HYBRIDIZATION AND RENATURATION KINETICS OF NUCLEIC ACIDS

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INTRODUCTION

When two DNA or RNA molecules contain sequences of nucleotides that are complementary to one another, they are able to come together to form a double helix with all of the bases paired. If both strands are DNA, the process is usually called renaturation or reassociation; if one strand is DNA and the other is RNA, the process is called hybridization. These essentially identical processes form the experimental basis for determining relatedness of nucleic acids. Renaturation and hybridization techniques have been used in thousands of papers over the past 15 years. This review is restricted to studies involving the kinetics of the renaturation and hybridization reactions.

The pioneering work of Ross & Sturtevant (1, 2) on the kinetics of formation of helices with RNA homopolymers, Marmur & Doty (3) on the kinetics of renaturation of DNA [for a review, see (4)], and Nygaard & Hall (5) on the kinetics of DNA-RNA hybridization led to the development by Britten & Kohne (6) and Wetmur & Davidson (7) of systematic frameworks for the interpretation of experimental results. The literature of renaturation and hybridization kinetics in this period has been included as a subject in several reviews (8–11). Our emphasis is on the continued development of an integrated and quantitative understanding of the many facets of the kinetics of renaturation and hybridization of nucleic acids. A recent methods paper by Britten, Graham & Neufeld (12) provides complementary information for practitioners in this area of research.

RATE CONSTANT DETERMINATIONS

A number of methods are available for following the time dependence of the fraction of nucleic acid bases in the native helical or denatured coil forms. For example, chromatographic or enzymatic methods are required for most tracer experiments. Nevertheless, because the absorbance method has been central to the study of
synthetic oligonucleotides and homopolymers, as well as important for the establishment of a quantitative description of the kinetics of renaturation and hybridization of naturally occurring nucleic acids, we defer discussion of the other methods until the subject matter requires their introduction. Some of the data presented below were obtained using other methods but could just as well have been the result of absorbance measurements.

Denaturation of a native nucleic acid is accompanied by an increase in the 260 nm absorbance of the nucleotide bases called hyperchromicity. Renaturation is accompanied by the opposite phenomenon of hypochromicity. The basic assumption in using this hypochromic shift as a monitor for the time course of renaturation is that, at a given temperature, any decrease in absorbance is proportional to an increased number of bases in the helical form. This assumption undergoes its most stringent test in experiments with oligonucleotides. Formation of one base pair need not be accompanied by an increase in the stacking interactions between adjacent bases along either of the two interacting chains. Formation of each additional base pair in sequence will contribute one stacking interaction between adjacent base pairs. Although more complicated formulas exist and have been shown by Applequist & Damle (13) to be in agreement with experimental results, a simpler formula has been suggested by Applequist (14) that relates the hypochromicity of a helix of length \( n \), \( H(n) \), to that of a helix of infinite length, \( H(\infty) \):

\[
\frac{H(n)}{H(\infty)} = 1 - \frac{1}{n}.
\]

This result has been shown by Scheffler et al (15) to fit experimental data for \( n > 7 \) and implies that the hypochromicity is simply proportional to the number of stacking interactions. The proportionality between hypochromism and bases paired is certainly true for homopolymers and even highly sheared complex nucleic acids under the conditions routinely employed for renaturation and hybridization experiments. Under certain conditions, the helix formation by oligomers may be described by the all or none approximation, which permits only fully denatured and fully native species to coexist in solution. Under these conditions, although less hypochromidty accompanies helix formation, the proportionality between hypochromism and bases paired remains valid.

**Temperature-Jump Experiments**

The methodology for performing temperature-jump experiments was worked out by Eigen and his co-workers and is described in Eigen & De Maeyer (16). This type of “chemical relaxation spectrometry” (17) has been applied to double helices of oligoadenylic acid formed at acid pH and to oligoriboadenylic acid plus oligoribouridylic acid by Pörschke & Eigen (18); to oligoribouridylic and polyribouridylic acid plus m\(^6\)m\(^9\) adenosine by Hoffmann & Pörschke (19); to self-complementary block copolymers containing equal length regions of riboadenylic acid and ribouridylic acid by Craig et al (20); to similar self-complementary copolymers that form guanine-cytosine base pairs by Pörschke et al (21). Detailed thermodynamic studies (21, 22) of the self-complementary copolymers have resulted
in the determination of conditions in which the all or none approximation holds very well. The kinetic studies have been performed under these conditions.

With most temperature-jump and stopped-flow apparatuses, the output of a photomultiplier is directly displayed on an oscilloscope. The signal observed above the dark current, measured with a blocked light beam, is proportional to the transmittance. For all renaturation and hybridization experiments, we are interested in the difference between the absorbance at time \( t \), \( A_t \), and the absorbance at infinite time, \( A_\infty \). If \( S_t \) and \( S_\infty \) are the corresponding signals resulting from light striking the photomultiplier, then we find

\[
A_t - A_\infty = \log_{10} \frac{S_\infty}{S_t}.
\]

2.

A temperature-jump experiment involves a rapid and small perturbation from equilibrium of a reaction with a finite enthalpy. Let \( (A) \) and \( (A') \) be the nucleotide concentrations of the complementary strands in equilibrium with \( H \). \( (H) \) is the base pair concentration of helical molecules. Let \( k_2 \) be the second-order rate constant for helix formation and \( k_1 \) be the first-order rate constant for helix dissociation. The equilibrium

\[
A + A' \rightleftharpoons H
\]

3.

is perturbed by increasing the temperature resulting in an increase in \( A \) and \( A' \) of \( \Delta A \) and a decrease in \( H \) of \( \Delta H \). If \( A \) and \( A' \) are equal and \( \Delta A \) is much smaller than \( A \), the rate equation takes the form

\[
\frac{d\Delta A}{dt} = [2k_2(A) + k_1] \Delta A.
\]

4.

which is a simple exponential reaction. A semilogarithmic plot of \( A_t - A_\infty \) versus time will be described by a single relaxation time \( \tau \), where

\[
\tau^{-1} = 2k_2(A) + k_1.
\]

5.

Again using the equilibrium expression

\[
\frac{k_2}{k_1} = \frac{(H)}{(A)^2}
\]

6.

and substituting the total nucleotide concentration, \( C_0 \), where

\[
C_0 = 2(A) + 2(H).
\]

7.

the following expression is obtained:

\[
\tau^{-2} = 2k_2k_1C_0 + k_1^2.
\]

8.

Thus, if a number of experiments are performed with varying total nucleotide concentration, a plot of \( \tau^{-2} \) versus \( C_0 \) will lead to independent determinations of the dissociation rate constant, \( k_1 \), and the renaturation or helix formation rate constant,
$k_2$. For the special case of self-complementary molecules, the corresponding relaxation expressions are

$$\tau^{-1} = 4k_2(A) + k_1$$  \hspace{1cm} (9)

and

$$\tau^{-2} = 8k_1k_2C_0 + k_1^2.$$  \hspace{1cm} (10)

Using these expressions, we find that rate constants for helix formation between self-complementary oligomers may be directly compared with the rate constants for renaturation and hybridization of complex nucleic acids. Data obtained from temperature-jump experiments are discussed below.

**Stopped-Flow Experiments**

Stopped-flow studies involve the rapid mixing of two potentially reactive species. Most of the experiments with nucleic acids have been performed on homopolymers whose length is substantially greater than the oligomers studied by chemical relaxation spectrometry. Stopped-flow experiments have been reported by Ross & Sturtevant (1, 2), Blake et al. (23), and Pörschke & Eigen (18) with polyriboadenylic acid plus polyribouridyllic acid. Lee & Wetmur (24, 25) have studied this reaction as well as the reactions between all deoxyribohomopolymers and ribohomopolymers containing the naturally occurring nucleotides as well as inosinic acid. Because of the use of longer molecules and the renaturation conditions, all of these reactions are irreversible. The rate equation reduces to

$$-\frac{d(A)}{dt} = k_2(A)(A').$$  \hspace{1cm} (11)

By defining $(P)$ as the total single-stranded nucleotide concentration for the case of $(A) = (A')$, we find that equation 11 reduces to the form given by Wetmur & Davidson (7) for complex nucleic acid renaturation:

$$-\frac{d(P)}{dt} = \frac{k_2}{2}(P)^2.$$  \hspace{1cm} (12)

which has a solution

$$\frac{1}{f_{ss}} = \frac{A_0 - A_\infty}{A_t - A_\infty} = \frac{k_2C_0t}{2} + 1.$$  \hspace{1cm} (13)

where $f_{ss}$ is the fraction of bases in single-stranded form and $A_0$ is the absorbance of entirely denatured molecules at the renaturation temperature. In most cases, $A_0 - A_\infty$ is taken to be the hyperchromicity found upon melting the native structure. Although this assumption may overestimate the true $A_0 - A_\infty$ value appropriate for the renaturation temperature, the error introduced is small and uniformly in the same direction. Again using the signals resulting from light striking a photomultiplier as described in equation 2, we find that a plot of $1/\log_{10}(S_0/S_t)$ versus time will give a slope, $m$, where
Experiments with Complex Nucleic Acids

Renaturation or hybridization kinetics experiments with complex nucleic acids take place on a much longer time scale than experiments with synthetic oligonucleotides or homopolymers. Reactions may be initiated by cooling a sample from a temperature above the melting temperature to the renaturation temperature. An alternate method for DNA renaturation (7) but not DNA·RNA hybridization involves base denaturation of the DNA at pH 13, neutralization while cold with NaH₂PO₄, and rapid electrolytic heating of the denatured DNA to the renaturation temperature. This last method is most applicable to the study of rapidly renaturing DNA. The simplest general apparatus involves placing a water-jacketed cell or cell holder in a standard recording spectrophotometer and attaching two recirculating constant-temperature baths to the cell or cell holder through a series of stopcocks. One bath is maintained at a temperature above the melting temperature of the DNA while the second is maintained at the renaturation temperature. A reaction is initiated by switching from the first bath to the second. Because of the high temperatures and long times involved, the baths should be filled with antifreeze and the cuvettes should be tightly stoppered. Renaturation and hybridization kinetics experiments have been reported for numerous nucleic acids. References to particular studies are given when the results are cited to illustrate the general principles developed below.

One method of plotting rate data is to use the general second-order rate equation and solution given above in equations 12 and 13. When a recording spectrophotometer is used to obtain the data, the absorbances in equation 13 are directly read from the chart. \( A₀ \) and \( Aₜ \) are the absorbances of denatured and native DNA, respectively. The \( A₀ \) value is obtained by melting the DNA. If \( Aₜ \) is not available, \( Aₜ \) may be calculated by assuming a 36% hyperchromicity for the nucleic acid. This value of 36% is very close to being a constant independent of base composition (except for homopolymers) or solvent composition. If a new solvent is being tested, a native DNA should be melted to establish the percent hyperchromicity. \( C₀ \) may be obtained spectrophotometrically. A solution of 48 micrograms/ml of native sodium DNA has an absorbance of 1 at 260 nm and a nucleotide concentration of \( 1.47 \times 10^{-4} \) M. A plot of \( 1/fₜ \) versus time will give a line whose slope is \( (k₂C₀)/2 \) leading to determination of the renaturation rate constant.

An alternate method of data presentation developed by Britten & Kohne (6) has also gained wide usage and is known as a Cot plot for the abscissa of \( \log_{10}Cot \). Cot, meaning \( C₀t \), is also a very useful method of describing reaction progress in a concentration independent form. Equation 13 may be inverted to give

\[
fₜ = \frac{1}{1 + \left(\frac{k₂}{2}\right)Cot}.
\]
A plot of $f_{ss}$ versus $\log_{10} \text{Cot}$ for a simple second-order reaction produces an inverted sigmoid curve with an inflection point at the midpoint of the reaction, $t_{1/2}$, where $f_{ss}$ is 0.5. The curve in the region of the midpoint is to a very good approximation a straight line. The rate constant may be determined from the $\text{Cot}_{1/2}$ value obtained when this line crosses $f_{ss}$ of 0.5:

$$k_2 = \frac{2}{\text{Cot}_{1/2}}. \quad 16.$$  

This form of data presentation may be the method of choice when a nucleic acid contains repeated sequences and does not renature with second-order kinetics, because the compression of the time scale allows short-time and long-time data points to be placed on the same graph. For simpler systems such as those considered in the next few sections of this review, however, the standard second-order rate plot is a simpler method of obtaining $k_2$ and is sensitive to small deviations from second-order kinetics. A second advantage of the log time scale is the ability to present data for nucleic acids of very different renaturation rate constants on the same graph. If two reactions both follow second-order kinetics, the Cot plots should be parallel. Second-order rate plots and Cot plots are illustrated in Figure 1 for the case of two ideal reactions of different renaturation rate constants.

Recently Morrow (26) and Britten et al (27) have looked into new plotting methods that take into account renaturation between circularly permuted fragments of DNA of varying size. The deviation of rate plots from second-order behavior is considered below.

The assumption has been made so far that renaturation and hybridization reactions follow second-order kinetics, at least initially. Implicit in this assumption is a statement that the rate of renaturation is limited by a nucleation event and that the subsequent base pairing into a helix is a fast process. The only way to distinguish

![Figure 1](image-url)  

**Figure 1** Rate plots used for analyzing hybridization and renaturation kinetics showing two rates differing ten-fold: (a) Standard second-order rate plot; (b) Cot plot. (---): Part of reaction not plotted by both methods.
kinetic order for renaturation and hybridization is to examine the concentration dependence of the reaction. Deviation of rate plots from second order arises from molecular heterogeneity. Subirana & Doty (28) first reported that, although the second-order rate constant is nearly independent of concentration, the rate constant decreases by as much as a factor of 2 for a 100-fold increase in DNA concentration. This result has been confirmed by all subsequent investigators except Studier (29), who studied renaturation in low-salt solutions and followed the reaction by a different method, which might have measured a different phenomenon than base-pair formation following a rate-limiting nucleation step. Rau & Klotz (30) have developed a theory to explain the dependence of the second-order rate constant on concentration, but which somehow also fits complete rate plots without taking into account molecular heterogeneity. Until either this theory is extended to take into account other known and previously explained phenomena or new evidence is developed that requires this theory, the origin of the weak dependence of $k_2$ on $C_0$ cannot be considered to be established. For practical purposes, most experiments are done with simultaneous control runs with DNA of known renaturation rate, and the dependence of $k_2$ on $C_0$ is ignored.

LENGTH AND COMPLEXITY EFFECTS ON RATES

Now that some experimental methods have been described that permit determination of $k_2$, we may discuss the interpretation of $k_2$ values. Following Wetmur & Davidson (7), the concept of a nucleation rate constant, $k_N$, is introduced.

The Theoretical Nucleation Rate Constant

The rate-determining step for renaturation or hybridization is the formation of a base-paired nucleation site between two homologous strands. This step is followed by a rapid zippering reaction in which the remaining complementary bases are paired. Assume that the nucleation sites (one or at most a few nucleotides) are evenly distributed along the nucleic acid chains with a density $\beta$ per nucleotide. Two definitions must now be introduced.

1. **Complexity:** The complexity, $N$, is the total number of DNA·DNA, DNA·RNA, or RNA·RNA base pairs present in nonrepeating helical products of a renaturation or hybridization reaction. The complexity for most double-stranded DNA viruses or bacteria is essentially the same as the number of base pairs in the intact haploid genome.

2. **Length:** The length, $L$, is the actual number of bases per single-stranded nucleic acid molecule present in a renaturation or hybridization reaction mixture.

Figure 2 is a simple (small $L$ and $N$) illustration of complexity and length. Letters are used to designate nucleotides. When the DNA of complexity and length 26 in Figure 2a is cleaved to length 13 in Figure 2b, the complexity is still 26. Unlike $N$, which depends only on the genetic composition of the source nucleic acids, $L$ is a function of the method of nucleic acid preparation. The DNA in Figure 2a might be expected to react twice as fast as the DNA in Figure 2b,
because twice as many bases will be paired per successful nucleation. That is, the rate constant $k_2$ should be directly proportional to $L$. Figure 2c shows a different DNA of complexity 13 with the same length, 13, as the DNA in Figure 2b. The DNA in Figure 2c should react twice as fast as the DNA in Figure 2b because the concentration of nucleation sites is twice as high. That is, the rate constant $k_2$ should be inversely proportional to $N$. The formal statement of these results follows.

The concentration of any one nucleation site is $\beta P/2N$. The rate of formation of one such site is $k_N (\beta P/2N)^2$. The number of loci able to act as nucleation sites is $\beta N$. The total rate of nucleation at all sites is then given by $k_N \beta^3 P^2/4N$. Assuming no permutation of sequences in the molecules in solution, we find that $2L$ bases will be paired per nucleation event. If a DNA is cut by a restriction endonuclease, this result will be true. If other degradation processes are employed, a permutation of sequences is produced that leads to a reduction in the yield of bases paired per nucleation. This problem is considered below in the section on products of renaturation reactions. The rate equation for the nonpermuted case is

$$ \frac{dP}{dt} = k_N \frac{\beta^3}{2N} \frac{L}{P^2}. \quad 17. $$

When this result is compared with the experimental rate equation 12, the following relationship between $k_2$ and $k_N$ is obtained:

$$ k_2 = k_N \frac{\beta^3}{N} \frac{L}{P}. \quad 18. $$

**Nucleic Acid Complexity**

Britten and co-workers observed and reported [Britten & Kohne (6)] that DNAs from different organisms sheared to the same length reacted with a rate constant inversely proportional to complexity in agreement with the argument outlined in Figure 2 and presented in equation 18. Wetmur & Davidson (7) confirmed this important result. The inverse $N$ dependence of $k_2$ holds over at least six orders of magnitude of $N$ for complex DNAs. The rate constants reported for the mammalian DNAs were those of the single copy sequences represented by the slowest renaturing fraction of the DNAs. Repeated sequences are considered below as a separate topic.

Figure 2  Length and complexity effects on hybridization and renaturation rates. (a) Length and complexity 26; (b) length 13, complexity 26; (c) length and complexity 13.
In order to establish the complexity correlation, it was necessary to obtain the complexities of the DNAs by some other means. This means involved assuming that the total haploid DNA content of a double-stranded DNA virus, bacterium, or eukaryotic cell, excluding fast-renaturing DNA from eukaryotic cells, was present in only one copy. This assumption proved to be true for the systems studied in both laboratories. One method of determining total DNA content in cells or viruses is the diphenylamine reaction (31). For viral nucleic acids only, it is often relatively simple to isolate the genome intact and to measure it by electron microscopy using the Kleinschmidt technique (see 32). The number of base pairs in the DNA is directly proportional to the measured length. The proportionality constant is a function of mounting conditions, so internal control DNAs of known length are recommended. Since the establishment of the inverse N dependence of \(k_2\), it has been possible to determine \(N\) for a DNA from a new source by measuring \(k_2\) values for this DNA and a DNA of known \(N\) under identical conditions. Renaturation kinetics remains the only method for determining complexity for cellular nucleic acids.

Recently, two other methods have been developed for determining complexities of viral nucleic acids. One method was developed in response to the problem of determining the complexity of the single-stranded RNA in the segmented genomes of RNA tumor viruses. Duesberg et al (33) have used the analysis of the most complex fragments of RNA fingerprints to establish \(N\) by assuming that the majority of these fragments would be present only once per single copy sequence. A method for determining the complexity of covalently inserted viral DNA (or complementary DNA) takes advantage of the quantitative gel electrophoresis systems for determining the lengths of restriction endonuclease fragments [Helling et al (34)]. If the entire genome of a transformed cell is cut with an appropriate restriction endonuclease, run on a gel, and hybridized with radioactive viral nucleic acid, then an upper limit to the complexity of the inserted DNA is obtained from the sum of the lengths of the restriction fragments (unique) that hybridized to the radioactive nucleic acid. Appropriate enzymatic and counting procedures may be used to correct for the end fragments that contain some nonviral DNA.

**Nucleic Acid Length**

Wetmur & Davidson (7) determined the relationship between \(k_2\) and the length of the single strands of DNA in solution, \(L\). DNA may be sheared in a syringe by blending, by sonication, by use of a high pressure press (12), or the single strands may be randomly degraded by a DNase or heat treatment followed by alkaline hydrolysis. This last procedure has recently been quantitated by Lindahl & Nyberg (35) and Lindahl & Andersson (36). Restriction endonucleases are now available for producing nonoverlapping (noncircularly permuted) fragments of DNA of various sizes. Restriction endonuclease digestion should become the method of choice for many renaturation kinetics experiments. Molecular weights of fragmented denatured DNAs prepared by any method may be determined by alkaline band velocity sedimentation using the equation of Studier (37) or by electron microscopy using the formamide technique and \(\phi\times174\) DNA as an internal control (32).
Hutton & Wetmur (38) determined the relationship between $k_2$ and the length of single-stranded RNA in a DNA·RNA hybridization reaction. RNA fragmentation was achieved by brief alkaline hydrolysis. RNA length determinations are more difficult than DNA length determinations. The electron microscopic methods of Delius et al (39) using the T4 phage gene 32 product or the $\text{CS}_2\text{SO}_4$-dimethyl-sulfoxide density gradient system (see 38) are quantitative methods free of the problems of variation in RNA structure that prevent the use of a calibrated transport method.

The DNA renaturation kinetics results show that $k_2$ is proportional to the square root of the length of fragmented DNA strands when both complementary strands are degraded the same amount. This result was shown (7) to hold over three orders of magnitude in $L$, beginning with $L$ of about 100. The square root dependence is not predicted by equation 18. The result implies that $k_N$ is inversely proportional to the square root of the length of the DNA strands or, in other words, that the availability of sites for nucleation decreases with increasing strand length. This result has been interpreted (7) as being a consequence of an excluded volume effect where the reacting complementary strands fail to penetrate one another completely. No other interpretation has yet proved consistent with experimental data, although experiments that conclusively prove or disprove the excluded volume argument have not been performed.

In some DNA renaturation experiments and in almost all DNA·RNA hybridization experiments, the lengths of the two complementary strands are not the same. Wetmur (40) studied the case of DNA strands of different length and found that both excluded volume theory and experiment were in agreement with a dependence of $k_2$ on the square root of the length of the shorter of two reacting single strands. Limited results with homopolymers (24) and extensive results with DNA·RNA hybridization (38) are in agreement with this dependence. The experiments have been performed only over a factor of 33 difference in strand length and cannot be assumed to hold beyond this limitation, although most experiments of interest are contained within this spread of lengths. Experiments have been performed by McConaughy & McCarthy (41) and by Niyogi (42) that show that oligonucleotides of 15 or more bases from complex nucleic acids will form specific hybrids with long complementary DNA. Kinetic results with this system could be used to extend the correlation of rate constant with DNA strand lengths where the complementary strands vary in strand length as well as to better integrate synthetic oligonucleotide, homopolymer, and complex nucleic acid studies. Melchior & Von Hippel (43) have described solvent systems with no melting temperature dependence on base composition, which should be ideal for such an effort.

In order to include the experimental length dependence in equation 18, the simplest expedient is to define a length-independent nucleation rate constant, $k_N^*$, which also includes the $\beta^2$ term. The result is

$$k_2 = k_N^* \frac{L_s^{0.5}}{N}$$

where $L_s$ is the length of the shorter of the two reacting complementary strands.
\(k'_n\) is now primarily a function of solvent (e.g., salt, pH, temperature) as well as gross nucleic acid composition (ribose or deoxyribose; base composition). Variables that affect \(k'_n\) are discussed in detail below.

**Products of Renaturation Reactions**

As mentioned above, when DNA molecules are sheared or otherwise degraded by means other than the use of restriction endonucleases, the resulting circular permutation will affect the yield of bases paired following a nucleation event. Miller & Wetmur (44) have examined the questions of yield and the nature of the products formed at various stages of a renaturation reaction. A theory was derived relating the length distribution function of the degraded DNA to the expected yield early in a renaturation or hybridization reaction. The initial yield per nucleation will vary from 0.67 times that obtained optically with nonoverlapping strands for molecules of identical length to 0.41 for products of random degradation such as produced by a nonspecific endonuclease or by hydrolysis. For example, the value with sonicated DNA is about 0.6. This number may be used to relate rate constants obtained by hydroxyapatite chromatography, which measures nucleation events and not bases paired, with optical or endonuclease S1 measurements of renaturation rates. We point out that \(k_2\) values measured optically and reported in the literature are not corrected for reduced yields due to degradation and permutation.

A second result of the circular permutation and finite length distribution of DNA molecules is the deviation of rate plots from ideal second-order behavior, also mentioned above. Miller & Wetmur (44) showed that between 40 and 60% renaturation three-stranded products are formed with one strand completely base paired. This means that the average yield per nucleation for sonicated DNA will decrease up to 50% towards the middle of the reaction. The order of formation of more complex multistranded products is not known, but the experiments show that the extending strands of the three-stranded structures never became completely base paired. The curve-fitting procedure of Morrow (26) or the equations derived by Britten et al (27) may be used to fit rate plots if data throughout a reaction are needed as in the cases of DNA containing sequences with various degrees of repetition. If these procedures are used, they should really be modified on a case by case basis to fit the length distribution function for the degraded DNA in question. With some distributions, almost ideal second-order behavior is observed. For simple cases involving no variation in sequence repetition, the rate constant may easily be obtained from the first half of the reaction.

**VARIABLES AFFECTING NUCLEATION RATES**

**Temperature**

The rate of renaturation of complex nucleic acids or homopolymers goes to zero at the melting temperature, \(T_m\). As the temperature is lowered, the rate first increases, then plateaus, and finally decreases. This bell-shaped rate constant–temperature profile obtained by early workers (4) has been repeated many times with the same results. The term criterion (12) may be defined as the difference in temperature
between \( T_m \) and the renaturation temperature. Stringent (small) or open (large) criteria are chosen depending upon the amount of mismatched sequences that may be permitted in a particular experiment with eukaryotic DNA. The subject of mismatching is described in detail later. For most viral, bacterial, or organelle nucleic acid experiments where no mismatching is to be expected, or for eukaryotic nucleic acid experiments where mismatched products of closely related sequences are to be permitted, an open criterion of about 25°C will in general give the maximum renaturation rate. If an open criterion is chosen, there is a range of about 10°C within which the rate constants obtained will be essentially independent of temperature. Most experiments have been performed under these conditions.

A theoretical model to explain the temperature dependence of renaturation rates was derived by Saunders & Ross (45). This theory has been revised by others to include more variables (7, 46-48). The theory assumes a rate-determining nucleation step (involving one or more base pairs) followed by quasi-stationary state intermediate steps of additional base-pair formation. These assumptions lead to a bell-shaped curve for all reasonable values of the elementary rate constants involved. The neglect of strand alignment as a separate variable during the early stages of the reaction cannot be considered a serious drawback of the formulation. A complete and correct statement of the theory is given by Crothers et al (47). The same model, including degeneracies introduced to account for end effects, has been presented by Craig et al (20) for rate constants obtained from temperature-jump experiments with oligonucleotides. The rate-constant equations are complicated and will not be presented here. The results of the application of the theory to the temperature dependence of renaturation rates are two-fold. First, an estimate of the elementary rate constant for adding another base pair to a growing helix may be made. Second, the rate-determining step for helix formation may be estimated from the activation energy of \( k_2, E_2^+ \). The theory shows that

\[
E_2^+ = E^+ + \Delta H_c + (\alpha - 1) \Delta H_f,
\]

where \( E^+ \) is the activation energy for the formation of the \((\alpha + 1)\) base pair, the rate-determining step. \( E^+ \) must be positive because it involves an elementary reaction, and its value may be expected to be small. The small value is required to be consistent with the magnitude of the helix propagation rate constant calculated below. \( \Delta H_c \) is a complex quantity including the heat of formation of the first base pair. \( \Delta H_c \) has been estimated using thermodynamic data to be approximately zero. \( \Delta H_f \) is the heat of formation of a base pair next to a long helical region. \( \Delta H_f \) has been measured at values of the order of \(-8\) kcal/mole depending on nucleotide composition.

Using temperature-jump methods, Pörschke & Eigen (18) estimate a helix growth rate constant of \(10^7-10^8\) sec\(^{-1}\) for oligoriboadenyllic acid plus oligoribouridylic acid. They also conclude that the rate-determining step is the formation of the third base pair. Applying the same method to self-complementary block copolymers with the same nucleotides, Craig et al (20) find a helix growth rate constant of 0.5 to \(2 \times 10^7\) for the same rate-determining step. As the molecules increase in length, the rate-determining step consistent with \( E_2^+ \) becomes the formation of the
second base pair. Lee & Wetmur (24, 25) found that most homopolymer reactions studied by stopped flow gave results consistent with formation of the second base pair being the rate-determining step. In a temperature-jump study of block co-polymers, which also contain guanine-cytosine base-pairing capability, Pörschke et al (21) conclude that the rate-determining step involves formation of either the first or second base pair.

One conclusion from these studies is that the nucleation size as defined kinetically is much shorter than the length of nucleotides necessary to form a thermodynamically stable structure. Many abortive nucleation events must take place during the course of renaturation of a complex nucleic acid. A second conclusion is that the rate of base-pair formation is exceedingly fast compared to the rate of nucleation. This result must be true to obtain second-order kinetics and to prevent abortive nucleations from interfering with renaturation.

**Ionic Strength, pH, Denaturing Solvents**

The melting temperatures of nucleic acids depend on counterion concentration. To maintain constant criterion, the renaturation temperature must be adjusted to compensate for changes in the melting temperature. Because nucleic acids are polyanions, counterion concentration would also be expected to be critical for determining the equilibrium constant for formation of a nucleation complex. The effect of salt concentration on the rate of renaturation of DNA has been investigated in low (29) and moderate to high (7) salt concentrations. The rate in low-salt concentrations is proportional to the cube of the counterion concentration. The dependence of renaturation rate on salt concentrations becomes less steep in high-salt concentrations, but still increases by a factor of 2 between 0.4 and 1.0 M sodium ion. Britten (49, 50) has obtained an empirical equation to fit the salt dependence renaturation rates and has developed a useful table (12, page 364), which allows rate constants obtained at different salt concentrations to be compared. Manning (51) has fitted the existing data to a theory that leads to a length for the electrostatically interacting segments of about 16 bases each. This number is in reasonable agreement with estimates of the statistical segment length of denatured DNA (40). The theory would also predict, in agreement with experiment (21), that the ionic strength dependence of the renaturation rate of short oligonucleotides would be less than that observed for higher polymers. The theory also makes predictions for divalent cations, which have yet to be tested experimentally because, in practice, divalent cations are avoided in renaturation and hybridization experiments. These experiments need to be performed to completely test the theory. Divalent cations may catalyze degradation of nucleic acids both by themselves and as cofactors of nucleases. Renaturation and hybridization experiments in low salt should be performed with chelators such as ethylenediaminetetraacetate (EDTA) present in the solutions. The EDTA concentration must be kept low to prevent its absorbance at 260 nm from interfering with the rate measurement. For low concentrations of EDTA to be most effective, the pH should be closer to 8 than the values of around 7 routinely used with phosphate buffers. In high salt, the necessity of using chelators has not been proven.
The effect of pH on renaturation rates of DNA has been investigated (7). There is no pH effect in the range from pH 6 to pH 8. Most experiments are performed in this range.

DNA renaturations (52) and DNA · RNA hybridizations (53) have been carried out in formamide-containing solutions as a means of lowering the melting temperature and hence the renaturation temperature to prevent heat-induced nucleic acid degradation. Studies in solutions containing urea (12) have been performed in an effort to avoid the problems created by the optical properties of formamide. The rate of DNA renaturation has been studied in several other denaturing solvents including aqueous solutions of ethylene glycol (7), sodium perchlorate (7, 54), tetramethylammonium chloride (54), and tetraethylammonium chloride (54). Chang et al (54) found 2.4 M tetraethylammonium chloride to be an ideal solvent for studying renaturation by optical means because of its lack of absorbance at 260 nm, the low melting temperature (63°C), low optimum renaturation temperature (45°C), independence of these temperatures of nucleotide composition (43), and relatively high renaturation rate. Wetmur (55) has determined the $k_v$ in this solvent to be $2.25 \times 10^5$ liters/mole-sec, a value similar to that obtained in 0.5 M NaCl using the optimum criterion.

Viscosity

Subirana & Doty (28) and Thrower & Peacocke (56) observed a decrease in renaturation rates with increasing sucrose concentration. Subirana & Doty (28) also observed that sodium polyacrylate or native T4 DNA, although increasing the macroscopic viscosity as measured with a viscometer, had little or no effect on the rate of renaturation of DNA. Sucrose will change the microenvironment about the DNA bases whereas the polymers will have little or no effect on the local solvent structure. Sucrose may thus be said to increase the microscopic viscosity while the polymers only change the macroscopic viscosity. Walker & McCallum (57) have pointed out the importance of the observation that native DNA does not change renaturation rates in terms of renaturation of a probe DNA in the presence of excess heterologous DNA, a subject considered in a following section.

The observations concerning the effect of microscopic viscosity on DNA renaturation have been extended by Wetmur & Davidson (7) and Chang et al (54) to include sucrose, glycerol, ethylene glycol, and sodium perchlorate. The observation that the rate of renaturation is inversely proportional to viscosity in high-salt solvents at all criteria (54) supports the rate-temperature profile theory, which provides for only one mechanism for the reaction at all temperatures. An alternative theory (48) involving different mechanisms for renaturation reactions at open and stringent criteria is not consistent with the new experimental results (54). The organic solvents have an effect on the binding of counterions to DNA. In low salt, the rate increase due to increased counterion binding tends to offset the decrease resulting from the increased microscopic viscosity. The exact nature of the viscosity effect on the rate of base-pair formation has not been established, although the result implies that the reorientation of the two bases and concurrent base stacking and hydrogen bonding may be a diffusion-limited process. The weak temperature dependence found for
calculated helix growth rate constants (7, 18, 20) is consistent with this interpretation.

Chang et al (54) have observed and Wetmur (55) has studied in detail the effect of adding negatively charged and neutral dextran polymers to renaturation mixtures. When very high concentrations of the polymers are added, DNA renaturation is accelerated, particularly by dextran sulfate. A simple excluded volume theory predicts a rate acceleration, $R$, due to a polymer of intrinsic viscosity $[\eta]$ and concentration $C$ of

$$R = \exp(0.4\beta[\eta]C).$$

The theory assumes that the volume exclusion by the added polymers effectively increases the DNA concentration and hence the rate of renaturation. The experiments agree with theory. With dextran sulfate, the value of $\beta$ is found to be about 0.5 in 2.4 M tetraethylammonium chloride. The value of $R$ may be as high as 20. This rate acceleration should be particularly useful for decreasing the time necessary for renaturing or hybridizing very complex eukaryotic nucleic acids.

**Nucleic Acid Composition**

In addition to solvent effects on $k_r$, which have been discussed, $k_r$ may be a function of nucleic acid composition. The variables in this case are the base composition of the nucleic acid (normally expressed as guanine plus cytosine content, or GC percent) and the sugar, which may be either deoxyribose or ribose.

The dependence of $k_r$ on GC percent has been investigated with contradictory results. We suggest that the dependence, if any, is so small as to be able to be ignored. Wetmur & Davidson (7) investigated a series of DNAs of “known” complexity and varying GC content and found a trend of increasing $k_r$ with increasing GC content. The choice of DNAs was limited, however, and new information, concerning those DNAs, makes the GC content–$k_r$ correlation suspect. T4 DNA, a glucosylated DNA, was included in the series. Ignoring any effect of glucosylation on rate, but correcting the “known” complexity to agree with electron microscopy measurements made with internal standards, we find that newer measurements and calculations of $k_r$ (38) show T-even and T7 DNAs with the same $k_r$. SV40 DNA was also included in the series. It is now known that SV40 virus passaged at high multiplicity results in marked heterogeneity in the product SV40 genomes. The high kinetic complexity found for SV40 DNA may be a result of a real increase in complexity of the molecules compared to the “known” complexity obtained using electron microscope measurements of contour length. Studies with N1 DNA (58) show a difference in GC content as measured by melting temperature, buoyant density, or base composition analysis. The best value is 70% (59) and not 64%.

Seidler & Mandel (60) found a trend identical to that found by Wetmur & Davidson (7), but again with limited data. On the other hand, Gillis et al (61), measuring DNA content by chemical means (62), found a GC content dependence of $k_r$ that was small but opposite to that obtained by the other workers. Nevertheless, even if we ignore errors inherent in the chemical measurements as well as possible polyploidy effects, the GC dependence of $k_r$ is barely significant compared to the errors inherent in the initial rate, renaturation kinetics measurements. Lee & Wetmur
(25), in studies using homopolymers, also found a bias toward faster nucleation rate constants for adenine- rather than cytosine-containing polymers, but these results may be due to peculiar structural parameters of the homopolymer chains themselves. Overall, we suggest that the results of the several investigators are consistent, showing a very weak dependence of $k'_N$ on GC percent.

In considering the effect of the sugar, we are limited to data for DNA·DNA renaturation and DNA·RNA hybridization. RNA·RNA data have not been obtained in a controlled manner together with DNA·DNA or DNA·RNA data except for the special cases of homopolymers (25). The answer we now seek is the ratio of $k'_N$ for hybridization to that for renaturation. Melli et al (63) and Bishop (64) found that DNA·RNA hybridization for *E. coli* RNA to *E. coli* DNA was about 0.4 times as fast as DNA·DNA renaturation and derived equations to describe competing renaturation and hybridization situations. The equations and techniques are important and are discussed in detail in the section on tracer studies. The value of 0.4 is doubtful, however, in part because careful DNA and RNA molecular weight measurements were not made. Strauss & Bonner (65) have also studied the formulation of competing renaturation and hybridization experiments. In experiments with ribosomal RNA and DNA, again without appropriate length measurements, they found that the ratio of nucleation rate constants varied from 0.25 to 0.55 depending on the temperature of renaturation, with the highest value at the higher temperature where ribosomal RNA secondary structure would be expected to be completely eliminated. This temperature dependence of the ratio of nucleation rate constants must be considered whenever RNAs with significant secondary structure are to be used in competing hybridization and renaturation experiments. Hutton & Wetmur (38) studied the rate of hybridization of φx174 DNA with complementary RNA synthesized in vitro and isolated as a 1:1 hybrid with the DNA. At the same time, DNA renaturation controls were performed with T2 and T7 DNA. The measurement of RNA lengths and its effect on hybridization rates were reported on above. A value of 0.75-0.80 is obtained for the relative values of $k'_N$ for hybridization and renaturation. This high value is convenient because it means that RNA will compete quite well with DNA in competing hybridization and renaturation experiments provided that the DNA and RNA lengths are the same (66). This latter point cannot be emphasized too strongly, as many investigators have been led to erroneous conclusions because lengths affect both the initial rate and the yield of the hybridization reaction. This subject is discussed below.

**SPECIAL CASES**

**Circular Permutation and Terminal Redundancy**

Many bacteriophages contain genomes that are circularly permuted and terminally redundant. When the DNA of such a phage is denatured and renatured, circular molecules are produced that contain the two redundant end pieces as single-stranded segments. These segments are visible in the electron microscope. Lee et al (67) observed a nonrandom distribution of end segments for coliphage 15 and proposed that the bias in the distribution could be the result of an excluded volume
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Effect. Tye et al (68) and Orosz (69) found, however, that any bias is the result of a nonrandom distribution of circular permutations in the initial sample. The products with short intersegment spacings are the result of nucleations in the center of both reacting single strands as well as nucleations near the ends of both strands. Products with long intersegment spacings involve nucleations near the center of one strand and the end of the second strand. When we allow an excluded volume effect and consider all possible nucleation events, we find that complete calculations (69) show that the excluded volume effect will not be observed in the final product distribution of end segments. Thus circularly permuted, terminally redundant DNA molecules do not turn out to be a special case after all.

Base Mismatching

In addition to nucleic acid composition and solvent, variables that affect melting temperatures include length and mismatching. Length effects on renaturation rates have already been considered in assuming constant criteria, so length effects on renaturation temperatures do not need to be discussed as a separate topic. Mismatching, however, is a new variable that affects renaturation rates as well as melting temperatures. When discussing the magnitude of melting temperature changes, the units will be degrees centigrade per percent of base pairs mismatched. Uhlenbeck et al (70) have studied mismatching due to defects synthesized into otherwise self-complementary block copolymers. Gralla & Crothers (71) have studied similar molecules where the defect was between GC rather than AU base pairs. The results range from 0.5 to 2.0° per percent with the largest values for the polymers studied by Gralla & Crothers (71). Kallenbach & Drost (72) have studied long polyinosinic acid plus copolymers of inosinic acid or uridylic acid with cytidylic acid. The structures formed might be expected to be bulge defects with only one base removed from pairing rather than a complete base pair. The results are 0.7° and 1.1° reduction in melting temperature for I-U and I-I mismatches, respectively. Because of the limited criteria employed, rate measurements in this work cannot be compared with DNA renaturation studies. Chemical modification of DNA bases has been used to produce mismatching. Deaminated DNA has recently been studied by Hutton & Wetmur (73), Bonnet et al (74), and Marsh & McCarthy (75). Other modifications include DNA reacted with chloroacetaldehyde (76, 77), N-acetoxy-N-2-acetylaminofluorene (78), ultraviolet light (79), glyoxal (73), and DNA to ethidium bromide (80). A theory for melting temperature changes (78) predicts an upper limit of 1.5°C per percent. As an average, the melting temperature is reduced 1.1 ± 0.2°C per percent base pairs modified. Low values (70, 72, 79) involve some free energy contribution of the mismatched base(s) to the structure while the high value of 2.0° per percent (71) obtained by Gralla and Crothers is most likely a reflection of a sequence effect, which is averaged out in complex nucleic acids. Kinetic studies (73–76, 78, 80) show the reduction of $k_2$ at constant criterion to be less than a factor of two for a 10°C reduction in melting temperature. A theory, with no arbitrary parameters, has been derived (73) that relates rate to melting temperature changes. The theory fits experimental results quite well. The importance of choice of criteria has been pointed out by several authors (73–75). By changing
criteria, it is possible to select either for or against mismatched hybrids. Care must be taken in choosing conditions for a study involving mixed hybridization with closely related species (74, 75) or involving repeated sequences of eukaryotic DNA, which are not true repeats but involve formation of mismatched hybrids.

Repeated Sequences

In cases where a DNA sequence is longer than the fragment size used for a renaturation experiment and is repeated within a genome, the rate of renaturation of that sequence is accelerated in direct proportion to the number of reiterations. The faster rate is simply the result of an increased concentration of this DNA sequence relative to the concentration of individual sequences in the rest of the genome and requires no special interpretation. An example of such a repeated sequence is the ribosomal DNA of bacteria, which was first extensively studied by Kohne (81). In the course of applications of renaturation kinetics methods to plasmid DNAs, Wood & Luck (82), Wells & Birnsteil (83), and Christiansen et al (84) measured complexities that were not independent of DNA strand length. In such a case, it is necessary to distinguish between kinetic complexity, as measured by renaturation kinetics, and sequence complexity as actually present in the DNA. In general, if a new DNA is being studied to determine its complexity, the kinetic complexity should be determined at several strand lengths. If the same values are obtained for N at each L, and if no mismatching rate reduction is involved, then the kinetic complexity may be assumed to be the same as the sequence complexity. If N is a function of L, then the kinetic complexity is an upper limit to the true value of sequence complexity. Recently, Christiansen et al (85) found that the unusual kinetic effects observed for *Saccharomyces carlsbergensis* mitochondrial DNA disappeared if stringent criteria were employed. The simplest interpretation of these results is that a primarily AT low-melting sequence or an imperfectly repeated sequence is copied many times throughout the chromosome with a spacing such that these sequences appear within the length of most of the strands used in the renaturation experiment. Under these circumstances, helix formation by these sequences fails to result in zipper ing to completion and forms structures that interfere with further nucleation at nonrepeated sites. Since experimental conditions were found in this case where N was independent of L, the interpretation of the kinetics was greatly simplified. An investigator who finds unusual kinetics with a new system might look for similar simplifying conditions by changing the criterion as well as the solvent, with particular attention directed to varying concentrations of tetraethylammonium chloride (43).

Mouse satellite DNA has been studied by Waring & Britten (86), Flamm et al (87), Sutton & McCallum (88), Hutton & Wetmur (89), and Southern (90) with somewhat different results. Hutton & Wetmur (89) showed and Southern (90) confirmed that this DNA also behaves anomalously with respect to the length dependence of the kinetic complexity. If the kinetic complexity is extrapolated to short lengths, the kinetic complexity begins to agree with sequence complexity (89). However, Southern (90) has shown that there are several orders of sequence complexity within this particular DNA. Because of the difficulty of even defining
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sequence complexity, the short repeat lengths, and the mismatching found in re-natured structures, it is doubtful that renaturation kinetics can result in the determination of a single kinetic complexity equal to a well-defined sequence complexity for this particularly complicated system. Investigators wishing to apply renaturation kinetics to satellite DNAs of higher organism nuclei would be well advised to consider the complications encountered with mouse satellite DNA (89, 90).

TRACER EXPERIMENTS

In a tracer experiment, a labeled nucleic acid is reacted with an unlabeled nucleic acid, only a small amount of which may be complementary to the labeled nucleic acid. Examples of well-defined probes are isolated specific messenger, ribosomal and transfer RNAs, complementary DNA transcripts of such RNA molecules, or tumor virus nucleic acids and reverse transcripts. In some cases, complex nucleic acids such as DNA from a related or even the same species may be used as a probe. The experiment must be designed in such a way as to allow the reaction of the probe to be followed as a function of time.

Labeled Nucleic Acids

A requirement for the use of a tracer is that the probe molecule must be different from the rest of the nucleic acid. The most common label is radioactivity. $^{32}$P, as phosphate, and $^{14}$C or $^{3}$H, as a nucleoside, may be introduced in vivo during nucleic acid synthesis. Labels may also be introduced in vitro by chemical methods or by synthesis of complementary molecules. Reverse transcriptase may be used to obtain a radioactive complementary DNA copy of an RNA that is quite long and complete [Efstratiadis et al (91)]. The use of reverse transcripts of RNA molecules has the advantage of allowing DNA, which is less labile or subject to inadvertent enzymatic digestion, to replace RNA in the hybridization reaction. Radioiodination of DNA also works very well and leads to very little base mismatching using the conditions described by Orosz & Wetmur (92). DNA may also be made fluorescent (76) and may act as a probe (77), although significant base mismatching is introduced by this method.

Filter Hybridization

A number of methods have been developed that involve immobilization of one of the complementary strands on a solid support. The method of filter hybridization introduced by Gillespie & Spiegelman (93) following Nygaard & Hall (5) has been the most widely employed. Kinetic and equilibrium studies (94–98) have shown reduced nucleation rate constants, displacement reactions, and an unusual length dependence using this technique. For these reasons, rates obtained by filter methods cannot be directly compared to rates obtained in solution. Before returning to solution reactions, we should point out that the filter hybridization method, in spite of many papers to the contrary (see, for example, 99), may be as sensitive as solution methods for detecting small quantities of complementary nucleic acids in the presence of a vast excess of noncomplementary DNA (100, reviewed in 101).
A different solid support method described by Shih & Martin (102) has shown great sensitivity although kinetic studies are incomplete. Finally, kinetic studies have recently been performed on the technique of in situ hybridization by Szabo et al (103) that show results analogous to filter hybridization experiments.

**Solution Hybridization**

The only difference between tracer experiments in solution and the optical renaturation and hybridization experiments described in the bulk of this review is that the renaturation of the probe molecule with complementary nucleic acid may take place in competition with renaturation of the complementary nucleic acid with unlabeled sequences identical to the probe. This situation exists when either a DNA or RNA probe is used with bulk unlabeled DNA. If the nucleation rate constants and molecular lengths are the same for the probe and bulk nucleic acids and the probe is sufficiently dilute to contribute essentially no additional concentration of its own particular sequence, the rate of reaction of the probe will be the same as the rate of reaction of the same unlabeled sequences with the complementary nucleic acid. Gelb et al (99) have used this technique to determine the number of copies of a tumor viral DNA inserted into a chromosome. The ratio of the probe rate constant to that of the bulk DNA is a measure of the number of copies. We should point out two other experimental conditions where the analysis is slightly different but the resulting rate constants are the same as described throughout this review. These conditions both occur when DNA is used as a probe to look for complementary messenger RNA. First, the reaction will be pseudo first order if RNA is in excess because the RNA cannot react with itself. Second, the reaction cannot go to completion because the RNA is complementary to only one half of the DNA. The reaction will be described by

\[
\frac{f_{ss} - (f_{ss})_\infty}{1 - (f_{ss})_\infty} = \exp(-k_2 R_0 t),
\]

where \((f_{ss})_\infty\) is the fraction of single strands at infinite time and \(R_0\) is the RNA concentration. The number of copies may be determined by taking the ratio of \(k_2\) for the DNA · RNA reaction to the \(k_2\) of the renaturation of the probe with itself.

Melli et al (63) described the case of bulk DNA and probe DNA or RNA where the rate constant for the probe reaction is different from the rate constant for the same unlabeled sequences. The result is important if the nucleation rate constants are not exactly the same, as with DNA · DNA and DNA · RNA reactions, and when the length of the probe is shorter than the length of the bulk nucleic acid. The result takes the form

\[
\frac{1}{f_{ss}} = \left\{\frac{k_2 \text{Cot}}{2} + 1\right\}^{k_p/k_2},
\]

where \(k_p\) is the rate constant for the probe reaction and \(k_2\) is the rate constant for the unlabeled homologous sequences, both reacting with the unlabeled complementary DNA. In general, \(k_p\) is less than or equal to \(k_2\). The initial rate for
the probe reaction is essentially governed by $k'_p$, because the complementary unlabeled sequences are still in high concentration. Using initial rates and assuming the reaction will go to completion, the ratio of $k'_p$ to $k_2$ for the total bulk DNA will give a number ($k'_p/k_2$) times the correct value for the number of copies of DNA complementary to the probe. A more important problem develops later in the reaction. Because the bulk DNA renatures faster, eventually the probe runs out of unlabeled complementary DNA with which to react. The reaction appears to fail to go to completion. This result may lead one to the erroneous conclusion that only a part of the probe sequence is represented in the bulk DNA. A simple method for testing this conclusion is available and should never be omitted before assuming incomplete homology of the probe to the bulk DNA. More denatured DNA is added late in the reaction to see if the remaining probe DNA is able to react further. A separate but parallel problem occurs when the probe is shorter than the bulk DNA. Not only does the complication of two rate constants appear but also displacement reactions are possible (97) during the later phases of the reaction. If at all possible, probe nucleic acids should be made at least the same length if not slightly larger than the bulk DNA in order to minimize the problems described above, which lead to apparent deviations from normal kinetic behavior.

**Separation of Native and Denatured Nucleic Acids**

At various points in a tracer study, it is necessary to decide how much of the probe nucleic acid is native and how much is denatured. With the fluorescent probe method (77), the proportion of native and denatured DNA may be determined from the polarization of the fluorescence. This method, however, has not yet been shown to be as sensitive as radioactive probe methods. When a radio-tracer is used, a separation method is required to isolate all of either the native or denatured nucleic acid at each step. Hydroxyapatite chromatography is a standard method. Martinson (104–106) has studied the chromatographic parameters. A description of detailed methods for application to renaturation studies, including pitfalls which may be avoided, is given in the methods paper by Britten et al (12). Other methods are also described in some detail, including the useful methods of Cs$_2$SO$_4$ density gradient sedimentation and enzymatic digestion of denatured nucleic acids. Cs$_2$SO$_4$ density gradient sedimentation may be used to separate DNA·RNA hybrids from other nucleic acids and to isolate hybrid DNA molecules with one iodinated strand (92). Ribonuclease has been used for years for digesting denatured RNA. Endonuclease S1 from *Aspergillus oryzae* may be used to degrade denatured DNA. Leong et al (107) found the S1 nuclease method plus TCA precipitation to be equivalent in sensitivity to hydroxyapatite chromatography. It is our experience that better data are obtained if Sephadex G-100 is used to separate the native DNA from the oligonucleotides following enzymatic digestion (C. L. Sprouse and J. G. Wetmur, unpublished observation). All fractions are then counted.

The rate constants obtained by enzymatic digestion procedures are the same as those obtained using optical methods. With hydroxyapatite chromatography or other methods that lead to isolation of partially native molecules, more radioactivity
is found per nucleation event. As pointed out above, the larger renaturation rate constants obtained by hydroxyapatite chromatography may be converted to optical rate constants using the equations of Miller & Wetmur (44).

**Applications**

A detailed discussion of the application of tracer methods to biological problems is beyond the scope of this review. A number of specific eukaryotic messages have been studied, some of which have been found to be the products of multiple gene copies (108). The reader is referred to studies of immunoglobin messages for examples of state of the art studies (109, 110). Similarly, many studies have involved tumor virus probes. The reader is referred to experiments with three different viral systems for examples of state of the art studies (101, 111, 112). Finally, renaturation and hybridization kinetics have been applied to the problem of the determination of the structure of eukaryotic chromosomes and their transcription products. Two reviews are available on these subjects (113, 114). For studies involving specific eukaryotes, the reader is referred to a series of recent papers in the field (115–120) that include the most modern kinetics techniques developed for studying these specific problems.

**SUMMARY**

Except for the special cases of filter or other solid support hybridizations and certain repeated sequence renaturations discussed above, the rate constant for renaturation or hybridization may be described using equation 19. $k_2$ depends on the measurement method, as described above, but measurements made using different techniques may be compared without difficulty. $k_N$ is a function of criterion, solvent composition, and mismatching as described above. Optimum $k_N$ values in 0.4 M NaCl for synthetic oligonucleotides (20), homopolymers (24, 25), and complex nucleic acids (38) without mismatching are given in Table 1. All of the $k_N$ values are the same within a factor of 4, with complex DNA having the highest value.

**Table 1** Length-independent nucleation rate constants

<table>
<thead>
<tr>
<th>Polymer</th>
<th>T (°C)</th>
<th>$10^5 k_N$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA + DNA (50% GC)</td>
<td>67</td>
<td>1.7</td>
</tr>
<tr>
<td>DNA + RNA (42% GC)</td>
<td>67</td>
<td>1.3</td>
</tr>
<tr>
<td>A$_5$U$_6$ + A$_5$U$_6$</td>
<td>30</td>
<td>1.5</td>
</tr>
<tr>
<td>A$_7$U$_7$ + A$_7$U$_7$</td>
<td>30</td>
<td>1.7</td>
</tr>
<tr>
<td>Poly A + Poly U</td>
<td>30</td>
<td>0.5</td>
</tr>
<tr>
<td>Poly A + Poly dT</td>
<td>42</td>
<td>1.2</td>
</tr>
<tr>
<td>Poly dA + Poly U</td>
<td>21</td>
<td>1.2</td>
</tr>
<tr>
<td>Poly dA + Poly dT</td>
<td>48</td>
<td>1.2</td>
</tr>
<tr>
<td>Poly G + Poly C</td>
<td>90</td>
<td>0.5</td>
</tr>
<tr>
<td>Poly G + Poly dC</td>
<td>88</td>
<td>0.65</td>
</tr>
<tr>
<td>Poly dG + Poly C</td>
<td>88</td>
<td>0.5</td>
</tr>
<tr>
<td>Poly dG + Poly dC</td>
<td>78</td>
<td>0.5</td>
</tr>
</tbody>
</table>
of all. This comparison shows that the sequence complexity of DNA has no inhibitory effect on $k'_n$. The lower $k'_n$ values for the homopolymers may be the result of structure formation in these repeating polymers, especially the purine-containing polymers, which interferes to some small degree with nucleation. Craig et al. (20) find a rate constant, $k_2$, of about $7 \times 10^4$ liters/mole-sec at 30°C for both A6U6 and A7U7 self-association in 0.25 M NaCl using nucleotide concentrations. After correcting for complexity and length (which are equal here), $k'_n$ is found to be about 2.4 and $2.6 \times 10^5$, respectively. After correcting to 0.4 M NaCl (multiply by 1.6) and nonpermuted products (divide by 2.5), one obtains values of $k'_n$ of 1.5 to $1.7 \times 10^5$. The fact that these values are higher than those obtained for polyadenylic plus polyuridylic acid may reflect the decreased electrostatic repulsion between strands, which might be expected, as described above, for oligonucleotide reactions. Nevertheless, the data for short oligonucleotides are in remarkable agreement with the data for polymers. The general conclusion is that equation 19 has almost (excluding certain repeated sequences) universal validity for describing the solution kinetics of hybridization and renaturation of nucleic acids, including the solution-tracer experiments which are most commonly performed to obtain data of biological relevance.

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