence of metazoans. Recent studies have dated the existence of the repeat pattern at the earlier unicellular stage of life development (Borchsenius, et al., 1978; Hinnebusch, et al., 1980). The pattern remains as an integral and major part of the genomes of the most complex of organisms. Below follows a phylogenetic survey of the organisms whose genome organization was found to contain, at least in part, a "*Xenopus*-like" DNA sequence pattern.

The "*Xenopus* pattern" of DNA organization was discovered in the primitive ciliate *Tetrahymena pyriformis GL* (Borchsenius, et al., 1978) and in the dinoflagellate *Crypthecodinium cohnii* (Hinnebusch, et al., 1980). The

fraction of the Tetrahymena genome which is arranged in the "Xenopus pattern" is only about a third of that usually found. The reason is that 90% of its DNA is single-copy. The flagellate Euglena gracilis also has a repeat pattern with 1500 base pair unique segments, but the dispersed repeats are longer than average for the pattern. 67% of the repeats average 4900 base pairs, while a third average 1000 base pairs (Rawson, et al., 1979).

The plant kingdom also has phyla in which studies have discovered representative organisms which have their DNA arranged in the "Xenopus pattern". Phylum Myxomycophyta, the slime molds, contain such members. Among these are Physarum polycephalum of the class Myxomycetea (Hardman, et al., 1980) and Dictyostelium discoideum of the class Acranea (Firtel and Kindle, 1975). The average length of the interspersed repeats in Physarum is 590 base pairs, about twice as long as the Xenopus average, but the pattern is the same. The higher vascular plants of the phylum Tracheophyta also have representative members with the "Xenopus pattern" of organization. Several members of the class Angiosperma were studied. The pattern was found in the monocotyledons rye (Secale cereale) (Smith and Flavell, 1977) and wheat (Triticum aestivum) (Gurley, et al., 1979) and in the dicotyledons soybean (Glycine max), cotton (Gossypium hirsutum), tobacco (Nicotiana tabacum) (Zimmerman and Goldberg, 1977) and mung bean (Vigna radiata) (Murray, et al., 1979). The pattern in the french bean Phaseolus vulgaris and the green gram P.

aureus is the same, except for the lengths of the interspersed repeated sequences which are 1900-2300 base pairs long (Seshadri and Ranjekar, 1980).

The survey of the animal kingdom has been far more expansive. Seven phyla in the subkingdom Metazoa, or animals with tissues, have been found to contain members with the "Xenopus pattern" of sequence organization. In the phylum Coelentrata, the jellyfish Aurelia aurita was found to belong to this group (Goldberg, et al., 1975). In the protostomial group, the primitive acoelomate Cerebratulus lacteus, the ribbon worm of the phylum Nemertea also belongs, as does the genus Urechis of the phylum Echiuroidea (Goldberg, et al., 1975). Several members of the phylum Mollusca were classified in this category. Among them were the gastropod mollusc Aplysia californica (Angerer, et al., 1975), the surf clam Spissula solidissima and the oyster Crassostrea virginica (Goldberg, et al., 1975). Among the phylum Arthropoda, two classes studied are represented. In the class Crustacea, the horseshoe crab Limulus polyphemus (Goldberg, et al., 1975) is included, and in the class Insecta, the members which were found to have the "Xenopus pattern" of organization were the firebat Thermobia domestica (French and Manning, 1980), the silkmoth Antheraea pernyi (Efstratiadis, et al., 1976), and the housefly Musca domestica (Crain, et al., 1976a). These species have the largest genome sizes of the insect phylum.

In the deuterostomial group, two phyla were found to have members whose genomes contain interspersed middle repetitive

sequences. They were the phyla Echinodermata and Chordata. Of the echinoderms, these species were the sea urchin Strongylocentrotus purpuratus (Graham, et al., 1974) and the common pacific starfish Pisaster ochraceous (Smith and Boal, 1978). Of the chordates, besides the amphibian Xenopus laevis in which it was first discovered (Davidson, et al., 1973b) and after which the pattern is named, the classes Reptilia and Mammalia have been extensively investigated. Of the species found to have "Xenopus-like" genome organization were the reptiles Python reticularis, Caiman crocodilus and Terrapene carolina (Epplen, et al., 1979) and the mammals Mus musculus (mouse) (Ginelli, et al., 1977), Rattus rattus (rat) (Bonner, et al., 1974; Pearson, et al., 1978; Wilkes, et al., 1978), Sus scrofa (pig) (Avvedimento, et al., 1976), Bos taurus (cow) (Mayfield, et al., 1980) and Homo sapiens (human) (Schmid and Deininger, 1975).

b. The "Drosophila Pattern" of Genome Organization

Manning, et al. (1975) found an exception to the "Xenopus pattern" of organization when they studied the DNA sequence organization of Drosophila melanogaster electron microscopically. Drosophila also has about 70% single-copy sequences. 18% of the DNA is highly reiterated, while 12% of the genome belongs to the middle repetitive frequency. The

latter grouping has about 40 classes of sequences each reiterated about 70 times. At least one third of these middle repeats are interspersed fairly uniformly among single-copy sequences, such that the average length of the interspersed repeat is 5.6 kb. The distribution ranges from less than 0.5 to 13 kb, with some segments possibly much longer. The average length of the unique segments between middle repeated sequences is greater than 13 kb pairs. Crain, et al. (1976b) confirmed this sequence pattern in _Drosophila_ by studying the reassociation kinetics of different sized fragments, by endonuclease S1 resistance of reassociated repeated sequences, and by the measurement of the hyperchromicity of repetitive fragments as a function of length. They agreed with the findings of Manning, et al. (1975) that there are about 2800 relatively long middle repetitive sequences interspersed with very long single-copy segments. Young and Hogness (1977) discovered a group of interspersed repeated structural genes in _Drosophila_ coding for abundant poly-A containing mRNA. They termed it copia. After finding copia in the inverted orientation, Carlson and Brutlag (1978) suggested an analogy between copia and transposable genetic elements in bacteria.

In the plant kingdom, the flax, _Linum usitatissimum_, a dicotyledon of the class _Angiospermae_ in the phylum _Tracheophyta_ was found to have this type of organization (Cullis, 1981). As discussed above, other members of this class exhibit a "_Xenopus_ pattern" of genome organization. This type of inconsistency manifested itself repeatedly.

In the animal kingdom, the nematodes *Caenorhabditis elegans* (Schachat, et al. 1978) and *Panagrellus silusiae* (Beauchamp, et al., 1979) lack short interspersed repeats, and have instead a longer repeated pattern similar to the one described above. A rather interesting curiosity also exists. *Drosophila* and three other members of the class *Insecta* in the phylum *Arthropoda* have the long repeat pattern described above, and yet, three other members of the same class exhibit the "*Xenopus* type" short interspersed repeats as discussed in the previous section. The insects which were found to have the "*Drosophila* pattern" of organization are *Chironomus tetanus* (Wells, et al., 1976) the honeybee *Apis mellifera* (Crain, et al. 1976a), and the flesh fly *Sarcophaga bullata* (Samols and Swift, 1979). All three have small genomes compared to the rest of the insect class. In the phylum *Chordata* the exceptions to the short interspersed pattern are found in two classes. The *Aves* class surprisingly consists of members with long period repeats in their genomes, in contrast to their very close evolutionary relatives, the reptiles. The birds found to exhibit the "*Drosophila* pattern" of repeats are the chicken *Gallus domesticus* (Eden and Hendrick 1978, Epplen, et al., 1978), the duck *Cairina domestica* and the pigeon *Columba livia domestica* (Epplen, et al. 1978). The most surprising member of this group classified by genome organization is a mammal. The syrian hamster of the genus *Mesocricetus* was found to exhibit the long repeat pattern of *Drosophila* (Moyzis, et al., 1977),

although unpublished statements to the contrary have been voiced.

The phylum Eumycophyta, the fungi, in the plant kingdom seem not to have interspersed repeats of any kind. The genomes of the organisms which were analyzed consist of 90-98% unique sequences with the rest being ribosomal repeats clustered in tandem. The organisms studied are the Ascomycetes Aspergillus nidulans (Timberlake, 1978) and Neurospora crassa (Krumlauf and Marzluf, 1979) and the Basidiomycete Schyzophylum commune (Dons and Wessels, 1980).

It has been suggested (Crain, et al., 1976a) that organisms with smaller amounts of DNA in their nuclei have the long pattern of repeats analogous to Drosophila. This trend has roughly held up, although results are preliminary. They seem to be reversed in birds, however, where an inverse relationship exists between genome size and the amount of single-copy DNA interspersed with repeated elements (Epplen, et al., 1979).

3. Sequence Organization of the Human Y Chromosome

The human Y chromosome is about the size of chromosome 21 or 22, each of which represents about 0.8% of the DNA in a cell (Mendelsohn, et al., 1969). The sequences which code for the development and differentiation of the testis are

localized on the short arm of the Y chromosome, since isochromosomes of long arms do not cause testis development (Jacobs and Ross, 1966). The plasma membrane histocompatibility-Y (H-Y) antigen has also been localized to the short arm of the Y chromosome (Koo, et al., 1977).

Cooke (1976) discovered that 70% of the DNA present in the Y chromosome exists as tandem repeats on the long arm of the chromosome. Two prominent male specific bands are visible when Hae III digested male DNA is electrophoresed on an ethidium bromide-agarose gel. The sizes of the bands are about 3400 and 2400 base pairs. By densitometric determination, these bands represented 0.4 and 0.2% of the DNA respectively, with the sequences in the larger band repeated 6.2×10^3 times and in the smaller one 4.4×10^3 times. Eco R1 digestion yielded the same sized but less prominent bands, while other restriction enzymes did not cause the appearance of male specific bands, indicating some sequence divergence in these repeats. The sequence divergence is supported by the existence of less intense dimers and trimers of the larger segment when Southern blotted with cRNA homologous to the 3400 base pair band. The 2400 base pair band is not homologous to these transcripts. The 3400 base pair sequences, but not the 2400 base pair sequences are enriched in the lighter fractions of a Ag-Cs_2SO_4 gradient, the regions expected to contain satellite III and IV DNA (Corneo and Ginelli, 1971). Bostock, et al. (1978) identified and localized these repeats to human satellite III. Kunkel, et al. (1976) isolated these

sequences by hybridization to vast excesses of female DNA and hydroxylapatite chromatography. Kunkel, et al. (1977) determined that these sequences had no evident role in male determination.

Each 3.4 kb molecule had at least two distinguishable types of reiterated sequences (Kunkel, et al. 1979). One type of sequence consists of at least two families which are highly reiterated and are not confined to the Y chromosome. The other type of sequence is composed of a minimum of 39 families, each moderately reiterated, and specific for the Y chromosome. The Y-specific and non-Y-specific sequences are interspersed with each other in the same 3.4 kb molecule, such that on the average three 800 base pair lengths of Y-specific sequences alternate with four 250 base pair non-Y-specific sequences. They also found that the population of 3.4 kb molecules is heterogeneous.

The pattern of repeated sequences in the Y chromosome is not consistent with either of the two patterns described above. Most of the repetitive sequences are tandem, and no repetitive sequences have yet been found interspersed among unique ones. Again, one of the aims of this project is to isolate sequences unique to the Y chromosome. From the above discussion, it is evident that these are of both the single copy and repetitive type.

CHAPTER V. PUTATIVE METHODS FOR ISOLATION OF SPECIFIC SEQUENCES WITHOUT A PROBE

A. INTRODUCTION

Several methods exist for isolating a specific sequence from the genome of a eukaryotic organism if a probe specific for that sequence, or a protein product of that sequence can be isolated (Shapiro, et al., 1974; Woo, et al., 1975; Kramer, et al., 1976; Tilghman, et al., 1977). There are no methods available, though, for isolating a sequence for which there is no probe. In this section, I outline several putative methods to deal with this issue. All of the methods I will describe will approach the problem by removing the rest of the genome, leaving behind the sequence of interest. In order to remove all of the DNA from a genome, save for a specific sequence, two criteria must be fulfilled. First, there must exist a genome which does not contain the sequence of interest. Second, the DNA which is deleted for that sequence must be modified in such a way that hybrid molecules between it and the genome of interest are removable in some manner. This can be accomplished in a continuous manner or by a stepwise batch process.

1. A Continuous Method for Gene Isolation

Shih and Martin (1973) developed a general method whereby a specific sequence can be removed from a mixture of sequences by hybridization to a probe on a solid support. They linked *in vitro* transcribed SV40 mRNA to cellulose by the carbodiimide method of Gilham (1968). They packed the RNA-cellulose into a water-jacketed column which was maintained at an optimum temperature for DNA/RNA hybridization in their solvent system. A mixture of 10^4 to 1 *E. coli* DNA to ^{32}P SV40 DNA was circulated through this column, through another high temperature denaturing column and back to the RNA-cellulose column again. SV40 sequences denatured in the high temperature column, and hybridized to the mRNA sequences on the cellulose column. The self-hybrids were pumped through the denaturing column again, only to return to hybridize to the sequences on the cellulose. Shih and Martin (1973) were able to remove between 40 to 45% of the ^{32}P labeled SV40 sequences from the solution, with negligible contamination by the *E. coli* sequences.

This procedure demonstrates a prototype of a continuous extraction method. In order to isolate a specific sequence for which no probe exists, the DNA which is missing the sequence to be isolated is the species which is to be

crosslinked to the column. The DNA containing the sequence to be enriched should be radioactively labeled and circulated through the column until the DNA on the column is saturated. At this point a fresh column must be substituted into the loop and circulation continued.

a. Advantages

The advantages of a continuous extraction method are obvious. The system is self-consistent, with no other steps necessary. All of the DNA-cellulose can be made and several columns can be packed at one time and stored. The fragments in solution need not hybridize to the column at any particular calculated efficiency since unhybridized fragments have further chances to do so in later passes. The DNA fragments in solution need not remain intact to be isolated, and can therefore be labeled to a very high specific activity in vitro. This fact also permits fragmentation to be achieved by sonication rather than by costly treatment with restriction endonucleases. After considerable enrichment, if so desired, the circulating DNA can be relabeled specifically, without labeling any of the extracting DNA and hindering the enrichment of counts specific for the desired sequence. In addition, no volume changes take place.

b. Disadvantages

There are also several rather serious disadvantages to the system. Renaturation rates of eukaryotic single-copy sequences are very slow due to their complexity. In order to carry out the kind of experiments described above, if a column is bound with 1 mg/ml of mammalian DNA, the calculated half-time of reaction for single-copy sequences in 1 M NaCl will be 30 hours under ideal conditions. Linking 1 mg/ml DNA to a column would be difficult, though, since the cellulose would have to be too fine for a reasonable flow rate to be achieved. Also, according to Flavell, et al. (1974), actual rate constants with DNA bound to solid supports are about one tenth of those calculated. This phenomenon is due to several steric factors. There are no DNA renaturation rate acceleration methods available to date that are compatible with a recirculating column. The calculated efficiency of extraction with a column is also an overestimate due to some loss of DNA covalently bound to the column material.

2. Batch Methods for Gene Isolation

Once the concept of modifying the extracting DNA and removing heteroduplexes from the presence of unmodified homoduplexes is established, many techniques can be conceived to carry out the task. The methods can be classified in several ways. It is easiest to view the techniques as divided between ones requiring the fragments carrying the sequence of interest to renature and to remain intact or be lost, and those methods which do not require either of these two criteria.

a. Batch Methods Requiring Fragment Renaturation and Integrity

1) Renaturation and Purification Efficiency

Since the renaturation of the fragments carrying the sequence of interest is required, the stoichiometric ratio of the two DNA species must be equal to allow heteroduplex and homoduplex formation at approximately the same rates. Even though the quantity of DNA from the genome with the sequence

of interest is reduced in each step, the concentration of homoduplex fragments carrying the sequence of interest remains the same. Thus, they renature at the same rate as extracting DNA which is replaced to its original concentration after each step.

The mathematics of purification with a batch process with a 1:1 initial ratio of the two species is now described. When two competing reactions are occurring at the same approximate rate in solution, the reaction can be written as

$$A + A \xrightarrow{k_a} A^2 \qquad (20)$$

$$A + B_o \xrightarrow{k_b} AB \qquad (21)$$

where A is the DNA with the sequence of interest, B_o is the modified extracting DNA and $k_a = k_b$. Statistically, one half of A goes to A^2 and one half to AB. If A^2 is further purified and the concentration of B_o is artificially restored after each step, the enrichment of A in step n can be expressed as

$$[A]_n = \frac{[A]_{n-1}}{[A]_{n-1} + [B_o]} \times [A]_{n-1} \qquad (22)$$

The enrichment of species A with a 1:1 mixture of the two species is outlined in table 4.

In actuality, when the DNA is of mammalian origin, the actual enrichment per step is different from that calculated. The repetitive sequences, which represent about 30% of the DNA, renature with other than their homologs and form mismatched duplexes with unaligned ends. These are removed when some of the methods are used. Thus, the first enrichment is greater than twofold, and subsequent steps are more efficient than calculated.

2) Advantages

Several methods can be used which employ easy, one step separation procedures. An unlabeled, low complexity double-stranded internal standard can be introduced in the beginning of the experiment and followed optically while the counts decrease. The standard would not be removed by the extraction procedures and, therefore, any decrease in its concentration could be used to monitor experimental losses due to DNA damage

TABLE 4

HYPOTHETICAL ENRICHMENT OF A DESIRED SEQUENCE USING
A BATCH PROCESS FOR PURIFICATION

	B_o[a]		A[b]
	1		1
step 1		↓	
	1		1/2
step 2		↓	
	1		1/6
step 3		↓	
	1		1/42
step 4		↓	
	1		1/1806
step 5		↓	
	1		1/3.2x106

[a] the DNA lacking the desired sequence is restored to its original concentration in each step.

[b] as the DNA containing the desired sequence decreases in concentration, the sequence of interest becomes enriched.

or laboratory manipulation. The standard will be double-stranded most of the time and would not, therefore, interfere with renaturation of the labeled species if the PERT method were used for renaturation. Rates in solution would not be affected. The product at any step can be relabeled as the counts decrease below useful levels. The internal standard can be a T even phage which cannot be catalytically iodinated in vitro due to the glucosylation of the cytosines.

3) Disadvantages

The initial one to one ratio of the two species necessitates at least five extraction steps to enrich a sequence 10^6 fold. The time involved in the hybridization and extraction is augmented by several dialysis and concentration steps in each batch extraction step. If hybridization is carried out to 7 half times in each step, the 87.5% yield in each step leads to a 50% yield after five steps if a hundred percent efficiency is achieved in recovering the product. The actual extent of renaturation past the first half time is less than predicted due to the factors discussed in chapter 1 and exemplified in chapters 2 and 3. This further contributes to lowered yields. Such is not the case, however, with restriction fragment renaturation.

A second problem is the considerable heat damage to DNA

which takes place when incubated at 70°C for long periods of time. Lindahl and Nyberg (1972) have measured the rate of depurination of DNA at various temperatures and pH values. At pH 7 and 70°C, the average size of single-strands of DNA after 300 minutes of heating is 4×10^6 daltons, and after 3000 minutes (50 hours) the strands are 4×10^5 daltons or 1200 bases long. The problems of slow renaturation rates have been solved to a great extent as presented in chapters 2 and 3. It is still necessary, though, to incubate mammalian DNA at fairly high concentrations for 12 to 20 hours in each step. The 60 to 100 total hours of heating in the five steps necessary for purification reduces the average size of the strands to about 800 bases. Most of the restriction fragments will remain intact. However, this number is an average, and a substantial number of the fragments will break from heating. The broken fragments are either removed or a larger number are created with each step, depending on whether the procedure relies on the removal or the destruction of the heteroduplexes. PERT renaturation cannot be used for extended periods because the phenol oxidation products destroy DNA at a finite rate. Besides, at the concentrations of DNA employed, PERT rates are no faster than in one molar NaCl solutions.

A third problem which occurs is radiation damage to the DNA. Krisch and Sauri (1975) and Krisch, et al. (1976) found that the disintegration of each ^{125}I atom incorporated into a cytosine residue results in a double-stranded break. The break is a combination of the Auger effect (vacancy cascade)

and bond breakage by hydroxyl radicals and hydrogen atoms created in the solution by gamma rays (Michaels and Hunt, 1978). The breakage of the homologous strand takes place mostly within 15-20 angstroms of the ^{125}I site, but can be detected up to 70 angstroms away (Martin and Haseltine, 1981). Each ^{125}I decomposition also causes 0.2 single-stranded breaks in addition to the two comprising the double-strand break in extracted T4 DNA molecules (Krisch and Sauri, 1975). The rate of single-stranded break formation is concentration dependant as T4 DNA in the phage head has 8 times as many such single-strand breaks per decomposition as in solution. The single-strand breaks are caused by hydroxyl radicals and hydrogen atoms, whose lifetimes in solution are about one and seventy nanoseconds respectively (Michaels and Hunt, 1978). The mean free path of these entities during these times are about 45 and 400 angstroms respectively. The double-stranded T4 DNA molecule has a radius of gyration of about 10,000 A and a center to center distance in a 250 ug/ml solution of about 10,000 A. Such a solution is therefore filled with molecules. Most of the single-strand breaks occur within the molecule containing the labeled cytosine whose ^{125}I molecule decomposes because the path traveled by the radical is far less than the radius of the molecule. In a solution containing 1 mg/ml of 500 nucleotide single-stranded Hae III fragments, the radius of gyration of the molecules is 160 A on the average, while the center to center distance is 800 A. This distance is within the range traveled by some of the hydrogen atoms in

their lifetime, since the values given are averages, but the likelyhood of a radical-DNA interaction is less than in the case of T4 DNA. Repine, et al. (1981) have concluded that most of the single-strand breaks from radiation damage are caused by hydroxyl radicals. They were able to prevent 80% of the single-strand breaks by addition of the hydroxyl radical quencher dimethyl sulfoxide to a concentration of 0.28 M. Commerford (1980) suggests storing ^{125}I DNA at 0-4°C in 20% ethanol (by volume) to minimize radiation damage.

At the level of labeling used in the gene isolation experiments, approximately 20% of the molecules contain one labeled cytosine, and very few molecules contain more than one labeled cytosine. The decomposed molecule no longer counts for assay purposes, since it is no longer radioactive. Thus, the loss in yield is only caused by single-stranded breaks on other molecules caused by radicals, which, from the above discussion, is small, and from the removal of labeled molecules which hybridize to molecules which sustained a double-stranded break when the ^{125}I atom they contained decomposed. The rate of damage is therefore fairly slow. If 20% of the molecules have iodocytosines, then in 60 days 10% of them sustain double-stranded breaks. In the period of 2 weeks that it takes to complete a gene isolation experiment, a very small fraction of the molecules is damaged.

In order to enable the formation of perfect homoduplexes restriction enzymes must be used to fragment the DNA. A fourth problem could be that this procedure could be quite

expensive due to the large number of units necessary to cleave the near milligram quantities of mammalian DNA at the start of experiments. However, the costs are not so great as to mitigate against the use of restriction endonucleases.

A final difficulty with the requirement of intact restriction fragments can be genetic polymorphism at specific loci in mammalian genomes among different individuals (Jeffreys, 1979). Further evidence of genomic change has been found in restriction patterns of human gastrointestinal carcinoma genomes (Humphries, 1981). If genetic rearrangement is a general phenomenon with mammalian genomes, then the same restriction enzyme would yield different size fragments containing the information of interest flanked by different sequences depending on its location. Rearrangements could lead to loss of that information in the procedures described in this section.

4) Description of Methods

(a) Modification and Mismatch Cleavage

In order to apply this procedure the DNA with the sequence to be isolated is cut with a restriction enzyme which recognizes four bases. The restriction fragments will thus be small enough (512 base pair weight average) to insure the existence of some fragments which contain only sequences of interest. The DNA can also be sonicated to achieve a limited enrichment with a system less complex than mammalian (Sprouse and Wetmur, unpublished results). The DNA is then homogeneously labeled _in vitro_ with ^{125}I by the procedure of Chan, et al. (1976) to minimize chain scission. They found the probability of bond breakage, p, to be proportional to the percent cytosine which became iodocytosine ($p = 6.5 \times 10^{-5} \times$ %C as IC). p is also equal to 1/<x> where <x> is the average length of the strands remaining after the reaction. Thus, <x> $= 1.5 \times 10^{4}$ bases per %C as IC. Under conditions generally employed for labeling, about 0.5% of the cytosines are iodinated, such that DNA segments about 30,000 bases long are cleaved once. This implies that restriction fragments of the size discussed above remain intact.

The DNA deleted for the sequence of interest is extensively sonicated and subsequently deaminated by the

method of Hutton and Wetmur (1973b) to about 10-15%. This results in a drop of about 7°C in the T_r of the heteroduplex and a negligible hybridization rate difference. After at least three, and preferably seven calculated half-times of renaturation for the single-copy sequences in solution, the mixture of the two species is incubated with endonuclease S1. The entities cleaved will be the single stranded ends of the restriction fragment/sonicated fragment heteroduplexes and sonicated homoduplexes, as well as sites of 3 or more adjacent mismatches in the heteroduplexes (Dodgson and Wells, 1977). Alternatively, prior to endonuclease S1 digestion, the temperature can be elevated to partially melt the mismatched heteroduplexes but leave intact the unmodified homoduplexes. 2.4 M Et_4NCl can be used as the solvent during the melting step to insure specific melting of the heteroduplexes. After a dialysis, the original concentration of deaminated extracting DNA is replaced, the mixture is denatured, and the procedure is repeated.

In addition to the general list of advantages, there are two more reasons to use this procedure. The separation time of the heteroduplexes from the homoduplexes is short (about 30 minutes for endonuclease S1 digestion), and the volume remains more or less constant.

In addition to the general list of disadvantages, this method has more drawbacks. Endonuclease S1 cleaves bases (nibbles) at the ends of double-stranded molecules which open and close at a finite rate (Shenk, et al., 1975). The method

also amplifies heat and radiation damage by cleaving the single-stranded regions of intact/cleaved restriction fragment heteroduplexes and creates more cleaved segments. These continue to amplify the problem of loss of DNA of interest in subsequent steps.

(b) Benzoylated-Naphthoylated DEAE (BND) Cellulose Chromatographic Separation of Tailed Heteroduplexes from Restriction Fragments

In this procedure, the DNA with the sequence to be isolated is again restricted with an enzyme recognizing four bases, and labeled _in vitro_. In addition to iodination 5' end labeling with [γ-^{32}P]ATP and T4 polynucleotide kinase can also be used for this procedure (Chaconas and Van deSande, 1980). The extracting DNA is sonicated. After denaturing and renaturing a one to one mixture of the two DNA species, the product is loaded onto a hydroxylapatite column at 70°C and washed with 0.075 M potassium phosphate buffer to remove very small fragments and purely single-stranded molecules (Britten, et al., 1974). When no further counts come off, the column is washed with 0.5 M phosphate buffer to elute the molecules with double stranded regions. These molecules are then loaded onto a BND column in 0.6 M NaCl at room temperature (Sedat, et al., 1967) and fractions are collected. Only the blunt-ended

restriction fragment homoduplexes will come through. After dialysis into H_2O and either ethanol precipitation or lyophilization, the sonicated extracting DNA is replaced and the procedure is repeated.

This method requires the least modification of the DNA and offers the advantage of an easy separation in each step in a short time. Along with the general disadvantages, this method has some other drawbacks. The separation at each step is less than 100% efficient, and therefore more than five steps are necessary. Large volumes of DNA in concentrated NaCl solution, created as the DNA is eluted off the column, are difficult to manage or reduce.

(c) Metrizamide Equilibrium Centrifugation of DNA Bound to Single-Strand Binding Proteins

The third procedure in this category is similar to the previous one in its method of differentiating heteroduplexes from restricted homoduplexes. Both procedures rely on the fact that heteroduplexes have single-stranded regions at the ends of the molecules. If Single-Stranded Binding Protein (SSB) (Sigal, et al., 1972) or T4 Gene 32 Protein (Alberts and Frey, 1970) is added to the DNA in stoichiometric quantities, the molecules with single-stranded ends will have different buoyant densities in metrizamide equilibrium gradients

(Birnie, et al., 1973). Native DNA has a buoyant density of about 1.12 in metrizamide, while proteins have a bimodal distribution of about 1.28 and 1.48 gm/ml. The reason for the bimodal banding pattern is unknown. Native DNA will float in most metrizamide gradients while proteins will band near the middle of the tube. Metrizamide is not highly hydrated in solution, so that the water activity of metrizamide solutions is close to one. Therefore, the macromolecules banded in metrizamide are nearly fully hydrated and the density required for isopycnic centrifugation is much lower than required for CsCl banding. The DNA concentrations in fractionated metrizamide gradients can be quantitated with methyl green (Peters and Dahmus, 1979).

This procedure permits volumes to remain constant or to diminish with each separation step. In addition to the general disadvantages cited before, though, the method does require miligram quantities of SSB in each step, the isolation of which is tedious and expensive even with overproduction with a cloned SSB gene on a plasmid. The separation step takes 24 hours and requires ready availability of a high speed rotor and ultracentrifuge.

b. Batch Methods Which Bypass the Need for Intact or Fully Renatured Fragments

1) Cesium Chloride Density Centrifugation

In this procedure, the DNA of interest is fragmented by sonication, and radioactively labeled by either iodination, nick translation or with ^{32}P on the 5' ends. The extracting DNA is sonicated and density labeled by in vitro iodination with cold KI by the procedure of Orosz and Wetmur (1974). If 70% of the cytosines become iodocytosine a significant separation in a CsCl density gradient can be achieved from unlabeled molecules. Because fragments of DNA containing the sequence of interest are carried along to further purification steps even if they do not renature, a large excess of extracting DNA can be used in each step. The buoyant density of networks of renatured molecules in which the majority of strands are density labeled is close to that of density labeled molecules, and will therefore band separately from unlabeled homoduplexes. The tubes can either be dripped, or the light band can be removed through the side wall of the tube with a needle and a syringe to avoid contamination by the heavy or hybrid band. Ethidium bromide is present to help visually localize the bands (Radloff et al., 1967),

especially if an unlabeled heterologous carrier is included. After the lighter band is removed, more density labeled DNA is added and the procedure is repeated.

This method seems to be superior to most of the ones previously described. Besides not being affected by fragmentation and incomplete renaturation, the procedure requires fewer steps than methods in the previous section because larger initial ratios of extracting DNA to the DNA of interest are used. With this procedure also, as with previous methods, the DNA can be relabeled after later purification steps without labeling any of the extracting DNA. The appropriate ethidium bromide concentration needs to be found where single and double strands have identical buoyant densities. The volume after each step remains the same or shrinks with this procedure.

The only disadvantages with this system are the 24 hour centrifugation steps in each enrichment procedure as well as relying on a ready access to a high speed rotor and an ultracentrifuge, and possible mixing of the bands when gradients are dripped. If the fragments are too small, the bands obtained will be broad and separation will be inefficient. In such instances more steps will be required. It may also be necessary to pass the DNA over a Sephadex G 100 column and continue the experiment with the DNA that was excluded.

2) Mercury Substitution and Thiol Sepharose Chromatography

The DNA with the sequence of interest is sonicated and labeled as in the previous method. The extracting DNA is also sonicated. It is then mercurated with sodium acetate buffered mercuric acetate by the procedure of Dale, et al. (1975). The mercury gets incorporated into the 5 position of cytosines almost exclusively. Native and single-stranded molecules label at the same rate. The Tm of mercurated DNAs drops at most about 0.2-0.4°C per every percent of the cytosines labeled (Dale and Ward 1975). Since the incorporation of as few as one mercury atom in 200 bases permits the retention on a sulfhydryl column under conditions necessary for this procedure, the T_r is not affected very much. Walbot (1978) found that the stability of mercurated DNA decreases with increased temperature, such that up to 10% of the mercury is lost in one hour of heating at 60°C. She found that addition of formamide, minimizing exposure to sulfhydryl reagents and avoiding temperatures above 35°C can minimize demercuration. Brown and Balmain (1979) found that a markedly decreased renaturation rate can be abolished in the presence of 1 mM 2-mercaptoethanol. They found almost no demercuration when mercury-DNA was incubated up to 300 hours at 43°C in formamide. but extensive demercuration after 48 hours when mercury-DNA was incubated at 60°C in phosphate buffer.

The two species are renatured taking into account the

above data. The renatured DNA is poured over a sulfhydryl-sepharose column and fractions are collected. More mercurated DNA is added to the effluent and the procedure is repeated. Unfortunately, it is not clear how current rate acceleration methods would behave under the above conditions. Without acceleration of renaturation rates, the mercury will be lost even at lower temperatures.

3) Separation of Poly dA-Tailed Heteroduplexes on a Poly dT Column

The sonicated fragments of the DNA which contains the desired sequence are labeled homogeneously with ^{125}I. The extracting DNA is sonicated and polyadenylated with deoxy ATP and terminal transferase (Roychoudhury and Wu, 1980). The mixture is renatured and poured over a poly dT-cellulose column. The effluent is concentrated, more poly A-tailed extracting DNA is added and the procedure is repeated.

This method has the same advantages as all of the techniques which do not require fragment renaturation or integrity to work. In addition the separation method is quick and a large ratio of poly dA-tailed DNA to the DNA being extracted can be used cutting down on the number of separation steps.

The disadvantages are few with this technique. The

column can lose some bound DNA resulting in less enrichment per step than expected. Large volumes, which have to be reduced can accumulate using columns.

In the following section, I will describe the use of several of these batch techniques, from both categories, in an attempt to isolate Y chromosome-specific sequences from male rat DNA. The modification and mismatch cleavage and the BND chromatography techniques in the category requiring fragment integrity and renaturation were attempted. In the other category, the CsCl banding technique was tried.

B. EXPERIMENTAL

1. Materials

All chemicals were reagent grade. Endonuclease S1 was obtained from Sigma and assayed as in Hutton and Wetmur (1973a). Lyphozyme Hae III was obtained from BRL and solubilized as directed. Restriction endonuclease DPN II was prepared by a modification of the method of Lacks and Greenberg (1977). Dextran Sulfate M_r = 500,000 was obtained from Pharmacia. Benzoylated-Naphthoylated DEAE cellulose and *E. coli* DNA type VIII were purchased from Sigma. Hydroxylapatite Biogel HTP DNA grade was purchased from Bio-Rad. DNA was sonicated as in chapter 2. $Na^{125}I$ carrier free was purchased from Amersham and $|\gamma\text{-}^{32}P|$ATP was purchased from New England Nuclear. Polynucleotide kinase was purchased from New England Biolabs and Calf Alkaline Phosphatase from Boehringer Manheim.

2. Preparation of Rat DNA

DNA was prepared from the livers of male and female Fischer rats by an extensive modification of the procedure of Marmur (1961). Rats were sacrificed and the livers were removed into ice cold rinse buffer (2 M NaCl, 0.01 M EDTA pH 8.0). The livers were minced with scissors in a Buchner funnel and washed with rinse buffer. To 5 grams of liver tissue, 4 ml of SDS buffer (0.6% sodium dodecylsulfate, 0.01 M EDTA pH 8.0 0.01 M Tris HCl pH 7.85) were added. The livers were then crushed in a B&B Motor and Control Corporation (N.Y.) tissue homogenizer at 4°C. The volume was subsequently brought to 12 ml per 5 gm of tissue with SDS buffer. 2.5 ml 8 M CsCl were added per 12 ml volume and the mixture was centrifuged in an SW27 rotor at 17,000 rpm for 2 hours. The clear layer which formed between pelleted and floating derbris was removed and extracted once with an equivalent volume of chloroform. It was then centrifuged at 12,000 rpm for 20 minutes. The top, clear layer was banded in CsCl at a density of 1.55 gm/ml with 300 ug/ml ethidium bromide at 40,000 rpm for 24 hours at 17°C. The band was removed and extracted 3 times with 1-butanol to remove the ethidium bromide. The DNA was then dialyzed into the desired buffer.

3. Radiolabeling

DNA was radioiodinated as in chapter 2 by the method of Chan, et al. (1976). Molecules were end labeled with $[\gamma-^{32}P]$ATP by the procedure of Chaconas and Van deSande (1980).

4. Density Labeling with Iodine

Female DNA was iodinated according to the procedure of Orosz and Wetmur (1974). A 1.1 ratio of KI to cytosine was used at 80°C for 10 minutes. A 70% rate of cytosine iodination was consistently achieved as measured by absorbance at 310 nm.

5. Deamination

DNA was deaminated according to the procedure of Hutton and Wetmur (1973). An incubation time of 20 seconds resulted in approximately 15% deamination as measured by T_m depression.

C. RESULTS

1. Deamination and S1 Cleavage

Experiments were carried out with deaminated sonicated female rat DNA and ^{125}I labeled male rat DNA that was renatured and restricted with Dpn II. Typically, a mixture of female and male DNA at a ratio of about 3 or 5 to one with a total concentration of about 8 ug/ml was renatured in a phenol emulsion in 2 M NaSCN and 8% phenol at room temperature for 48 to 72 hours. The phenol emulsion was subsequently dialyzed into endonuclease S1 digestion buffer (0.1 M NaOAc pH 4.17, 0.2 M NaCl. 0.001 M ZnCl2) at 4°C and digested with about a 3 to 5-fold excess of encdonuclease S1. The product was dialyzed into 2 M NaSCN, an aliquot was removed for assay, and the procedure was repeated. When concentrations decreased substantially additional deaminated female DNA was added to bring the concentration to about 8-10 ug/ml. The procedure was repeated 2 or 3 times. After each pass, an aliqout was removed, dialyzed into 0.01 M NaCl and saved for assay. The assay samples were each hybridized with excess quantities of sonicated male and female DNA in solutions containing 15% dextran sulfate 10% dimethylsulfoxide and 2 M NaCl at 65°C for five days, and subsequently digested with endonuclease S1. They were then spotted on tlc strips and developed with 1 M

HCl.

No preferential protection was afforded the labeled species by either male or female DNA. The protection afforded did not follow a pattern. Use of modified DNA and endonuclease S1 was abandoned in favor of alternative procedures. The extent of renaturation at any point during the experimental assay was not quantitated. Neither the size of the fragments nor the amount of destruction was quantitated. These experiments were carried out prior to the studies on optimum conditions for DNA renaturation described in chapters 2 and 3.

2. BND Chromatography

Hae III digested ^{32}P end labeled male rat DNA fragments were denatured and hybridized to sonicated female rat DNA, mostly at one to one ratios. Both the PERT with 2 M NaSCN and 8% phenol and the 40% dextran sulfate and 1 M NaCl solution renaturation rate acceleration methods were used. The extent of renaturation was assayed after each hybridization step by hydroxylapatite chromatography with 0.075 M potassium phosphate buffer at 70°C. In some cases, the products of the renaturation steps were preparatively chromatographed on a hydroxylapatite column and fragments which contained double-stranded regions were used to continue the experiment.

These fragments represented the DNA which eluted from the column with 0.5 M sodium phosphate at 70°C after the latter had been washed with 0.075 M potassium phosphate buffer at 70°C.

The renatured DNA was chromatographed on a BND column with 0.6 M NaCl at room temperature. The effluent was added to sonicated female rat DNA of the same quantity as was present in the beginning of the experiment, concentrated, denatured and renatured. The procedure was carried out several times before it was terminated by one of its shortcomings. Aliquots from various passes were hybridized with excess quantities of male and female DNA fragments and assayed by hydroxylapatite chromatography. All radioactive determinations were made by Cerenkov counting. Different results were obtained if the DNA preparation were digested with T1 and pancreatic ribonuclease after restriction and labeling. Other experiments, not reported, were also carried out where the preparation was NaOH treated after labeling. Both of these procedures allow elimination of RNA introduced with the restriction endonuclease. One experiment is reported in table 5. Significant fractions of fragments which were at least partially renatured were eluted from hydroxylapatite columns at higher phosphate concentrations. When the eluents from the BND columns were assayed, about equal fractions of the fragments renatured with excess male and female DNA after the first step, but none appeared to do so after the second

TABLE 5

ATTEMPTED ISOLATION OF Y-SPECIFIC SEQUENCES USING BND CHROMATOGRAPHY

step no.[a]	renat. time (hrs)[b]	% eluted off HTP[c] in 0.075 M PO_4^{+3}	% eluted off HTP in 0.5 M PO_4^{+3}	% eluted off BND in 0.6 M NaCl	male assay[d] (% off HTP)	female assay[d] (% off HTP)
1	8	36	27[e]	28[e]	40	35
2	12	19	39[e]	26[e]	100	100

a Prior to the first step, the DNA was end labeled with ^{32}P, digested with pancreatic and T1 ribonucleases and ethanol precipitated. Counts were only associated with the DNA on a subsequent autoradiogram.

b The male rat DNA was renatured with a 1.5 to 1 ratio of female rat DNA in 1M NaCl with 40% dextran sulfate at 70°C.

c Hydroxylapatite.

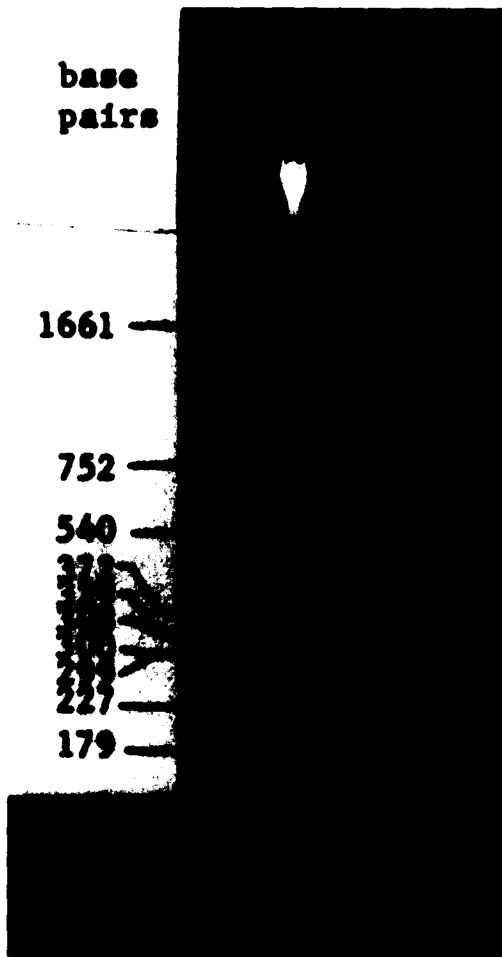
d Products were assayed by hybridizing with excess DNA for 21 hours in 1M NaCl and 40% dextran sulfate at 70°C and chromatographed on an HTP column in 0.075 M PO_4^{+3}.

e Preparative column.

step. The DNA from the first pass was incubated a total of 29 hours, and from the second step, a total of 41 hours, including the assay time. In several experiments, relabeling of purification products was attempted but not achieved. The entities either did not label, or the substances into which the label was incorporated did not behave like nucleic acid.

The starting material was reinvestigated. Neutral and denaturing 1% agarose gels of Hae III restricted male rat DNA were run before and after NaOH treatment. One such neutral gel is shown in figure 9a. The gel shows that the male DNA was restricted completely, and has a weight average of about 700 to 800 base pairs as determined by Hae III digested bacteriophage lambda size markers. Sonicated female rat DNA had a weight average of about twice that of male DNA (not shown). The restricted male rat DNA had several prominent bands which were visible over the background distribution of restriction fragments. Intact rat DNA appears to be of high molecular weight. End labeled DNA fragments appear to remain intact as observed by the bands which remain prominent in the autoradiogram shown (figure 9b.). Most of the counts incorporated are either in very small pieces which migrate to the bottom of the gel, or in species which did not enter the gel. The number average of the end labeled molecules in the autoradiogram appears to be about 300 to 400 base pairs. NaOH treatment of the labeled population followed by dialysis produces autoradiograms in which the label is only present in the DNA restriction fragment distribution (not shown).

Fig. 9. Size distribution of restriction endonuclease Hae III digested male rat DNA. (A) Hae III digested male rat DNA is found in the middle lanes and intact male rat DNA is found in the right hand lane of this 1% agarose gel. Hae III digested SV40 DNA was used for size marker in the left lane.
(B) Autoradiogram of ^{32}P labeled Hae III digested male rat DNA on a 1% agarose gel with Hae III digested SV40 DNA as size marker.


base
pairs
1661
752
540
227
179

base pairs
1661
752
540
372
329
308
300
299
227
179

3. CsCl Density Centrifugation

A greater effort was now applied toward development of a method which would not require intact wholely native DNA molecules. Conditions were investigated which would afford the optimal separation of two DNA species of different densities in a CsCl gradient. It was found that at a ratio of 5 to 1 favoring the denser species, the heteroduplex bands very slightly above the dense band, about 5 mm below the light band. Experiments were performed also to determine the approximate ethidium bromide concentration affording the widest separation between two bands of different densities. Of the concentrations investigated, 15 ug/ml ethidium bromide yielded the widest separation.

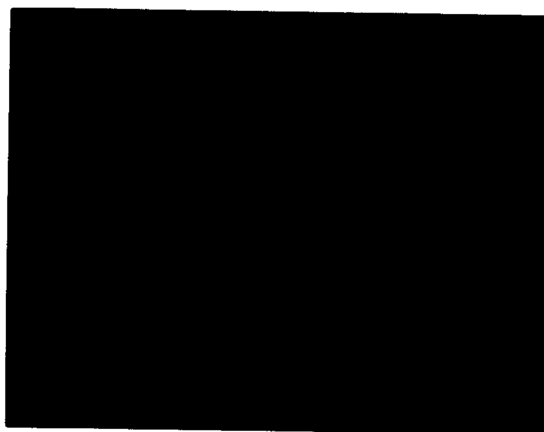
Experiments were carried out with *E. coli* DNA to determine the viability of this procedure. Sonicated *E. coli* DNA was density labeled *in vitro* to place iodine on 70% of the cytosines. A five to one ratio of heavy to light DNA was renatured for seven half-times to permit formation of networks of molecules. A trace amount of ^{125}I-labeled light *E. coli* DNA was included in the renaturation. Light and heavy homoduplexes were also formed with tracer radioactive molecules. Light and heavy homoduplexes were mixed with the heteroduplex and banded in CsCl with 100 ug/ml ethidium bromide. Two tubes of light and heavy homoduplexes were

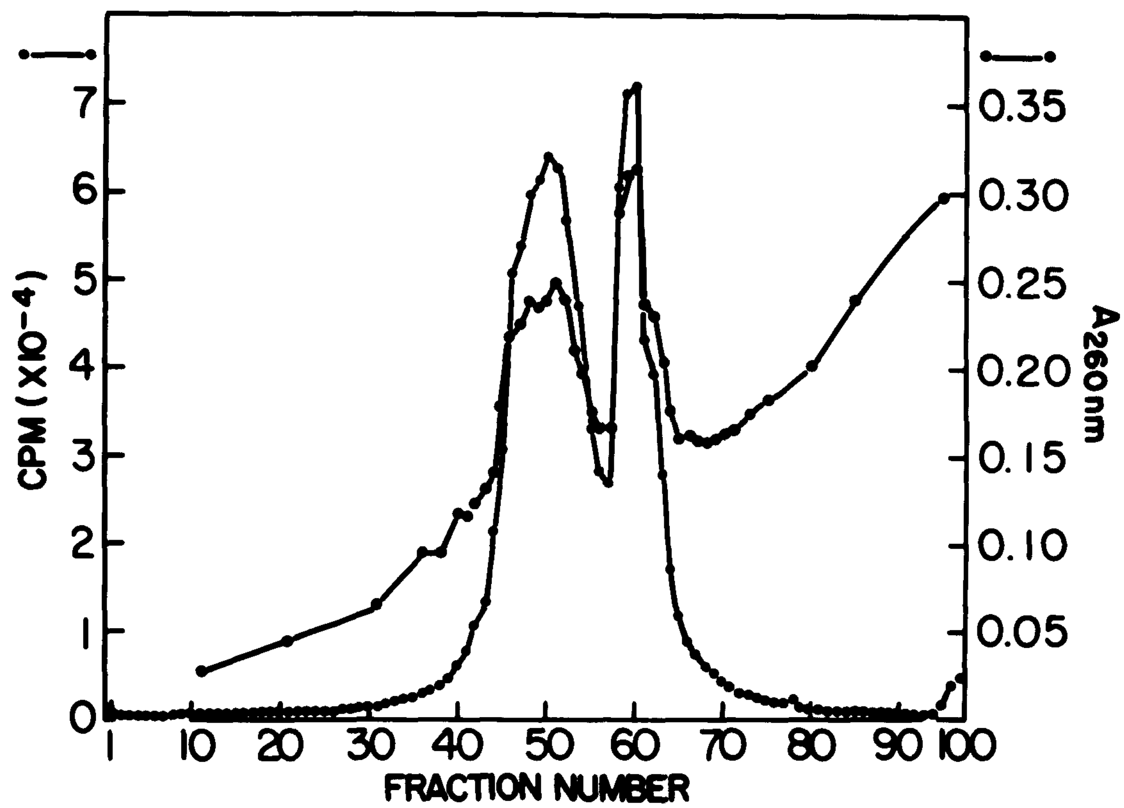
included as markers, with care taken to maintain the same volumes and densities.

Figure 10a is a photograph of a result. A substantial separation is achieved between the 5/1 heteroduplex band and the light homoduplex. The tube was dripped, the fractions were counted, and the absorbance at 260 nm was recorded. Figure 10b portrays the result. Some overlap exists resulting in about a 10% contamination of the light band by the hybrid band.

Experiments were also carried out with male and female rat DNA in order to attempt isolation of Y chromosome specific sequences. Male rat DNA which was sonicated and ^{125}I labeled to trace levels was hybridized to five-fold excess quantities of density labeled sonicated female rat DNA using either the dextran sulfate solution hybridization method of rate acceleration or the PERT system. Following dextran sulfate hybridization, the dextran sulfate was precipitated from solution with CsCl. After PERT renaturation, the solution was diluted with three volumes of 2 M NaCl and extracted at least three times with chloroform. Cesium chloride and ethidium bromide were added to a density of about 1.68 gm/ml and a concentration of 15 ug/ml respectively, and centrifugation was carried out in a swinging bucket rotor at 40,000 rpm for at least 24 hours. The tubes were fractionated by dripping from the bottom, or the upper band was extracted from the side with a needle and syringe. Appropriate dialysis and concentration

Fig. 10. Cesium chloride density gradient centrifugation of different densities of *E. coli* DNA. (A) The tubes shown represent 1) unmodified *E. coli* DNA homoduplexes 2) density labeled *E. coli* DNA homoduplexes and 3) a mixture of 1), 2) and a heteroduplex of a five to one ratio of density labeled to unmodified *E. coli* DNA. Trace amounts of light ^{125}I labeled *E. coli* DNA were included in all hybridizations. (B) Fractionation of the tube containing the heteroduplex band.




CPM (X10⁻⁴)
0
1
2
3
4
5
6
7
0.05
0.10
0.15
0.20
0.25
0.30
0.35
A260nm
1
10
20
30
40
50
60
70
80
90
100
FRACTION NUMBER

steps were carried out, dense female rat DNA of the original quantity was replaced, and the experiment was repeated. Aliquots after each enrichment step were saved to assay by hybridization to excess male and female DNA fragments and hydroxylapatite chromatography.

The results achieved with mammalian DNA were not nearly as successful as those obtained with *E. coli*. The bands under the best conditions were wide and diffuse with considerable overlap. One such example is provided in figure 11.

In a typical experiment, the PERT was used to hybridize the species. Following 42 hours of shaking at room temperature, 23% of the counts comigrated with the HCl solvent front on a tlc strip. The tube containing the equilibrium centrifugation had one wide, diffuse band, which when fractionated showed a single peak with a high background as seen in figure 12a. The identical experiment renatured with dextran sulfate at 70°C for 11.5 hours did not yield a peak at all, either visually or by counts when it was fractionated (figure 12b). Typically, most experiments renatured with dextran sulfate at 70°C suffered considerable degradation and did not yield bands when subjected to equilibrium centrifugation in a CsCl gradient. In several instances a visible band formed in the gradient following renaturation in dextran sulfate at 70°C for many hours. However, when fractionated, no increase in counts was observed with the visible band. The profiles of one such experiment are shown

Fig. 11. Cesium chloride density gradient centrifugation of different densities of rat DNA with an unmodofied _E. coli_ DNA internal standard. This result represents the third step of a Y chromosome isolation experiment in which originally a five to one ratio of density labeled female rat DNA to light ^{125}I labeled male rat DNA were renatured in the presence of an unmodified native _E. coli_ DNA tracer. The original concentration of female rat DNA was restored after each step.

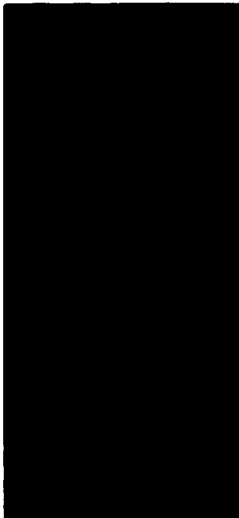
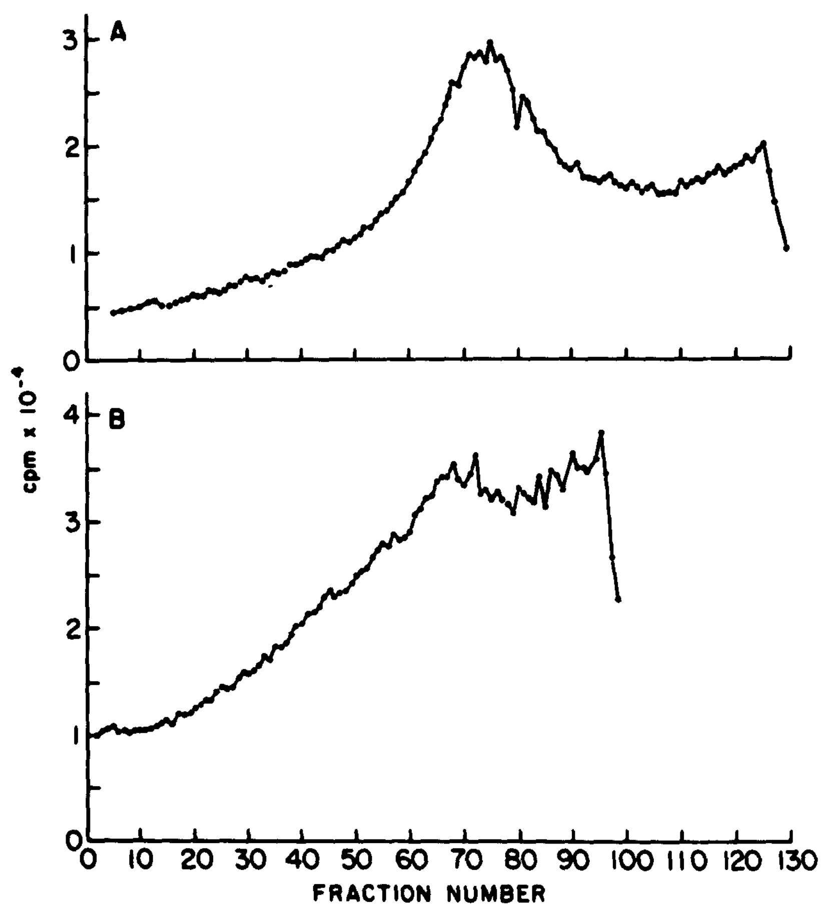
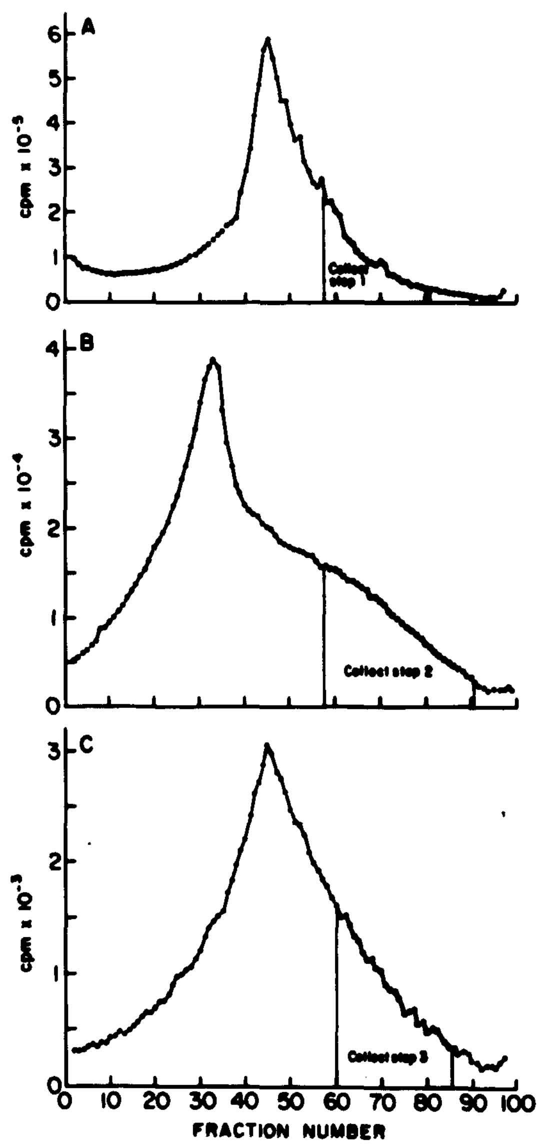


Fig. 12. Fractionation of CsCl gradients containing a five to one ratio of density labeled female rat DNA to ^{125}I labeled light male rat DNA which underwent centrifugation after (A) PERT renaturation for 42 hours at room temperature and (B) renaturation with 40% dextran sulfate and 1 M NaCl for 11.5 hours at 70°C.


A
B
cpm x 10^-4
0
1
2
3
4
0 10 20 30 40 50 60 70 80 90 100 110 120 130
FRACTION NUMBER

in figure 13. The gradient was formed with a density of 1.6 gm/ml and 100 ug/ml ethidium bromide. The tubes were fractionated from the bottom after each centrifugation. No separation of peaks was observed. Fractions were renatured at 70°C in 1 M NaCl and 40% dextran sulfate. The products were assayed by endonuclease S1 digestion of hybridized products of sample and excess male and female rat DNA fragments. Little protection was offered by either male or female DNA.

Fig. 13. Fractionation of the products of CsCl density gradient centrifugation of a typical Y chromosome isolation experiment. The initial ratio of the density labeled female rat DNA to the ^{125}I labeled light male rat DNA was five to one. The original female rat DNA concentration was restored after each purification step. The fractions indicated were collected for further enrichment. Renaturation was carried out in 1 M NaCl with 40% dextran sulfate at 70°C. The total heating time after the third step was 58 hours.


A
cpm x 10⁻³
Collect step 1
B
cpm x 10⁻⁴
Collect step 2
C
cpm x 10⁻³
Collect step 3
FRACTION NUMBER

D. DISCUSSION

1. Deamination and S1 Cleavage

In retrospect, the procedures followed were not optimal for achieving the desired goal. The male DNA was restricted after iodination and renaturation. The appropriate time would have been prior to labeling. A low complexity internal standard with a known restriction pattern should have been included prior to restriction and iodination to verify the integrity of the fragments. Following renaturation, the emulsion should have been diluted with 2 M NaCl instead of directly dialyzed into endonuclease S1 digestion buffer. The extent of destruction as well as the extent of renaturation should have been assayed after each renaturation step prior to endonuclease S1 digestion. Destruction could have been assayed by thin layer chromatography, and renaturation by hydroxylapatite chromatography. This should have also been done with the samples used for assaying enrichment.

By using the PERT to anneal male and female DNA fragments, the renaturation of Y chromosome specific fragments was hindered. Competition for the phenol interface by the heterologous high complexity DNA in excess slowed the renaturation of the Y-specific sequences and contributed to

their destruction by endonuclease S1. There is also a continuous rate of destruction of the DNA by six days of shaking in the presence of phenol. Deamination and S1 cleavage deserves reinvestigation.

2. BND Chromatography

The DNA preparation had large quantities of low molecular weight RNA species which, due to their many free ends, competed for end labeling with the DNA fragments. The concentration of the RNA species varied among the preparations used, as on some gels they were quite visible, while in other instances, they only became apparent after labeling and autoradiography. Following incubation with 0.5 M NaOH at 37°C for 2 to 4 hours, no trace of intact RNA fragments remained on autoradiograms. NaOH treatment also caused the disappearance of the radioactive species which had previously not entered the gel. The bands which had been visible in the DNA restriction fragment distribution remained undisturbed following NaOH treatment. These bands represented families of repetitive sequences interspersed within the genome (Meunier-Rotival, et al., 1982) and/or possibly mitochondrial DNA. Both species would be removed after one enrichment step.

Trace amounts of non-nucleic acid species were also labeled. These entities became increasingly more significant

in later steps because DNA-associated counts were being lost, while these species continued to be carried along if proper precautions were not taken. On occasion, where relabeling was attempted and failed, the only observable counts at that point were probably non-DNA associated.

Competition from unlabeled fragments probably slowed the apparent renaturation rate of the labeled species with the PERT. The reaction did progress, however, since in one experiment, after 38 hours of reassociation, 35% of the counts were acid soluble, and yet 47% of the counts remained bound to hydroxylapatite at low phosphate concentrations. That means that of the intact species, 72% had double-stranded regions. The major source of destruction came from heating the species in 1 M NaCl at 70°C in the presence of 40% dextran sulfate for long periods of time at pH 6.85. In all subsequent experiments, heat related damage was the main source of destruction. Loss of material was experienced when the molecules were fragmented to sizes which were too small to efficiently renature or to form stable duplexes. Non-DNA associated counts were enriched with every BND column chromatography step.

Proper execution of this method is possible. The DNA which is used should be restricted, purified with either NaOH digestion or RNAse treatment, phenol and chloroform extracted extensively, and then labeled. All non-DNA associated counts should be removed at every step, following every incubation or column chromatography. Duplex DNAs should be isolated by

hydroxylapatite chromatography before and after each BND chromatography step. The sizes of molecules should be followed by gel electrophoresis and autoradiography after each step to determine degradation. Degradation and the presence of non-nucleic acid species should also be followed by thin layer chromatography and endonuclease S1 digestion at every step. Renaturation should be carried out in tris buffer at pH 7.85 and 0.01 M EDTA to decrease the chances of DNA fragmentation. Molecules should be relabeled only after great pains have been taken to insure that the only species present are DNA fragments which do not stick to BND cellulose.

If all of these steps are carefully followed, this procedure may still fail due to the fragmentation of the DNA from heating in the presence of such huge quantities of dextran sulfate.

3. CsCl Density Centrifugation

As discussed above, considerable destruction of DNA takes place during solution renaturation at 70°C with 40% dextran sulfate for many hours. The fragments or nucleotides become distributed throughout the tube and mask any peaks which form. The DNA also becomes too short to band or to form networks which facilitate banding.

The bands which form with mammalian DNA are broad

diffuse and overlapping. Even if separation and enrichment are achieved with this method under these conditions, the enrichment would be far less than that which is theoretically predicted due to the contamination of the light band by the overlapping hybrid band. Mammalian DNA also has sattelite DNA which broadens the bands formed and decreases resolution. These regions are, however, comprised of repetitive sequences, and will therefore be removed from the light band in the first pass. They will not add to the lack of resolution of the light band past the first step. Single and double-stranded DNA band separately in CsCl. Conditions have to be found in which these two species are co-buoyant. This will probably be achieved by finding the ethidium bromide concentration at which the buoyant densities of single and double strands are identical.

In order to make this system work as with the other methods described above, the DNA has to be cleaned up and sized after each renaturation step by either gel filtration or velocity sedimentation. If the density gradient equilibrium centrifugation is carried out with large, intact DNA strands, sharp, narrow, nonoverlapping bands will be formed and good separation will be achieved. The degradation of single-stranded DNA in both the PERT reaction and especially in solution at 70°C in the presence of high concentrations of dextran sulfate needs to be investigated before this or any other of the above procedures can be carried out successfully.

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