STUDIES ON THE KINETICS OF RENATURATION OF DNA

Thesis by

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Dedicated to my wife, Bridie

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ABSTRACT

A temperature jump system is described for studying fast DNA renaturation reactions. The reaction is found to be second order as seen in the time course of the reaction and in the concentration dependence of the rate. The stepwise base-pairing model of Saunders and Ross is extended to allow varying DNA base composition. Rate constants calculated with this model are compared with experimental rate constants at varying temperatures.

T4 and T7 DNAs were fragmented by various procedures. The molecular weight of denatured DNA was determined by alkaline sedimentation. For a given DNA, fragmented into different molecular weights, the rate of renaturation is found to be proportional to the square root of the molecular weight. The rate of renaturation of DNA was measured in sucrose, glycerol, ethylene glycol and sodium perchlorate solutions. The melting temperature of DNA is changed by different amounts in each solvent. Nevertheless, the rate constant multiplied by the viscosity and divided by the renaturation temperature is found to be a constant. Thus, the rate determining step must be hydrodynamically limited.

The complexity of the DNA of an organism is defined as the total DNA complement of the organism. The rates of renaturation of SV40, T7, N1, T4, E coli and Ascites tumor DNA (non-repeated sequences) are inversely proportional to the complexity. After complexity correction, the rate of renaturation is found to depend

slightly on the GC content of the DNA. The stepwise renaturation model predicts this result.

A method is described for positively staining electron microscope grids prepared by the method of Kleinschmidt and Zahn.

Some properties of N1 DNA are described. The DNA has a buoyant density corresponding to 64% GC, a molecular weight of 33×10^6 and the property of reversible cyclization like lambdoid phage DNAs.

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CHAPTER 1

The Renaturation of DNA as a Second Order Reaction

Contents:

A. Introduction

The subject of the kinetics of renaturation of DNA was extensively reviewed by Marmur, Rownd and Schildkraut (1963). They also discussed the denaturation of DNA and the factors contributing to the relative stabilities of the helical and coil forms of DNA. The important facts, known at the time of this review, concerning the kinetics of renaturation of DNA are as follows:

1. The renaturation reaction is second order in DNA phosphate concentration.

2. The maximum rate of renaturation occurs at a renaturation temperature of 20 - 30°C. below the melting temperature (T_m) of the DNA.

3. Decreasing the molecular weight of the DNA results in a decrease in the rate of renaturation of the DNA.

4. The rate of renaturation decreases as the genetic complexity of the source of the DNA increases.

5. The rate of renaturation is very dependent on ionic strength below 0.4 M. sodium ion and is almost independent of salt concentration above this ionic strength.

Experimental methods for studying the physical properties of DNA and the rate of renaturation of DNA were discussed. The related problem of hybridization was considered in relation to the problem of renaturation of DNA.

Since the publication of this review, more data about the same subjects has been presented by Subirana and Doty (1966), Subirana (1966),

and Thrower and Peacocke (1966). The new finding in these papers seems to be that the rate of renaturation of DNA depends on the viscosity of the solvent.

A group of workers at the Carnegie Institution of Washington (Bolton, Britten, Cowie, Roberts, Szafranski, and Waring, 1965; Britten and Kohne, 1966; Waring and Britten, 1966; Britten and Kohne, 1967) have shown that the dependence of the rate of renaturation on DNA source is an inverse proportionality between rate and complexity. Complexity will be precisely defined in Chapter 4. These authors degraded all DNAs, used for comparison, to the same single strand molecular weight prior to denaturation.

In the first six chapters of this thesis, we shall consider the same factors as those in the Marmur, Rownd and Schildkraut (1963) review. In addition, in Chapter 5, between the chapter on the effects of source or complexity and the final chapter on solvent effects (including salt effects), we shall consider the effect of GC content on the rate of renaturation of DNA. We shall provide quantitative relations which permit the correlation of data taken in various high salt solutions and in solutions of differing viscosities or anions. We shall also demonstrate that the rate of renaturation is proportional to the square root of the molecular weight and verify the results of the Carnegie group concerning complexity effects over a range of complexities of 10^6. A theoretical model is proposed to explain the temperature and GC effects and to provide a basis for the consideration of complexity, molecular weight and viscosity effects. Excluded volume effects, intra-molecular

aggregation effects and inter-molecular aggregation effects are considered in terms of the mechanism of the renaturation reaction. Thus a possible theoretical foundation is provided to explain all the various effects of DNA and solvent parameters on the rate of renaturation of DNA.

Chapter 7 deals with electron microscopic observations of DNA. A review of theoretical and practical considerations is made, and some new results are presented. These results include improved methods of mounting and visualizing DNA.

Chapter 8 deals with the physical properties of the DNA of the bacteriophage N1 (host M. lysodeikticus). The DNA exhibits the same reversible cyclization as does λ DNA. A review of lysogenic phage DNAs is included in this chapter.

One of the electron microscopic procedures was used for preparation of electron micrographs presented in the chapters on renaturation kinetics. The DNA of N1 phage was employed in the study of the GC dependence of the kinetics of renaturation. In both cases results are stated in the chapters on renaturation kinetics that are not explained until the final two chapters. Independent historical introductions are given at the beginning of Chapter 7 and of Chapter 8.

B. Preparation of Bacteriophages and DNAs

T4 phage used for this work were obtained from two sources. The first source was a gift by Professor W. Dreyer of an acid (pH = 3.9) precipitated wet pack of T4 phage plus bacterial debris. The second source was growth of T4 phage on E coli B/5 in Hershey medium (Steinberg and Edgar, 1962). T4 phage were purified by differential centrifugation in 0.2 F. NaCl, 0.01 F. $MgCl_2$, 0.01 F. Tris OH plus HCl, pH 7.8. Alkaline band velocity sedimentation (Vinograd, Kent, Bruner and Weigle, 1963; Studier, 1965) showed that the freshly grown phage contained fewer single strand breaks in their DNA than the wet pack preparation.

T7 phage were grown on E coli B/5 by the method of Davison and Freifelder (1962a). After purification by differential centrifugation, T7 phage were further purified by banding in CsCl of density 1.50 plus 0.01 F. $MgCl_2$, 0.01 F. Tris OH plus HCl, pH 7.8 (Vinograd and Hearst, 1962).

N1 phage, grown on Micrococous lysodeikticus by the method of Scaletti and Naylor (1959), were a gift of Professor J. V. Scaletti. These phage were purified by the same procedure as T7 phage.

DNA was extracted from T4, T7, and N1 phage by the same procedure. Phenol, distilled under nitrogen, was stored at -20° C. Before use, this phenol and ether were equilibrated with 0.2 F. NaCl, 0.01 F. EDTA, 0.01 F. Tris OH plus HCl, pH 7.8. After extraction with phenol (Mandell and Hershey, 1960), the DNA was extracted repeatedly with ether (Davison and Freifelder, 1962b) until no phenol

absorbance was seen in the U. V. spectrum of the DNA solution.

E coli DNA was purchased from General Biochemicals, Inc. The DNA was dissolved and deproteinized by adding aqueous salt solution and chloroform and rocking the mixture for five days in the cold. The aqueous phase was centrifuged at 4000 rpm in a Sorvall Superspeed Centrifuge to remove DNA aggregates and aggregated proteins.

Ascites tumor DNA was a gift of Mr. C. S. Lee. It was prepared from isolated nuclei.

SV40 DNA was a gift of Mr. W. Bauer and Professor J. Vinograd.

Visking dialysis tubing was prepared for use by boiling twice for 15 min. in 0.1 F. EDTA, pH 7.8 and repeatedly in distilled water, each time rinsing the inside of the tubes with redistilled water. Tubing was rinsed and reboiled just before use.

Most DNA solutions were dialyzed in the cold against 0.875 F. NaCl, 0.01 F. EDTA, pH 7.8 and then exhaustively against 0.875 F. NaCl, 0.0002 F. EDTA, 0.005 F. Tris OH plus HCl, pH 7.8 or 0.0002 F. EDTA, 0.01 F. phosphate, pH 6.8 - 7.0. The solutions used for renaturation studies at salt compositions other than 1.0 F. sodium ion were dialyzed against the appropriate salt concentrations with one of the above buffers. All dialysis solutions and DNA solutions were maintained saturated with chloroform.

DNA solutions were stored frozen at -20° C. A solution containing T4 DNA, A_{260} = 2.0, 0.1 F. NaCl, 0.0002 F. EDTA, 0.005 F. Tris OH plus HCl, pH 7.8 was analyzed by neutral band velocity sedimentation

before and after freezing and thawing five times. This treatment resulted in damage to less than 20% of the molecules. $S_{20,w} = 65$ in both cases.

C. Denaturation of DNA

Two methods, alkaline denaturation and thermal denaturation, were used to denature DNA prior to following the renaturation reaction. Thermal denaturation has been previously employed to prepare DNA solutions for study of renaturation kinetics (Marmur and Doty, 1961; Subirana and Doty, 1966). The milder method of alkaline denaturation has been used in sedimentation velocity studies of high molecular weight DNAs (Studier, 1965) and for denaturation and subsequent renaturation of large molecules in preparation for electron microscopy (Thomas and MacHattie, 1964).

Alkaline denaturation was the method of choice for DNA solutions in which the DNA had a single strand molecular weight greater than 3×10^6. Thermal denaturation could be used for DNAs sheared into smaller size without danger of producing molecular weight changes through degradation. The process of degradation is discussed in some detail in Chapter 3.

The following method was used for DNA denaturation: One part of 1.0 F. NaOH was added to 8 parts of a DNA solution in 0.875 F. NaCl, 0.0002 F. EDTA with either 0.005 F. Tris OH plus HCl, pH 7.8 or 0.01 F. phosphate, pH 6.8. After 10 minutes at room temperature to assure denaturation, the solution was cooled to 0°C. One part of 2.0 F. NaH_2PO_4 was then added at 0°C. to neutralize the solution. If DNA solutions denatured in this manner were kept cold and if the renaturation reactions were studied within an hour, little or no renaturation occurred before increasing the temperature to the region of the renaturation

temperature. The final solution was 1.0 F. in sodium ion and 0.2 F. in phosphate buffer at pH 6.8. The use of NaH_2PO_4 for neutralization prevented local regions of high acidity in which DNA degradation might occur. Phosphate buffer is better than many other buffers as it is optically stable in the U.V. at high temperatures. A more important aspect of the use of alkaline denaturation is that alkaline band or boundary velocity sedimentation experiments were routinely performed to measure the molecular weight of the DNAs. Use of alkaline denaturation assures one that the molecular weight measured by sedimentation is the same as the molecular weight in solution. When this procedure was used, velocity sedimentation experiments performed on samples taken before and after renaturation experiments gave the same alkaline sedimentation coefficients.

When thermal denaturation was used, the solution was then cooled directly to the renaturation temperature for observation of the rate of renaturation. The standard denaturation procedure was to heat a DNA solution to 5° C. above the maximum of the melting region for ten minutes. Consistent results, including consistent alkaline sedimentation results, were obtained for DNAs sheared to a double strand molecular weight of 6×10^6 or lower. All methods of thermal denaturation employed broke larger DNAs into pieces of about this size (in terms of single strand length).

D. Optical Methods for Following Renaturation

The processes of denaturation and renaturation result in a change in the ultraviolet absorbance of DNA (Marmur and Doty, 1961) due to changes in the stacking of the DNA bases. Thus the degree of renaturation, where long sequences of DNA are either denatured or in register (Rich and Tinoco, 1960), may be followed by observing the absorbance of a DNA solution as a function of time.

Two types of equipment were used in this work for following the course of the renaturation reaction. The first, a modified temperature jump system used in conjunction with a Cary 14 spectrophotometer, was used for relatively fast renaturation reactions. The fastest half time observed was seven seconds. Solutions which have been denatured by alkali and kept cold to prevent renaturation can be rapidly heated to the renaturation temperature and maintained at this temperature. The second system involved using a Beckman 4 cell holder in a compartment maintained at temperature by a Haake bath. This system, for which the rate of temperature change is slower, could only be used for following slow renaturation rates.

In both systems, the DNA solutions were flushed with helium before heating to prevent the formation of bubbles of air which occur when air saturated solutions are heated. Helium becomes more soluble in water with increasing temperature above 30° C. and has only a small temperature coefficient of solubility in the range 0° - 80° C.

1. A Modified Temperature Jump System

A 1 cm. quartz cell was fitted with a machined teflon top, two platinum plates with platinum leads, a thermistor with insulated leads and a polyethylene coated stirring bar as shown in Figure 1-1. The openings for the leads were drilled through the fitted teflon top. The platinum plates were used in applying heating current to the solution in the cell, and the thermistor was used to monitor the temperature of the solution. As stirring facilitates temperature uniformity throughout the cell, moderate stirring was used for almost all rate measurements.

A box to hold the Cary cell was constructed as per Figure 1-2. The cell holder was isolated from the environment by polyurethane. The stirring motor was built into the box. The box mounts to the top of the sample compartment of a Cary 14 spectrophotometer in a reproducible manner. A system was available to pass air from a coil in a constant temperature bath through the cell holder compartment. This provided a means of slowly adjusting the ambient temperature before starting temperature jump experiments. If the air heating system were used, then less electrical power was needed to maintain elevated temperature in the cell. The power circuit is diagrammed in Figure 1-3a. The wave form is supplied by a Hewlett Packard Test Oscillator 650A and amplified by a Dynakit Mark III amplifier modified in the output stage to match the cell impedence at various salt concentrations. A frequency of 30 Kc was used to prevent electrolysis in the cell. A helipot precision potentiometer, R = 20, 000 ohms, was used to adjust the power necessary to maintain constant temperature in the cell.

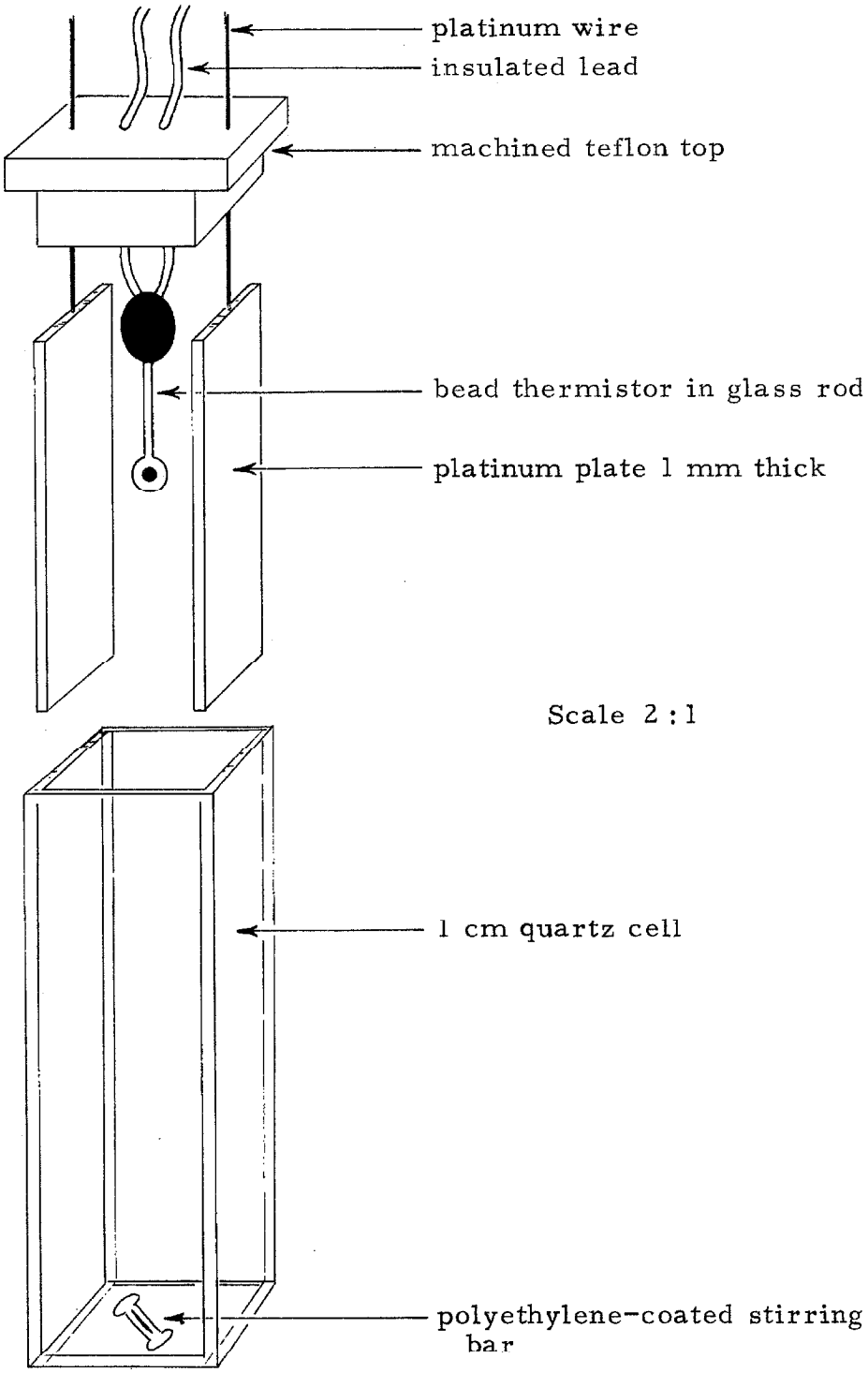


Figure 1-1. Cell used with modified temperature jump system.

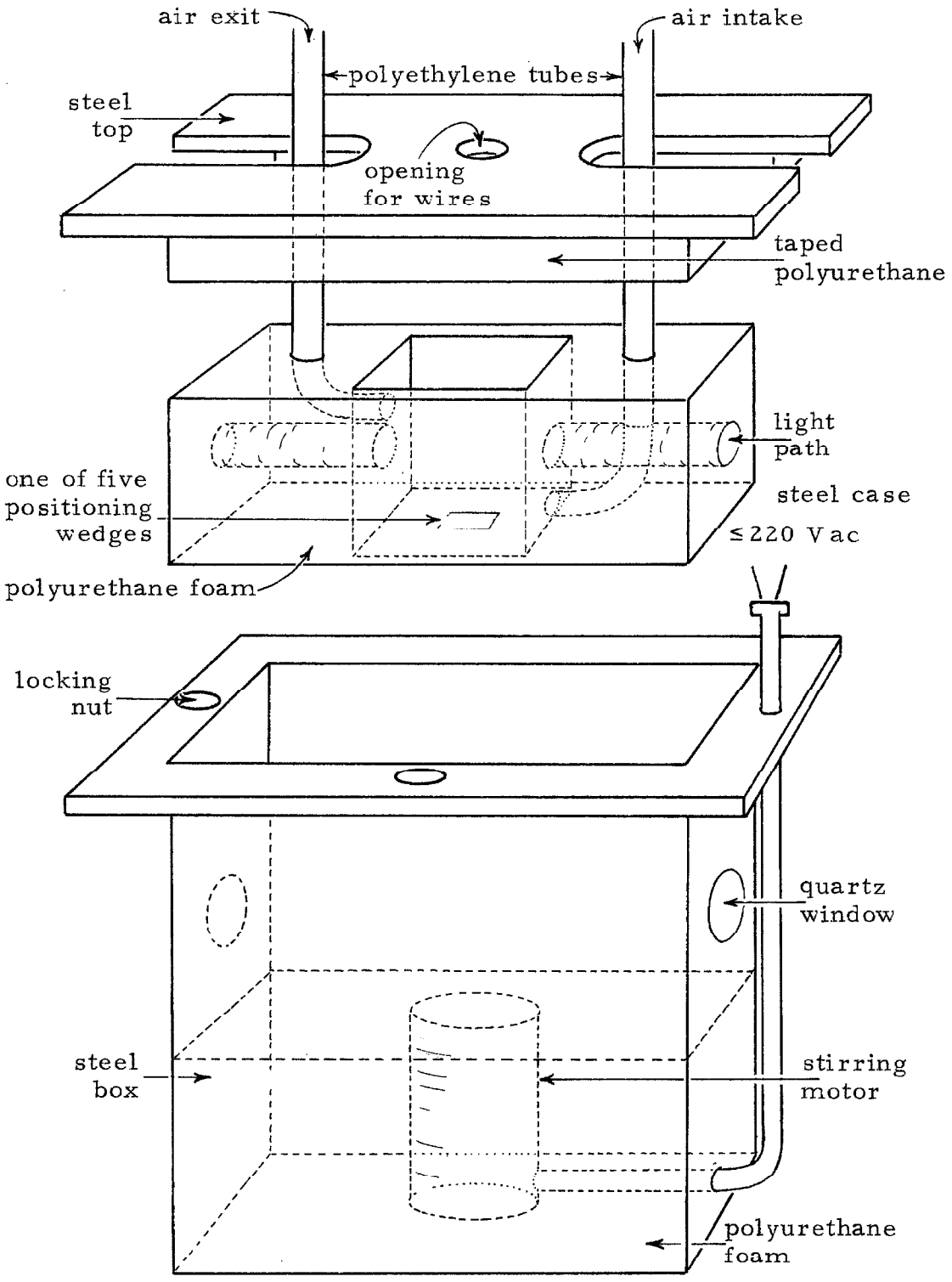


Figure 1-2. Box for temperature jump apparatus

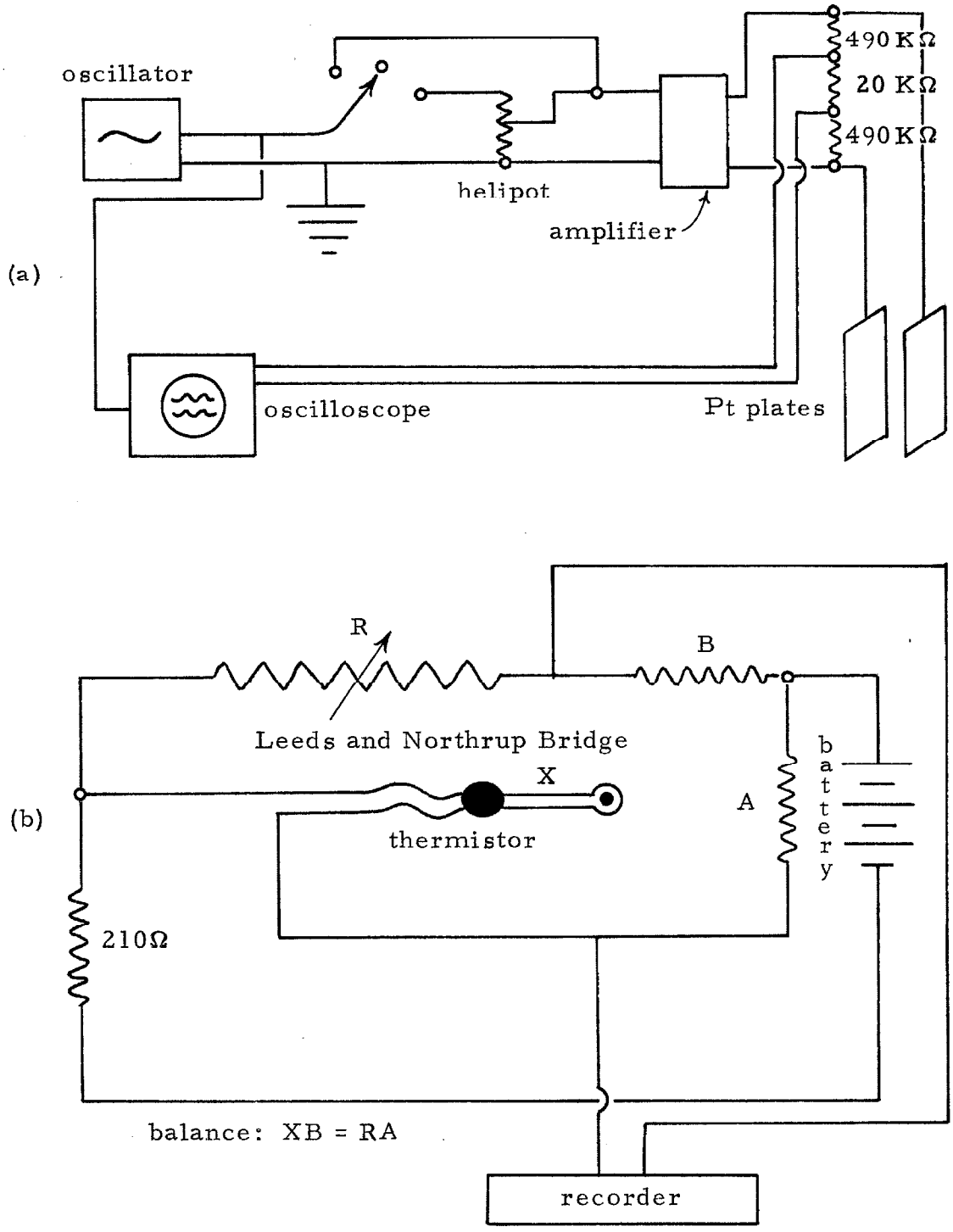


Figure 1-3. (a) Power circuit. (b) Temperature measurement circuit.

The output of the oscillator and amplifier was monitored with a Tektronix type 502 Dual Beam Oscilloscope. The maximum output of the amplifier was 625 volts or 60 watts, whichever was highest. A 1.5 ml. solution of moderate or high salt ($\geq$ 0.004 F. NaCl) may be heated at greater than three degrees per second without distortion of the output signal of the amplifier.

The temperature measuring system is shown in Figure 1-3b. A VECO Model 61A7 of T61A18 "general purpose bead thermistor in glass probe with extension leads" with a resistance of 1 megohm and a change of resistance of 5%/degree at 25° C. was used to monitor the temperature. The response time is less than one second. 22 volts across the thermistor results in an error of less than 0.1 degree. A 22.5 Volt Burgess 5156 battery was used to power the temperature monitoring system. All values were recorded with a Varian Model G-14 Strip Chart Recorder with minimal current flow across the recorder. On the 100 mv. scale, about 6° C. was full scale. The response time of the recorder is comparable to that of the thermistor.

A Cary Model 14 recording spectrophotometer was used to follow the optical density of the solutions. In no case did we approach the response time of the Cary. All work was done at the maximum of the DNA absorption spectrum. For dilute DNA solutions, a 0.1 - 0.2 O.D. slidewire was employed.

2. Gilford Type Systems

A Gilford Model 2000 Multiple Sample Absorbance Recorder with thermostated cell compartment was used for simultaneous measurement

of three sample absorbances relative to a blank and for measurement of temperature. The temperature was controlled with a Haake bath. The absorbance was zeroed on each sample such that the minimum (native) to maximum (denatured) absorbance range occupied most of the full scale.

The samples were melted at $\frac{2}{3}$° C. /minute until the range of melting was well passed and cooled as quickly as possible to the renaturation temperature. This process of cooling takes a minimum of seven minutes, so reactions could only be studied whose half times were of the order of 14 minutes or longer. For a second order reaction, the half time is inversely proportional to the initial concentration of reactants. In this particular case, if a sample had an initial absorbance of 0.2, rate constants faster than 90 liters/mole-second would be difficult to measure. We shall see that this limits one to sheared DNAs of complexity greater than or equal to that of T4 DNA.

A 4 position thermostated cell holder analogous to that of the Gilford system was constructed for use in a Cary 14 spectrophotometer. Similarly slow rates could be measured in an analogous manner. With 2.5 mm. width cells, solutions treated with alkali and neutralized in the cold could be delivered into the cells at T_m - 15° C. and the renaturation followed while the ambient temperature was cooled to T_m - 25° C. In this case, half times of the order of three minutes were within range. This method was not, however, employed if sufficient material existed for use of the modified temperature jump system.

E. The Second Order Renaturation Reaction

The renaturation reaction, in terms of moles of DNA phosphate, may be written as

$$P + P' \xleftrightarrow{k_2} PP' \qquad \text{where } (P) = (P')$$

If $P_T = 1.4 \times 10^{-4} A_{260,\,\text{native}}$ = total DNA phosphate in solution, and $A_\infty = A_{260,\,\text{native}}$,

$$(P) = \tfrac{1}{2} \frac{A - A_\infty}{A_0 - A_\infty} P_T$$

where A_0 = absorbance of DNA when all bases are unpaired (denatured state), and A = absorbance of DNA when $(P)/(P_T)$ is the fraction of unpaired bases in solution. If the renaturation reaction obeys second order kinetics so that

$$-\frac{d(P)}{dt} = k_2 (P)^2,$$

$$\text{then} \quad -\frac{d\frac{(A - A_\infty)}{(A_0 - A_\infty)} P_T}{2\, dt} = \frac{k_2}{4} \frac{(A - A_\infty)^2}{(A_0 - A_\infty)^2} P_T^{\,2}$$

Integrating from t = 0 to t = t,

$$-\int_{A_0}^{A_t} \frac{dA}{(A - A_\infty)^2} = \frac{k_2 P_T}{2 (A_0 - A_\infty)} \int_0^t dt$$

$$\text{thus} \quad \frac{A_0 - A_\infty}{A_t - A_\infty} = \frac{k_2 P_T t}{2} + 1$$

The absorbance data may be plotted as $(A_0 - A_\infty)/(A - A_\infty)$ versus time. The slope multiplied by 2 and divided by P_T would then be the second order rate constant.

All of our melting data, within an experimental error of 5%, are consistent with the hyperchromicity factor,

$$A_0 - A_\infty = 0.36\, A_\infty$$

Thus, $$\frac{1}{A_t - A_\infty} = k_2\, 1.95 \times 10^{-4}\, t + \frac{1}{0.36\, A_\infty}$$

One may thus plot $1/(A - A_\infty)$ versus time and divide the slope by 1.95×10^{-4} to obtain the second order rate constant.

It is necessary to know either A_0 or A_∞ to obtain the rate plot. With the Gilford type system, both A_0 and A_∞ are known, unless denatured material is added to the cells as is the case with the temperature jump system. In the latter cases, A_0 is obtained by melting the DNA after the renaturation reaction. The relation $A_\infty = A_0/1.36$ may be used to determine A_∞ to within experimental error. Melting curves were routinely obtained after all renaturation experiments performed.

Sample results from the modified temperature jump apparatus and the Gilford apparatus appear in Figure 1-4 and 1-5 respectively. A data plot of the results of the renaturation observed in Figure 1-4 appears in Figure 1-6. Note that the second order rate law is observed throughout the major part of the reaction.

Figure 1-4

A Renaturation Experiment with the Temperature Jump Apparatus

The absorbance of a solution of T4 DNA as a function of time. A_{260}, native = 1.02. The DNA was rapidly heated from 0°C. to 67°C. (T_m - 25°C.) and kept at 67°C. while the DNA renatured. The temperature was then slowly elevated to 100°C. The DNA melted. The temperature was lowered as rapidly as possible to T_m - 25°C. and kept constant. The DNA again renatured with the same rate.

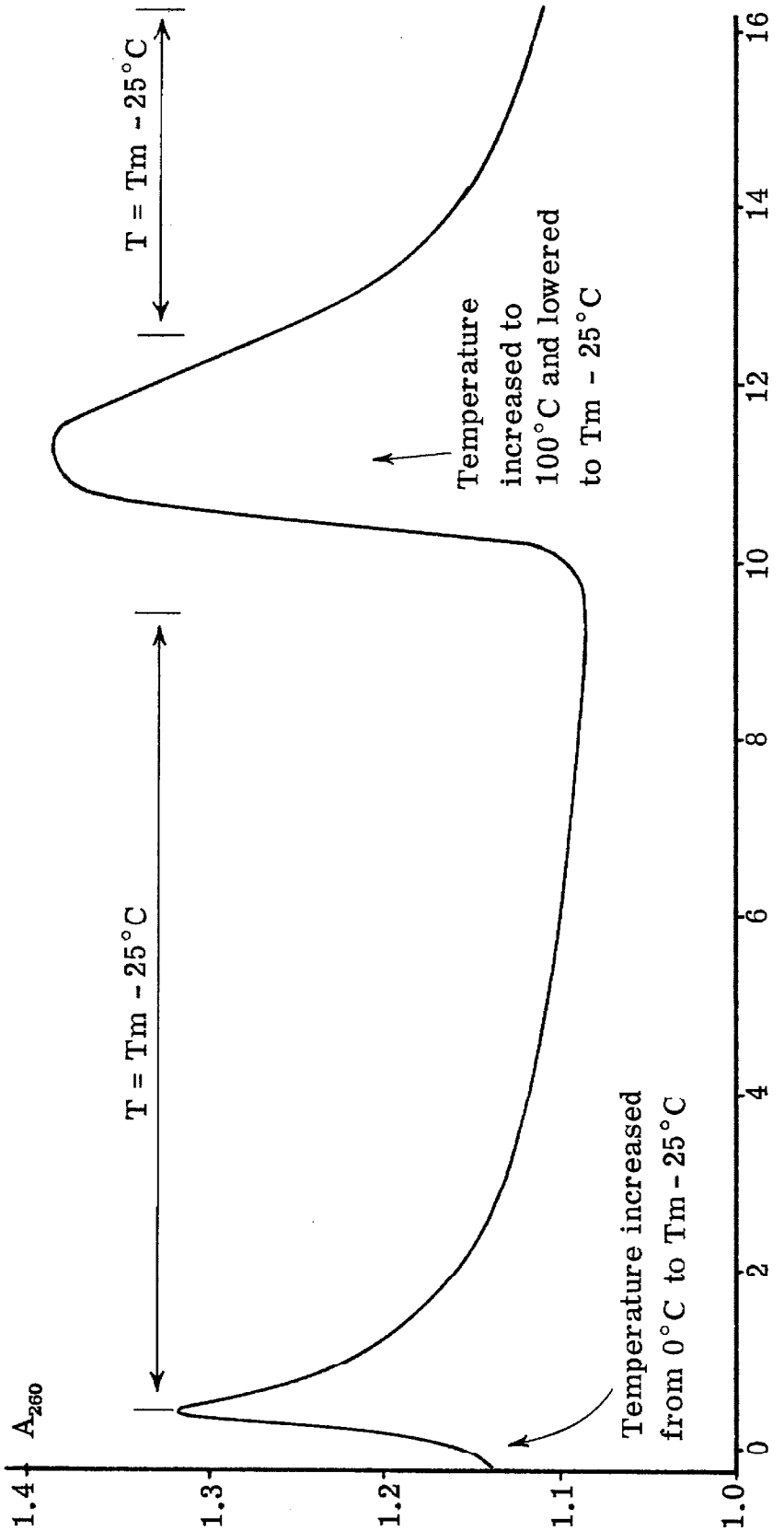

A_{260}
1.4
1.3
1.2
1.1
1.0
0
2
4
6
8
10
12
14
16
T = Tm - 25°C
T = Tm - 25°C
Temperature increased from 0°C to Tm - 25°C
Temperature increased to 100°C and lowered to Tm - 25°C

Figure 1-5

A Renaturation Experiment with the Gilford System

The absorbance of a solution of T4 DNA in 0.15 NaCl as a function of time. The DNA was melted from completely native to completely denatured. The temperature was lowered as quickly as possible to 60° C. and kept at 60° C. while the DNA renatured.

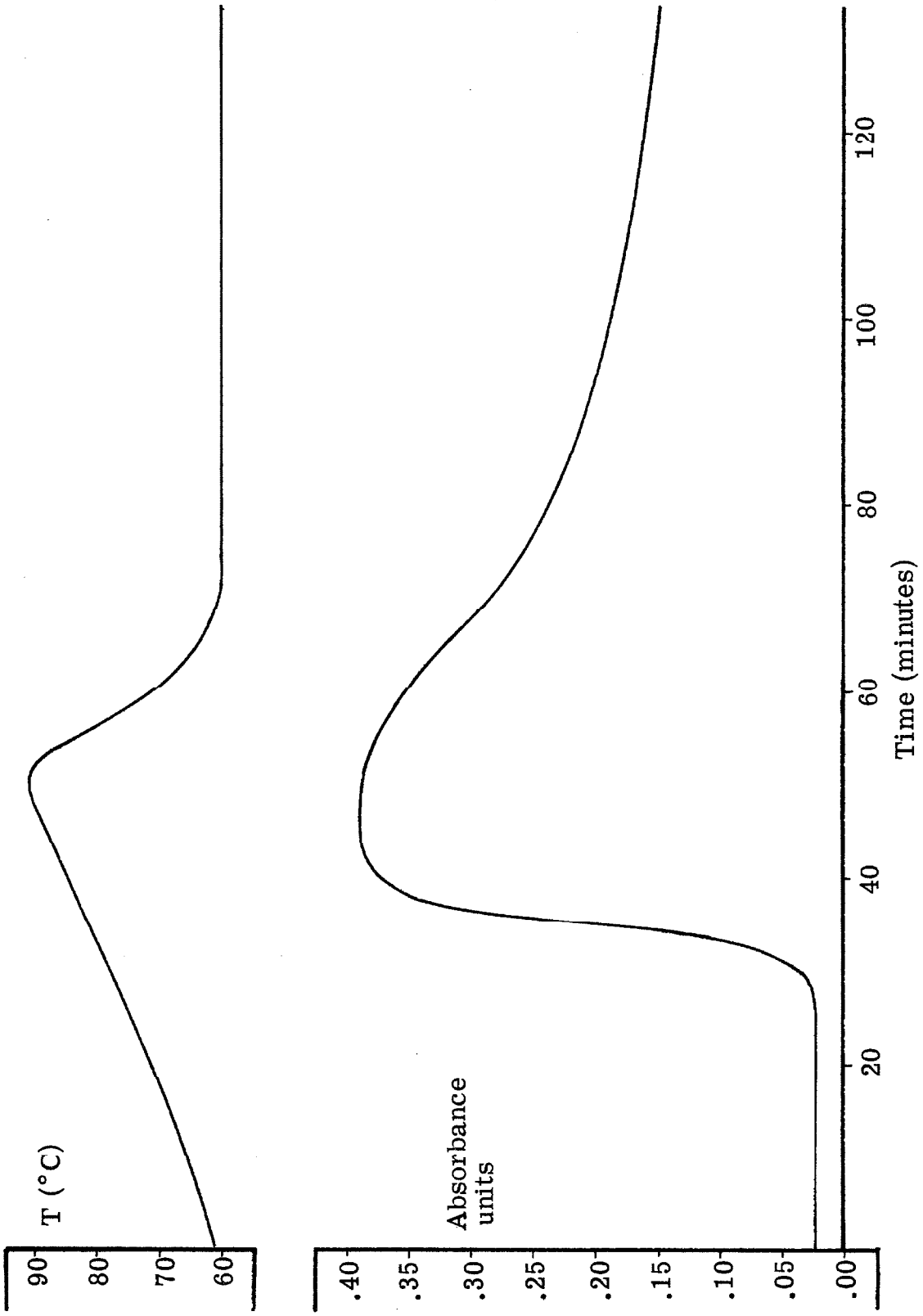
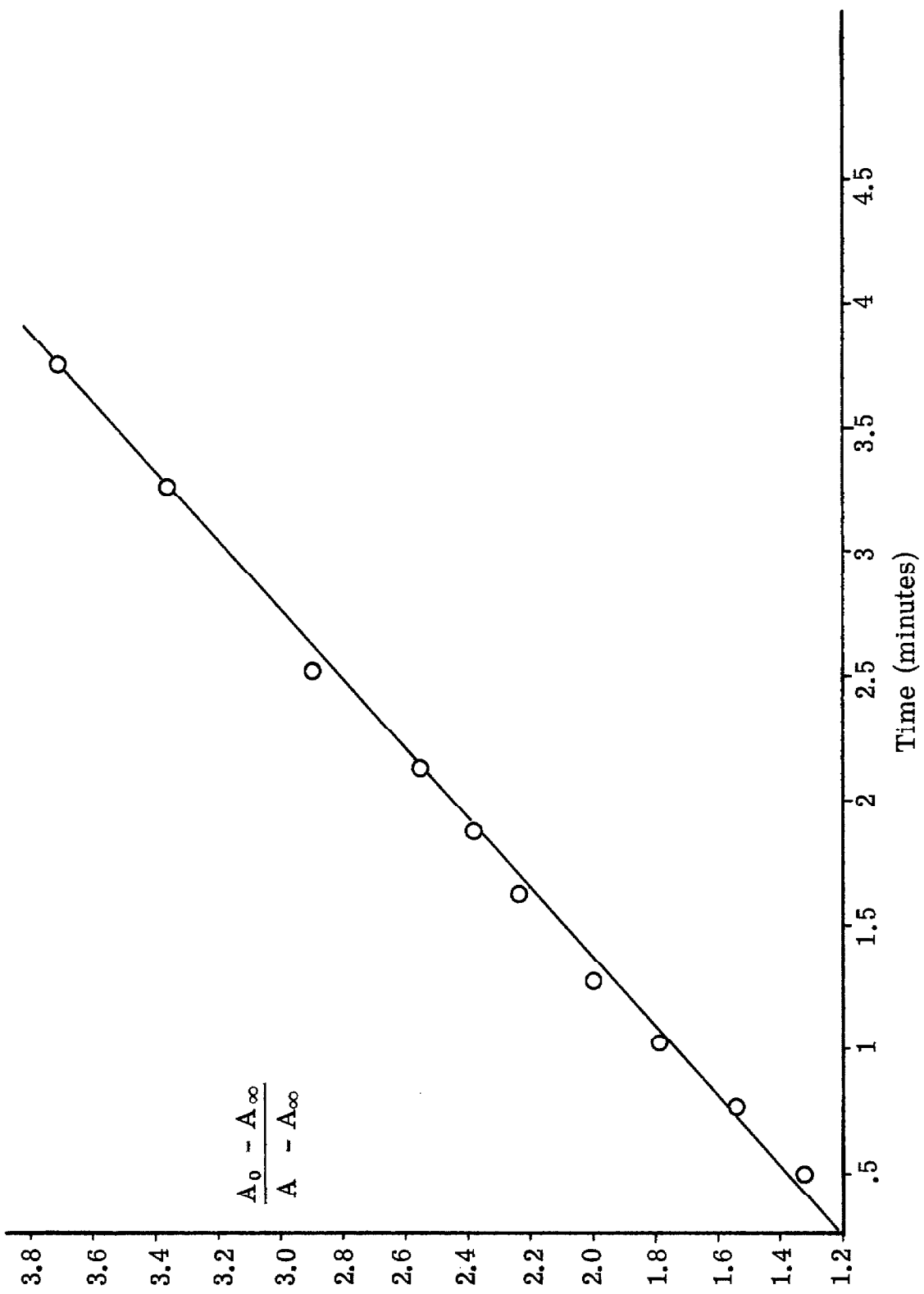

T (°C)
90
80
70
60
Absorbance units
.40
.35
.30
.25
.20
.15
.10
.05
.00
20
40
60
80
100
120
Time (minutes)

Figure 1-6

A Second Order Rate Plot for Renaturation of T4 DNA

The plot shown is for the reaction which was described in Figure 1-4. The slope of the plot is 1.18×10^{-2} sec^{-1}.

$$k_2 = \frac{2 \times \text{Slope}}{P_T} = \frac{2 \times 1.18 \times 10^{-2}}{1.02 \times 1.4 \times 10^{-4}} = 165 \text{ liters/mole-sec}$$


$\frac{A_o - A_\infty}{A - A_\infty}$
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
.5
1
1.5
2
2.5
3
3.5
4
4.5
Time (minutes)

F. Concentration Effects on the Rate of Renaturation

Concentration effects on the rate of renaturation have been studied by Subirana and Doty (1966), who found that the renaturation reaction approximately obeys second order kinetics. We have subsequently checked the second order nature of the reaction in 1.0 F. sodium ion at $T_m - 25°$ C. Tables 1-1 and 1-2 show results for T4 DNA of a single molecular weight and E coli DNA of a single molecular weight at various DNA concentrations. In both cases, there is a slight decrease in the second order rate constant upon increasing the concentration of DNA, but not a very marked decrease. The more exact T4 DNA results are averages of a large number of experiments. The straight line second order rate plot (Figure 1-6) and the independence of concentration observed for the second order rate constant are both in agreement with a second order reaction.

TABLE 1-1

Rate of Renaturation of T4 DNA versus Concentration

Concentration (μg/ml)	Rate Constant (liters/mole-sec.)
62	113
31	116
15.5	125
7.75	131

TABLE 1-2

Rate of Renaturation of E coli DNA versus Concentration

Concentration (μg/ml)	Rate Constant (liters/mole-sec.)
175	5.2
87	4.6
56	5.3
23	4.8
11.5	5.7

In connection with a study of the effect of molecular weight (sedimentation coefficient) on the rate of renaturation, an additional check of the effect of concentration was obtained for T4 and T7 DNAs. The results appear in Figures 1-7 and 1-8 respectively. There appears to be a small increase in the rate constant of renaturation of T4 and T7 DNAs with decreasing concentration, comparable to the effects seen in Tables 1-1 and 1-2. The dependence of the rate of renaturation on molecular weight will be discussed in Chapter 3. The important fact is that the alteration of concentration does not effect the molecular weight dependence of the rate of renaturation.

Although there may be a small deviation from second order kinetics as seen by decreasing rate constants with increasing concentration, the deviation is not large. Thus the reaction obeys second order kinetics quite well over a moderate range of concentrations, molecular weights and DNA complexities.

Figure 1-7

The Rate Constant for Renaturation of T4 DNA as Function of Alkaline Sedimentation Velocity

O: 70 μg/ul

■: 7 μg/ul

The dependence of rate constant on alkaline sedimentation velocity is not altered by a ten-fold change in concentration.

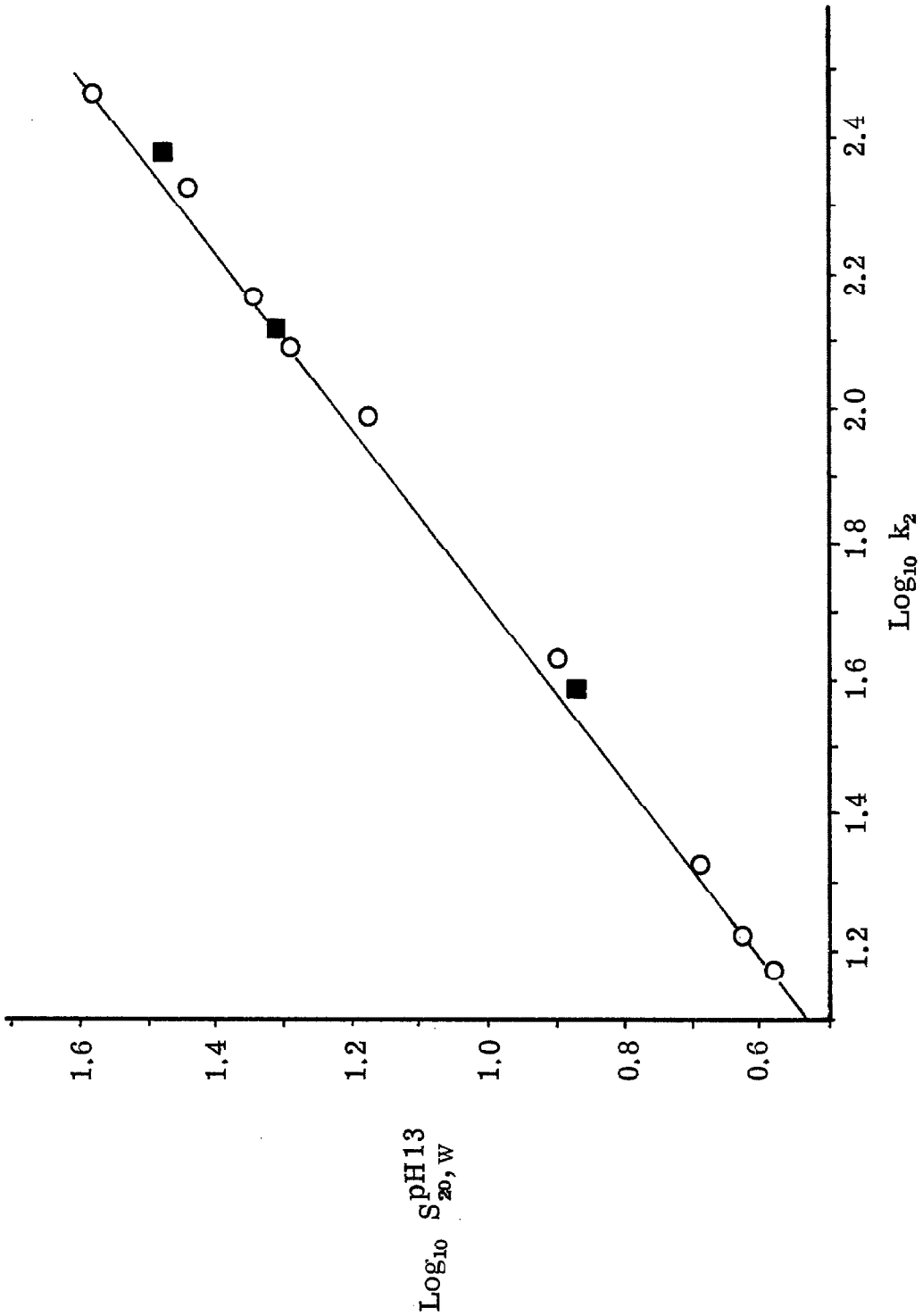

1.6
1.4
1.2
1.0
0.8
0.6
Log10 S20,W pH13
1.2
1.4
1.6
1.8
2.0
2.2
2.4
Log10 k2

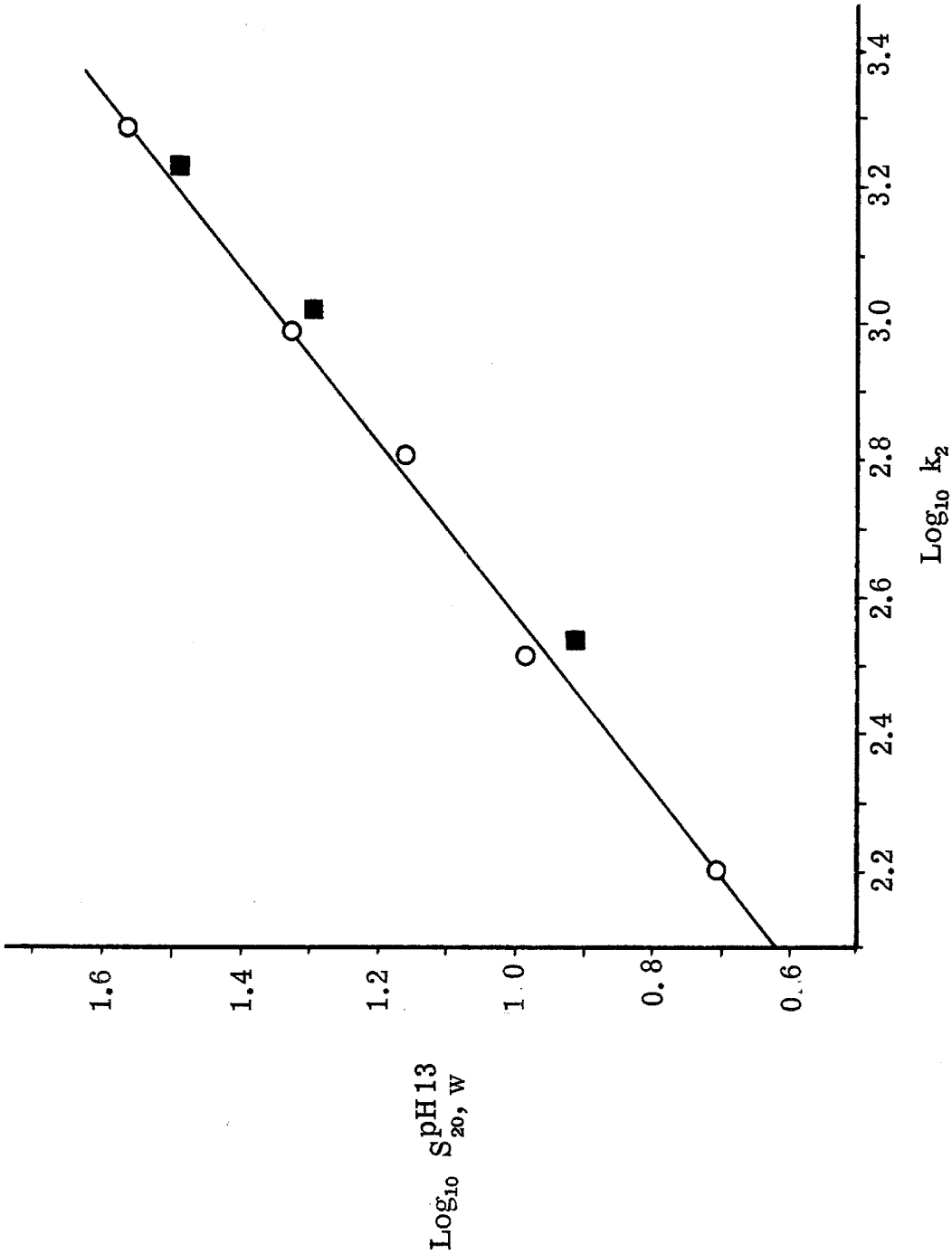
Figure 1-8

The Rate Constant for Renaturation of T7 DNA as Function of Alkaline Sedimentation Velocity

O: 70 μg/ul

■: 7 μg/ul

The dependence of rate constant on alkaline sedimentation velocity is not altered by a ten-fold change in concentration.

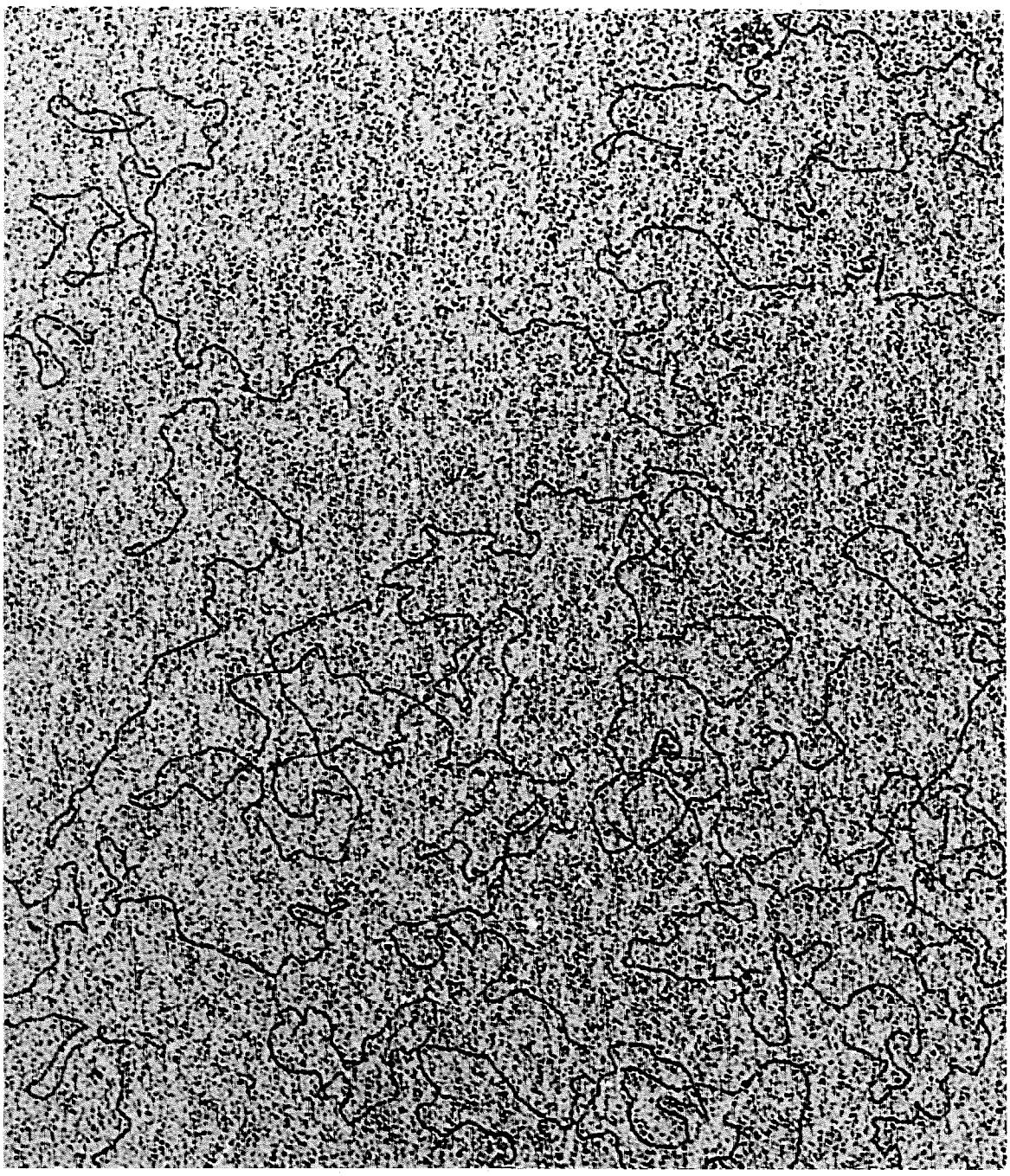

1.6
1.4
1.2
1.0
0.8
0.6
2.2
2.4
2.6
2.8
3.0
3.2
3.4
Log_{10} $S^{pH\,13}_{20,\,w}$
Log_{10} k_2

G. The Nature of the Products

The restoration of transforming activity, and physical, chemical and immunological properties of native DNA upon renaturation has been demonstrated (Marmur and Lane, 1960; Doty, Marmur, Eigner and Schildkraut, 1960; Levine, Murakami, Van Vunatus and Grossman, 1960). Electron micrographs of renatured T2 DNA (Thomas and MacHattie, 1964) show a completely native structure. To demonstrate that the products of renaturation at OD 1.0 in 1.0 F. sodium ion at $T_m - 25°$ C. are native DNA molecules without unusual form, T7 DNA, $S_{20,w}^{pH\ 13} = 35$ with some 30% trailing material, was renatured to 80% completion and observed by electron microscopy using a modified Kleinschmidt technique described in Chapter 7. The results show long individual strands corresponding to the length of T7 DNA, some small amount of denatured DNA, and some strands shorter than whole T7 DNA resulting from reactions involving broken DNA molecules. No unusual structures are observed. These results are consistent with the second order nature of the renaturation reaction which requires formation of entire molecules following a successful nucleation. See Plate 1-1.

PLATE 1-1

T7 DNA renatured at OD 1.0 in 1.0 F. Na^+ at T_m - 25° C. to 80% completion. The DNA, by alkaline band sedimentation, contained about 30% single strand broken material. Uranyl stained Kleinschmidt preparation. Magnification: x 16,000.



CHAPTER 2

The Effect of Temperature on the Rate of Renaturation

Contents: Page:

A. Temperature effects on the rate of renaturation--the plateau region.

The effect of renaturation temperature on the rate of renaturation has been studied by Marmur and Doty (1961) and Ross and Sturtevant (1962) and reviewed by Marmur, Rownd and Schildkraut (1963). Theoretical treatments have been given by several authors (Flory, 1961; Sanders and Ross, 1960; Kallenbach, Crothers and Mortimer, 1963). All experiments show a plateau region of maximum renaturation rate around T_m -25°C. Above and below this region a decrease is observed in the rate. A discussion of the theoretical interpretation of this effect is given later in this chapter.

Data from experiments performed with a sheared sample of T4 DNA in 1.0 M sodium ion are given in Table 2-1. The melting temperature of T4 DNA in this solvent is 92°C.

There is little change in the rate of renaturation of T4 DNA with temperature in the region T_m -16°C. to T_m -32°C. The same DNA heated 20 minutes at 100°C. gave a renaturation rate at 67.5°C. of 102 liters/mole-sec, so no degradation effects could have interfered with the results. The rate constant at T_m -37.5°C is less than that found in the plateau region. This could be due to stable nucleations which do not lead to the proper products. This possibility will be discussed below.

The existence of the large plateau region permits one to be somewhat inexact in achieving and maintaining the renaturation temperature. Control to within 2°C. is more than sufficient, although

TABLE 2-1

Renaturation Rates of T4 DNA in 1.0 F Sodium Ion

Renaturation Temp. (T_r)	$T_m - T_r$	Rate constant (liters/mole-sec.)
78	14	100
76	16	112
74	18	113
74	18	116
70	22	122
70	22	117
67.5	24.5	118
67.5	24.5	118
67.5	24.5	113
65	27	116
65	27	118
60	32	120
54.5	37.5	74
54.5	37.5	76

our systems provided for much better temperature control than this. All the experiments reported in the following chapters were done at T_m -24 ± 2°C.

B. Temperature effects on the rate of renaturation--high temperature region.

An examination of the effect of temperature on the rate of renaturation in the high temperature region between the plateau region and the melting region was made with a sheared sample of E coli DNA in the standard salt solution. The T_m of E coli DNA in 1.0 F. sodium ion is 98°C. The results appear in Table 2-2.

TABLE 2-2

Rate of Renaturation of E coli DNA versus Temperature.

T_r (°C.)	$T_m - T_r$ (°C.)	k_2 (liters/mole-sec)
92	6	1.5
87	11	3.1
83	15	4.8
82	16	5.3
77.5	20.5	6.0
77	21	5.6
73	25	5.8

The same plateau type region within 10% error of 5.8 liters/mole-sec can be seen from T_m -16°C. to lower temperatures. A decreasing rate with increasing temperature occurs from T_m -16°C. to higher temperatures, approaching a zero rate in the region of the melting temperature. Both the plateau and the higher temperature

region effects are discussed in the theoretical sections following.

A similar small temperature dependence before and through the plateau region in 0.4 and 1.0 F. NaCl has been observed by Thrower and Peacocke (1966).

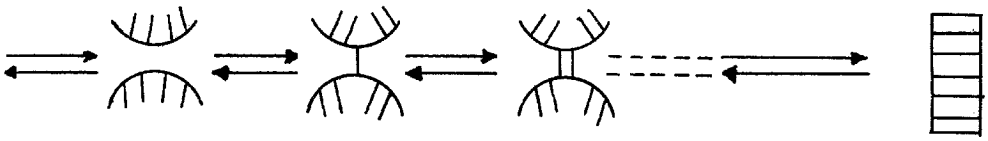
C. Theoretical interpretation of temperature effects on the rate of renaturation of DNA.

1. Statement of a model.

The effect of temperature on the rate of renaturation of homopolymer nucleic acid solutions has been treated experimentally and theoretically by several authors (Ross and Sturtevant, 1960; Ross and Sturtevant, 1962; Flory, 1961; Sanders and Ross, 1960; Kallenbach, Crothers and Mortimer, 1963). In the theoretical models, a steady state is assumed for all intermediates.

Consider the sequential pairing of the two DNA strands.

$$A + A' \underset{k_{0,-1}}{\overset{k_{-1,0}}{\rightleftharpoons}} C_0 \underset{k_{10}}{\overset{k_{01}}{\rightleftharpoons}} C_1 \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} C_2 \cdots\cdots \underset{k_{N,N-1}}{\overset{k_{N-1,N}}{\rightleftharpoons}} C_N$$



C_0 is the state where the potentially reacting bases are confined to a small volume. C_i $(i = 1,N)$ is the case where i base pairs have formed.

For the linear system beginning with C_0, two cases were tested without making the steady state approximation. In the first case, we let $k_{10} \gg k_{01}$ and $k_{N-1,N} \gg k_{N,N-1}$. In the second case, we again took $k_{10} \gg k_{01}$ and this time took N large enough so that the total equilibrium constant,

$$K_{eq} = \prod_{i=1}^{N} k_{i-1,i} \Big/ \prod_{i=1}^{N} k_{i,i-1}$$

was greater than 1. The results obtained were the same as those obtained making the steady state approximation that $dC_i/dt = 0$ for all intermediates. The mathematics for the rigorous kinetics model is given in Benson, pp. 39-42 (1960).

2. The steady state sequential pairing of the DNA bases.

It should be noted that once the first base pair of a DNA is formed, the DNA chain has two ways in which to grow. This complication is neglected in the simple model outlined above. Unlike previous theoretical treatments, however, account is taken of different rate constants for AT and GC base pairs.

For the sequence of first order reactions described above,

$$0 = \frac{dC_i}{dt} = k_{i-1,i}\, C_{i-1} + k_{ii}\, C_i + k_{i+1,i}\, C_{i+1} \quad \text{where } i=0, ---,$$

N-1 and where $k_{ii} = -k_{i,i-1} - k_{i,i+1}$

The determinant of the coefficients is given by

$$D = \begin{vmatrix} k_{00} & k_{10} & 0 & & & 0 \\ k_{01} & k_{11} & k_{21} & 0 & & \\ 0 & k_{12} & k_{22} & & & k_{N-1,N-2} \\ & 0 & & & k_{N-1,N-1} & \\ 0 & & & k_{N-2,N-1} & & \\ & & 0 & & & 0 \end{vmatrix}$$

D_i is the same determinant with column i replaced by the column:

$$\begin{bmatrix} -k_{-1,0}\,[A]\,[A'] \\ 0 \\ \vdots \\ 0 \\ -k_{N,N-1}\,C_N \end{bmatrix}$$

The steady state concentration of intermediate i is given by $C_i = D_i/D$. Let $[A] = [A']$. The overall rate is given by $-d[A]/dt = v = k_{-1,0}[A]^2 - k_{0,-1}C_0$. The rate may be rearranged to be

$$v = \left\{ \prod_{i=0}^{N} k_{i-1,i}\,[A]^2 - \prod_{i=0}^{N} k_{i,i-1}\,[C_N] \right\} / D \qquad \text{or}$$

$$v = \prod_{i=0}^{N} \left\{ k_{i-1,i} \left([A]^2 - [C_N]/K_{eq} \right) \right\} / D$$

then for (C_N/K_{eq}) small due to the large equilibrium constant (negligable back reaction),

$$K_{eq} = \frac{k_{-1,0}}{k_{0,-1}} \prod_{i=1}^{N} S_i.$$ (S_i is the equilibrium constant average for base pair i). Where $S_i > 1$ $(i = 2, \ldots, N)$, the apparent or initial rate may be given by $v = \prod_{i=0}^{N} k_{i-1,i}[A]^2 / D$ or (writing out the determinant)

$$v = \frac{\prod_{i=0}^{N} k_{i-1,i}\,[A]^2}{\sum_{i=0}^{N} \left[\prod_{i=0}^{j-1} k_{i,i-1} \prod_{i=j}^{N-1} k_{i,i+1} \right]}$$

Therefore, the second order rate constant is given by

$$k_2 = \left\{ \prod_{i=0}^{N} k_{i-1,i} \right\} \Bigg/ \left\{ \sum_{j=0}^{N} \left[\prod_{i=0}^{j-1} k_{i,i-1} \prod_{i=j}^{N-1} k_{i,i+1} \right] \right\}$$

Now consider the case where $k_{-1,0}$ is the rate constant for diffusion into a volume element, $k_{0,-1}$ is the rate constant for diffusion out of that volume element where D is the translational diffusion coefficient and σ_{AB} is the radius of the volume element in cgs units, then

$$\frac{k_{-1\rho}}{k_{0,-1}} = \frac{4}{3}\pi \frac{\sigma_{AB}^{3} N_{Avagadro}}{1000} \text{ liters/mole}$$

$$k_{0,-1} = 3D / \sigma_{AB}^{2} \text{ sec}^{-1} \quad \text{(Wang and Davidson, 1966b).}$$

Now let $S_i = \sigma_i s_i$ = the equilibrium constant for the formation of the ith base pair where s_i is the equilibrium constant of such a base pair (AT or GC) in the middle of a long DNA sequence and σ_i is any necessary correction for position in the sequence of reactions; e. g. , the formation of the initial base pair results in no stacking interaction, the formation of the second base pair may involve less stacking, etc. For the moment consider all $k_{i+1,i} = k_f/S_i$ where k_f is the forward rate constant for base pair formation independent of the step or the base

composition. The rate constant can then be rewritten as:

$$k_2 = k_{-1,0}\, k_f^N \Big/ \left\{ k_f^N + k_{0,-1}\, k_f^{N-1} \left[1 + \frac{1}{S_1} + \frac{1}{S_1 S_2} + \frac{1}{S_1 S_2 S_3} \cdots\right]\right\}$$

or k_2 (liters/mole-sec) can be rearranged to be

$$k_2 = \frac{4/3\ \pi\ \sigma_{AB}^3\ k_f\ N_{Avagadro/1000}}{\frac{k_f \sigma_{AB}^2}{3D} + 1 + \frac{1}{S_1} + \frac{1}{S_1 S_2} + \cdots\cdots + \frac{1}{S_1 S_2 \cdots S_{N-1}}}$$

If we do wish to allow for different forward rate constants (and therefore different backward rate constants as the ratio is constant)depending on the step in the reaction sequence:

$$k_{i-1,i} = k_f\ f(i)$$

$$k_{i,i-1} = k_f\ f(i)/S_i$$

where $k_f\, f(0) = k_{-1,0}$ and $k_f\, f(0)/S_0 = k_{0,-1}$

where f(i) is the difficulty of opening or closing the i^{th} base pair compared to a standard rate k_f, the rate constant takes the following form:

$$k_2 = \frac{4/3\ \pi\ \sigma_{AB}^3\ k_f\ N_{Avagadro/1000}}{\frac{k_f \sigma_{AB}^2}{3D} + \frac{1}{f(1)} + \sum_{i=2}^{N} \left(\frac{1}{f(i)} \prod_{j=2}^{i} \frac{1}{S_{j-1}} \right)}$$

If $\sigma(1) = \sigma$ and $\sigma(i) = 1$ for $i = 2, N$, then for all equal forward rate constants,

$$k_2 = \frac{4/3\,\pi\,\sigma_{AB}^3\, k_f\, N_{Av}/1000}{\frac{k_f\,\sigma_{AB}^2}{3D} + 1 + \frac{1}{\sigma s_1} + \frac{1}{\sigma s_1 s_2} + \ldots + \frac{1}{\sigma s_1 s_2 \ldots s_{N-1}}}$$

If $f(1) = \sigma$, or the altered rate constant, due to lack of stacking in the initial base pair formation step, is taken as the forward rate constant instead of the backward rate constant, then

$$k_2 = \frac{4/3\,\pi\,\sigma_{AB}^3\, k_f\, N_{Av}/1000}{\frac{k_f\sigma_{AB}^2}{3D} + \frac{1}{\sigma} + \frac{1}{\sigma s_1} + \frac{1}{\sigma s_1 s_2} + \ldots + \frac{1}{\sigma s_1 s_2 \ldots s_{N-1}}}$$

If we assume that $\frac{1}{\sigma} \gg 1$ and $\frac{1}{\sigma s_1} \gg \frac{k_f \sigma_{AB}^2}{3D}$, then for the general case, $f(1) = a$ and

$$k_2 = \frac{4/3\,\pi\,\sigma_{AB}^3\, k_f\, N_{Av}/1000}{\frac{1}{a} + \frac{1}{\sigma} \cdot \left(\frac{1}{s_1} + \frac{1}{s_1 s_2} + \ldots + \frac{1}{s_1 s_2 \ldots s_{N-1}} \right)}$$

If $a > \sigma s_1$, even though $a < 1$, the term $1/a$ does not contribute to k_2.

The case of equivalent forward rate constants was used for the computer study immediately following this section.

We will now show that our result is the same as that of Saunders and Ross (1960) for all equivalent backward rate constants. If we take the case of N steps all equivalent to base pair formation with k_i defined as k_{01} and all other forward rate constants as k_f and all backward rate constants, including k_{10}, $k_{0,-1}$ as k_b, we can rearrange our rate equation to be

$$v = \frac{k_i}{k_b} \cdot k_f [A] [B] \Big/ \left\{ \frac{k_f}{k_b} + 1 + \frac{k_b}{k_f} + \left(\frac{k_b}{k_f}\right)^2 + \cdots + \left(\frac{k_b}{k_f}\right)^{N-2} \right\}$$

and as $$\frac{1}{1-t} = 1 + t + t^2 + \cdots + t^{N-1} + \frac{t^N}{1-t}$$

we may rearrange the form to produce the equation

$$v = \frac{k_i [A] [B] \left(1 - \frac{k_b}{k_f}\right)}{1 - \left(k_b / k_f\right)^N} + \text{function } (C_N)$$

which is identical to the equation of Saunders and Ross (1960).

We have programmed the case of constant k_f and varying s_i in FORTRAN IV language for an IBM 7094 computer.

3. Evaluation of parameters in the theoretical equation for the second order rate constant for DNA renaturation.

For the case of equal forward rate constants, the rate constant was found to be given by

$$k_2 = \frac{(4/3\,\pi\,\sigma_{AB}^{3}\,N_{Av}/1000)\,(k_f)}{\frac{k_f}{3D/\sigma_{AB}^{2}} + 1 + \frac{1}{S_1} + \frac{1}{S_1S_2} + \ldots + \frac{1}{S_1 \ldots S_{N-1}}}$$

where $S_i = \sigma_i s_i$.

For the choice of an impact parameter of 5 Å = σ_{AB}, $\frac{4/3\,\pi\,\sigma_{AB}^{3}N_{Av}}{1000}$ $= 0.3\ \frac{\text{liters}}{\text{mole}}$. (This term is called VRATIO in the computer program.)

For any reasonable choice of $\frac{3D}{\sigma_{AB}^{2}}$, the term $\frac{k_f\sigma_{AB}^{2}}{3D}$ was insignificant. Thus, $\frac{3D}{\sigma_{AB}^{2}}$ (called VBACK in the program) was defined as a very large number.

$$k_2 \cong \frac{0.3\,k_f}{1 + \frac{1}{S_1} + \frac{1}{S_1S_2} + \ldots + \frac{1}{S_1 \ldots S_{N-1}}} \quad \text{liters/mole-sec}$$

We have previously stated that s_i was the equilibrium constant for an AT of GC base pair in the middle of a large DNA. s_{AT} and s_{GC} were determined as follows.

$$s_{AT} = A_{AT}\, e^{-\Delta H_{AT}/RT}$$

$$s_{GC} = A_{GC} e^{-\Delta H_{GC}/RT}$$

We defined $A_{AT} = A_{GC}$. s_{AT} equals 1 at the melting temperature of a 100% AT DNA in 1.0 F. sodium. T_m (100% AT) = 351°K. Similarly for the GC pairs, T_m = 392°K. Therefore, $\frac{\Delta H_{AT}}{\Delta H_{GC}} = \frac{351}{392}$. We defined ΔH_{AT} = -8000 cal/mole (Wang and Davidson, 1966a; Applequist and Damle, 1965, and references therein). Then ΔH_{GC} = -8935 cal/mole and A = 1.04×10^{-5}. (H_{AT} = HAT, H_{GC} = HGC, A_{AT} = SAT, A_{GC} = SGC in the program). The enthalpies may be thought to consist of a term for hydrogen bonding (about 1 kcal/bond) and a term for stacking, the term which is not present in the formation of the first base pair.

It is still necessary to define k_f and σ_i. We first defined

$$\sigma_1 = Be^{-\Delta H_{\sigma_1}/RT_m} = 0.001$$

where T_m = 371°K. Applequist and Damle (1965) found $4/3\, \pi\, \frac{\sigma_{AB}^3 N_{Av}}{1000} \times \sigma_1 = (2.8 \times 10^{-2} - 2.4 \times 10^{-4})$ for poly A self-association at pH 4.0. Our values are similar. Though the cases are not quite analogous, the similarity implies that our choice is reasonable. If $\sigma_1 s_1$ is to have no stacking interaction, then ΔH_{σ_1} must be 6000 cal/mole so that the total enthalpy of $\sigma_1 s_1$ is approximately that of hydrogen bond formation for AT or GC base pairs (2 or 3 kcal/mole).

B = 3.43. We also tested the case where both σ_1 and σ_2 were defined to be different from 1. In the second case,

$$\sigma_1 = Be^{-\Delta H_{\sigma_1}/RT_m} = 0.01$$

$$B = 34.3$$

and $\sigma_2 = Ce^{-\Delta H_{\sigma_2}/RT_m} = 0.1$ so that $\sigma_1\sigma_2$ (at T_m) = 0.001. [B = SSIG(1), C = SSIG (2), ΔH_{σ_1} = HSIG (1), ΔH_{σ_2} = HSIG (2) in the program]. We arbitrarily took ΔH_{σ_2} = 3000 cal/mole. Then C = 6.11.

We have determined k_2 (T) experimentally and can now predict S_i (T). Therefore, by fitting out results for various $k_f = F\, e^{-\Delta H_f/RT}$, we should be able to determine ΔH_f. (F = SF and ΔH_f = HF in the program). We should note that uncertainties in ΔH_{σ_1} contribute to uncertainties in ΔH_f. Also, uncertainties in σ_1 contribute to uncertainties in k_f. We have defined the maximum experimental rate k_2 as 4×10^5 liters/mole-sec in determination of a fit for F. The reason for this choice will be discussed in chapter 3.

The computer program was set up so that all possible sequences of AT and GC base pairs were tested up to a specified limit. Beyond that limit, an average S_i was defined by $S = s_{AT}^{1-f_{GC}}\, s_{GC}^{f_{GC}}$ where $f_{GC} = \frac{\%GC}{100}$ in the DNA being considered. All the rates obtained were weighted by the probability of finding such a sequence in a DNA of specified GC content. The limit for choice of AT or GC base pairs was taken as 6, as specification of more than 6 had no effect on the calculated rate constants.

4. Computer information.

Several more values had to be specified for the computer program. This information is not essential for following the text of this thesis. If desired, the reader may omit reading this section. The additional values were as follows:

PERGC is the GC% of any DNA.

The temperature range to be investigated is given by

TMAX is the maximum temperature studied in degrees centigrade (92°C. except for GC% < 34).

TINC is the increment by which the temperature is decreased between tests (3).

NTINC is the total number of temperatures investigated (18 to span 41-92°C.).

NSTEP is the number of steps of base pairing which are to be tested for AT and GC cases rather than to be specified by an averaged function of S. NSTEP = 6.

NSIG is the number of σ values to be specified. All others are defined as 1. Three numbers are set equal to either 1 or 2 as methods of control.

NTERM The series $\frac{1}{S_1} + \frac{1}{S_1 S_2} + \ldots\ldots + \frac{1}{S_1 S_2 \ldots S_{NSTEP}}$ is terminated if NTERM is 1 and continued if NTERM is 2 until the last term added no longer contributes to the series or 1000 steps are taken. $S_{NSTEP + i}$ for i greater than or equal to 1 is defined as $(s_{AT})^{\frac{\%AT}{100}} (s_{GC})^{\frac{\%GC}{100}}$. NTERM = 2.

NEQ Always set equal to 2 for DNA. For a first order reaction such

as the cyclization of λ DNA (Wang and Davidson, 1966a), NEQ is set equal to 1, the equilibrium constant is calculated, and the term $1 + 1/K_{eq}$ is multiplied by the apparent rate constant. In this latter case, VRATIO is dimensionless. NEQ = 2.

NOUT The program automatically tests all base sequences up to NSTEP and calculates rates for these initiation sequences. The values are averaged according to the GC content of the DNA and a total rate computed. The output from the computer always included the input data properly labelled and the total rate constants as a function of temperature. If the rates for the individual base sequences are also desired as output, set NOUT equal to 2, otherwise use 1 to suppress large numbers of superfluous pages of information. NOUT = 1.

The computer program is printed below. A sample of input data as a function of ΔH_f follows the program. Both σ cases are included. The first column on each card was and must be blank.

5. Results and discussion.

The best fit for the activation energy of the forward rate step is 7500 ± 2000 cal/mole with σ(2) = 1. With σ(2) ≠ 1, the best value is 7500 + 8500 - 3000 ± 2500 cal/mole or 13000 ± 2500 cal/mole. The use of two σ values did not alter either the GC dependence (see chapter 5) or the temperature profile to any significant degree. The output of average rate constant as a function of temperature for 34% and 49% GC DNAs with 7500 as the forward rate activation energy

Computer Program for Calculating Second Order Renaturation Rate Constants with the Sequential Base Pairing Model (5 pages)

```
$ID                 JGW, CH98298, 2
$IBJOB
$IBFTC  SSR         DECK
      DIMENSION  IATGC(100), HSIG(100), SSIG(100), ST(2), S(100)
      DIMENSION  TOTRAT(100)
C     INPUT AND OUTPUT OF ORIGINAL DATA
 1000 READ (5, 1) HAT, SAT, HGC, SGC, VRATIO
      WRITE (6, 5)
      KSAVE = 1
    5 FORMAT (1H1, 11H  INPUT DATA)
    1 FORMAT (1H, 5E14. 7)
      WRITE (6, 6)
    6 FORMAT (1HO, 51H ENTHALPY  AND PREEXPONENTIAL AT/GC AND VOLUME RATIO)
      WRITE (6, 3) HAT, SAT, HGC, SGC, VRATIO
    3 FORMAT (1HO, 5E14. 7)
      READ (5, 1) HG, SF, TMAX, TINC, VBACK
      WRITE (6, 7)
    7 FORMAT (1HO, 73H ENTHALPY AND PREEXPONENTIAL FORWARD, T (C)  MAX, TINC  1, BACK
     DIFFUSION RATE)
      WRITE (6, 3) HF, SF, TMAX, TINC, VBACK
      READ (5, 2) PERGC, NTINC, NSTEP, NSIG, NEQ, NTERM
    2 FORMAT (1H,  E14. 7, 514
      WRITE (6, 8)
    8 FORMAT (1HO, 87H PERCENT GC,  NUMBER OF T INCREMENTS,  STEPS, SIGMAS,  1OPTIONS
     OF EQUILIBRIUM,  TERMINATION)
      WRITE (6, 9)
    9 FORMAT (1H, 80H 1 IN OPTIONS MEANS USE EQUILIBRIUM AND TERMINATE SU 1M  AT N
     STEP,  OTHERWISE USE 2)
```

```
      WRITE (6,4) PERGC, NTINC, NSTEP, NSIG, NEQ, NTERM
      NXX = (2**NSTEP)*NTINC
    4 FORMAT (1HO, E14.7, 514)
      WRITE (6,10)
   10 FORMAT (1HO, 42H ENTHALPY AND PREEXPONENTIAL OF THE SIGMAS)
      PGC = PERGC/100.0
      PAT = 1.0-PGC
      DO1191 = 1, NTINC
  119 TOTRAT (1) = 0.0
      DO511 = 1, NSIG
      READ (5, 12) HSIG (1), SSIG(1)
   12 FORMAT (1H , 2E14.7)
   51 WRITE (6, 13)HSIG (1)
   13 FORMAT 1HO, 2E14.7)
      READ (5, 45) NOUT
  451 FORMAT (1H, 12)
      WRITE (6, 452)NOUT
  452 FORMAT (1HO, 57H OUTPUT SUPPRESSION 1 = YES, 2 = NO, THE INPUT CHOICE 1E
     IS , 12)
      DO100110 = 1, 2
      DO100109 = 1, 2
      DO100108 = 1, 2
      DO100107 = 1, 2
      DO100106 = 1, 2
      DO100105 = 1, 2
      DO100104 = 1, 2
      DO100103 = 1, 2
      DO100102 = 1, 2
      DO100101 = 1, 2
      IATGC(01) = 101
      IATGC(02) = 102
      IATGC(03) = 103
      IATGC(04) = 104
      IATGC(05) = 105
      IATGC(06) = 106
      IATGC(07) = 107
```

```
      IATGC(08) = 108
      IATGC(09) = 109
      IATGC(10) = 110
   14 FORMAT (1H1, 35H BASE PAIRS IN ORDER 1 = AT, 2 = GC)
   15 FORMAT (1HO, 1012)
      GOTO (251, 252), NOUT
  252 CONTINUE
      WRITE (6, 15) (IATGC(I), 1=1, 10)
      WRITE (6, 81)
  251 CONTINUE
   81 FORMAT (1HO, 33H CALCULATED RATES AT TEMPERATURES)
      T=TMAX+273. 16+TINC
      NGC=0
      D09991 = 1, 10
  999 NGC = NGC + IATGC(1)-1
      TAT=NSTEP-NGC
      TGC = NGC
      PTOT = (PAT**TAT)*(PGC**TGC
C     CALCULATION SECTION
      DO100J = 1, NTINC
      T=T-TINC
      RT=1. 986*T
      ST(1) = SAT*EXP (-HAT/RT)
      ST(2) = SGC*EXP (-HGC/RT)
      F = SF*EXP(-HF/RT)
      DO611 = 1, NSIG
      K = IATGC(1)
   61 S(I) = SSIG(I)*EXO(-HSIG(I)/RT)*ST(K)
      L = NSIG + 1
      DO621 = L, NSTEP
      K = IATGC(1)
   62 S(1) = ST(K)
C     OPTION OF EQUILIBRIUM CONSTANT
      ANUM = 1. 0
```

```
      GO TO (63,64),  NEQ
   63 AK = VRATIO
      DO651 = 1,NSTEP
   65 AK = AK*S(1)
      ANUM = 1.0 + AK
   64 VNUM = ANUM*F*VRATIO
C     CALCULATION OF THE DENOMINATOR
C     OPTION OF TERMINATION
      VDEN = (F/VBACK) + 1.0
      STEP = 1.0
      DO731 = 1,NSTEP
      STEP = STEP/S(1)
   73 VDEN = VDEN + STEP
      GO TO (71,72),  NTERM
   72 SAV = (ST(2)**PGC)*(ST(1)**PAT)
      L = NSTEP + 1
      DO741 = L,1000
      STEP = STEP/SAV
      VDEN = VDEN + STEP
      IF (STEP/VDEN-0.001)71,71,75
   75 CONTINUE
   74 CONTINUE
   71 V = VNUM/VDEN
      TOTRAT (J) = TOTRAT (J) + V*PTOT
      TC = T-273.16
      GOTO (351,352),NOUT
  352 CONTINUE
      WRITE (6.82) TC,V
  351 CONTINUE
      IF (KSAVE - NXX) 1001,1002,1002
   82 FORMAT (1HO,15H TEMPERATURE = ,F7.2,10X,17H RATE CONSTANT = ,E14.7,  121H
     LITERS/MOLE SECOND)
 1001 KSAVE = KSAVE + 1
  100 CONTINUE
 1002 WRITE (6,152)
```

```
  152 FORMAT (1H1, 38H THE TOTAL RATE CONSTANTS FOR THIS DNA)
      DO153J = 1, NTINC
      RQ = J
      T = TMAX + TINC* (1.0-RQ)
  153 WRITE (6, 82) T,  TOTRAT (J)
      GO TO 1000
      END
$DATA
$ENDJOB
```

Input Data for Renaturation Rate Constant Program

```
$DATA
 -8.0000000E+03+1.0400000E-05-8.9350000E+03+1.0400000E-05+3.0000000E-01
 +3.0000000E+03+1.2000000E+11+9.2000000E+01+3.0000000E+00+2.0000000E+10
 +5.0000000E+01+009+006+001+002+002
 +6.0000000E+03+3.4300000E+00
 +1
 -8.0000000E+03+1.0400000E-05-8.9350000E+03+1.0400000E-05+3.0000000E-01
 +3.0000000E+03+1.2000000E+11+9.2000000E+01+3.0000000E+00+2.0000000E+10
 +5.0000000E+01+009+006+002+002+002
 +6.0000000E+03+3.4300000E+01
 +3.0000000E+03+6.1100000E+00
 +1
 -8.0000000E+03+1.0400000E-05-8.9350000E+03+1.0400000E-05+3.0000000E-01
 +6.0000000E+03+7.2000000E+12+9.2000000E+01+3.0000000E+00+2.0000000E+10
 +5.0000000E+01+009+006+001+002+002
 +6.0000000E+03+3.4300000E+00
 +1
 -8.0000000E+03+1.0400000E-05-8.9350000E+03+1.0400000E-05+3.0000000E-01
 +6.0000000E+03+7.2000000E+12+9.2000000E+01+3.0000000E+00+2.0000000E+10
 +5.0000000E+01+009+006+002+002+002
 +6.0000000E+03+3.4300000E+01
 +3.0000000E+03+6.1100000E+00
 +1
 -8.0000000E+03+1.0400000E-05-8.9350000E+03+1.0400000E-05+3.0000000E-01
 +9.0000000E+03+4.3000000E+14+9.2000000E+01+3.0000000E+00+2.0000000E+10
 +5.0000000E+01+009+006+001+002+002
 +6.0000000E+03+3.4300000E+00
 +1
 -8.0000000E+03+1.0400000E-05-8.9350000E+03+1.0400000E-05+3.0000000E-01
 +9.0000000E+03+4.3000000E+14+9.2000000E+01+3.0000000E+00+2.0000000E+10
 +5.0000000E+01+009+006+002+002+002
 +6.0000000E+03+3.4300000E+01
```

```
 +1
 -8.0000000E+03+1.0400000E-05-8.9350000E+03+1.0400000E-05+3.0000000E-01
 +1.2000000E+04+2.6000000E+16+9.2000000E+01+3.0000000E+00+2.0000000E+10
 +5.0000000E+01+009+006+002+002+002
 +6.0000000E+03+3.4300000E+01
 +3.0000000E+03+6.1100000E+00
 +1
SENDJOB
```

appear on the following pages.

The results of theory and experiment suitably corrected in magnitude for the effects of DNA complexity and length, which are explained in chapters 3 and 4 , are plotted for two cases. The plateau region results with T4 DNA are shown in figure 2-1. The high temperature region results are shown for E coli DNA in figure 2-2.

The point observed at the lowest temperature for T4 DNA does not agree with the theoretical curve. It should be noted that we have throughout assumed that the entire hyperchromicity change in melting a DNA was the same as the change in hyperchromicity expected during renaturation. This is not quite true. Denatured DNA itself develops some hyperchromicity on cooling. An error of the order of 15% in the rate constant is introduced at the temperature of maximum renaturation rate due to this effect. As this effect increases as the renaturation temperature is lowered, it might lead to a choice of too high an activation energy for the forward process. As the cooling curve of denatured DNA is quite smooth near T_r, the choice of too high an activation energy for the forward rate would be expected to partly cancel this effect as a source of error. But at the lowest temperature point, the effect of extraneous hyperchromicity is larger and would account, in part, for the disagreement of the experimental and theoretical curve. In addition, at such a low temperature, conformational changes of the denatured DNA might be anticipated and would themselves affect the rate. This effect is discussed in great detail below.

Computer Output of Input Parameters for Renaturation Rate

Constant Calculations for T4 and E Coli DNAs

A. T4 DNA

INPUT DATA

ENTHALPY AND PREEXPONENTIAL AT/GC AND VOLUME RATIO

-0.8000000E 04 0.1040000E-04-0.8935000E 04 0.1040000E-04 0.3000000E-00

ENTHALPY AND PREEXPONENTIAL FORWARD, T (C) MAX, TINC, BACK DIFFUSION RATE

0.7500000E 04 0.7500000E 14 0.9200000E 02 0.3000000E 01 0.2000000E 11

PERCENT GC, NUMBER OF T INCREMENTS, STEPS, SIGMAS, OPTIONS OF EQUILIBRIUM, TERMINATION 1 IN OPTIONS MEANS USE EQUILIBRIUM AND TERMINATE SUM AT N STEP, OTHERWISE USE 2

0.3400000E 02 18 6 1 2 2

ENTHALPY AND PREEXPONENTIAL OF THE SIGMAS

0.6000000E 04 0.3430000E 01

OUTPUT SUPPRESSION 1 = YES, 2 = NO, THE INPUT CHOICE IS 1

B. E COLI DNA

INPUT DATA

ENTHALPY AND PREEXPONENTIAL AT/GC AND VOLUME RATIO

-0. 8000000E 04 0. 1040000E-04-0. 8935000E 04 0. 1040000E-04 0. 3000000E-00

ENTHALPY AND PREEXPONENTIAL FORWARD. T (C) MAX, TINC, BACK DIFFUSION RATE

0. 7500000E 04 0. 7500000E 14 0. 9200000E 02 0. 3000000E 01 0. 2000000E 11

PERCENT GC, NUMBER OF T INCREMENTS, STEPS, SIGMAS, OPTIONS OF EQUILIBRIUM, TERMINATION 1 IN OPTIONS MEANS USE EQUILIBRIUM AND TERMINATE SUM AT N STEP, OTHERWISE USE 2

0. 4900000E 02 18 6 1 2 2

ENTHALPY AND PREEXPONENTIAL OF THE SIGMAS

0. 6000000E 04 0. 3430000E 01

OUTPUT SUPPRESSION 1 = YES, 2 = NO, THE INPUT CHOICE IS 1

Calculated Renaturation Rate Constants for T4 DNA

THE TOTAL RATE CONSTANTS FOR THIS DNA

TEMPERATURE = 92.00	RATE CONSTANT =	0.1540512E	03	LITERS / MOLE SECOND
TEMPERATURE = 89.00	RATE CONSTANT =	0.8483463E	05	LITERS / MOLE SECOND
TEMPERATURE = 86.00	RATE CONSTANT =	0.1301836E	06	LITERS / MOLE SECOND
TEMPERATURE = 83.00	RATE CONSTANT =	0.1642453E	06	LITERS/ MOLE SECOND
TEMPERATURE = 80.00	RATE CONSTANT =	0.1913615E	06	LITERS / MOLE SECOND
TEMPERATURE = 77.00	RATE CONSTANT =	0.2128816E	06	LITERS / MOLE SECOND
TEMPERATURE = 74.00	RATE CONSTANT =	0.2294581E	06	LITERS / MOLE SECOND
TEMPERATURE = 71.00	RATE CONSTANT =	0.2416062E	06	LITERS / MOLE SECOND
TEMPERATURE = 68.00	RATE CONSTANT =	0.2497435E	06	LITERS / MOLE SECOND
TEMPERATURE = 65.00	RATE CONSTANT =	0.2543389E	06	LITERS / MOLE SECOND
TEMPERATURE = 62.00	RATE CONSTANT =	0.2558182E	06	LITERS / MOLE SECOND
TEMPERATURE = 59.00	RATE CONSTANT =	0.2546341E	06	LITERS / MOLE SECOND
TEMPERATURE = 56.00	RATE CONSTANT =	0.2511861E	06	LITERS / MOLE SECOND
TEMPERATURE = 53.00	RATE CONSTANT =	0.2458319E	06	LITERS / MOLE SECOND

TEMPERATURE = 50.00 RATE CONSTANT = 0.2389345E 06 LITERS / MOLE SECOND

TEMPERATURE = 47.00 RATE CONSTANT = 0.2308047E 06 LITERS / MOLE SECOND

TEMPERATURE = 44.00 RATE CONSTANT = 0.2217073E 06 LITERS / MOLE SECOND

TEMPERATURE = 41.00 RATE CONSTANT = 0.2118891E 06 LITERS / MOLE SECOND

Calculated Renaturation Rate Constants for E coli DNA

THE TOTAL RATE CONSTANTS FOR THIS DNA

TEMPERATURE = 92.00	RATE CONSTANT =	0.1804024	E 06	LITERS / MOLE SECOND
TEMPERATURE = 89.00	RATE CONSTANT =	0.2257501	E 06	LITERS / MOLE SECOND
TEMPERATURE = 86.00	RATE CONSTANT =	0.2619207	E 06	LITERS / MOLE SECOND
TEMPERATURE = 83.00	RATE CONSTANT =	0.2908417	E 06	LITERS / MOLE SECOND
TEMPERATURE = 80.00	RATE CONSTANT =	0.3124287	E 06	LITERS / MOLE SECOND
TEMPERATURE = 77.00	RATE CONSTANT =	0.3302523	E 06	LITERS / MOLE SECOND
TEMPERATURE = 74.00	RATE CONSTANT =	0.3419316	E 06	LITERS / MOLE SECOND
TEMPERATURE = 71.00	RATE CONSTANT =	0.3489645	E 06	LITERS / MOLE SECOND
TEMPERATURE = 68.00	RATE CONSTANT =	0.3519219	E 06	LITERS / MOLE SECOND
TEMPERATURE = 65.00	RATE CONSTANT =	0.3513104	E 06	LITERS / MOLE SECOND
TEMPERATURE = 62.00	RATE CONSTANT =	0.3477019	E 06	LITERS / MOLE SECOND
TEMPERATURE = 59.00	RATE CONSTANT =	0.3415113	E 06	LITERS / MOLE SECOND
TEMPERATURE = 56.00	RATE CONSTANT =	0.3332217	E 06	LITERS / MOLE SECOND
TEMPERATURE = 53.00	RATE CONSTANT =	0.3232036	E 06	LITERS / MOLE SECOND

TEMPERATURE = 50.00 RATE CONSTANT = 0.3118140E 06 LITERS / MOLE SECOND

TEMPERATURE = 47.00 RATE CONSTANT = 0.2993606E 06 LITERS / MOLE SECOND

TEMPERATURE = 44.00 RATE CONSTANT = 0.2861188E 06 LITERS / MOLE SECOND

TEMPERATURE = 41.00 RATE CONSTANT = 0.2723283E 06 LITERS / MOLE SECOND

Figure 2-1

The Rate Constant for Renaturation of T4
DNA as a Function of Temperature

O: experimental values

■: theoretical values - 34% GC
(Theoretical Rate Constant $\times$ 118/256 $\times$ 10^3)

The discrepancy at 54.5°C. between the theoretical and experimental values is discussed in the text.

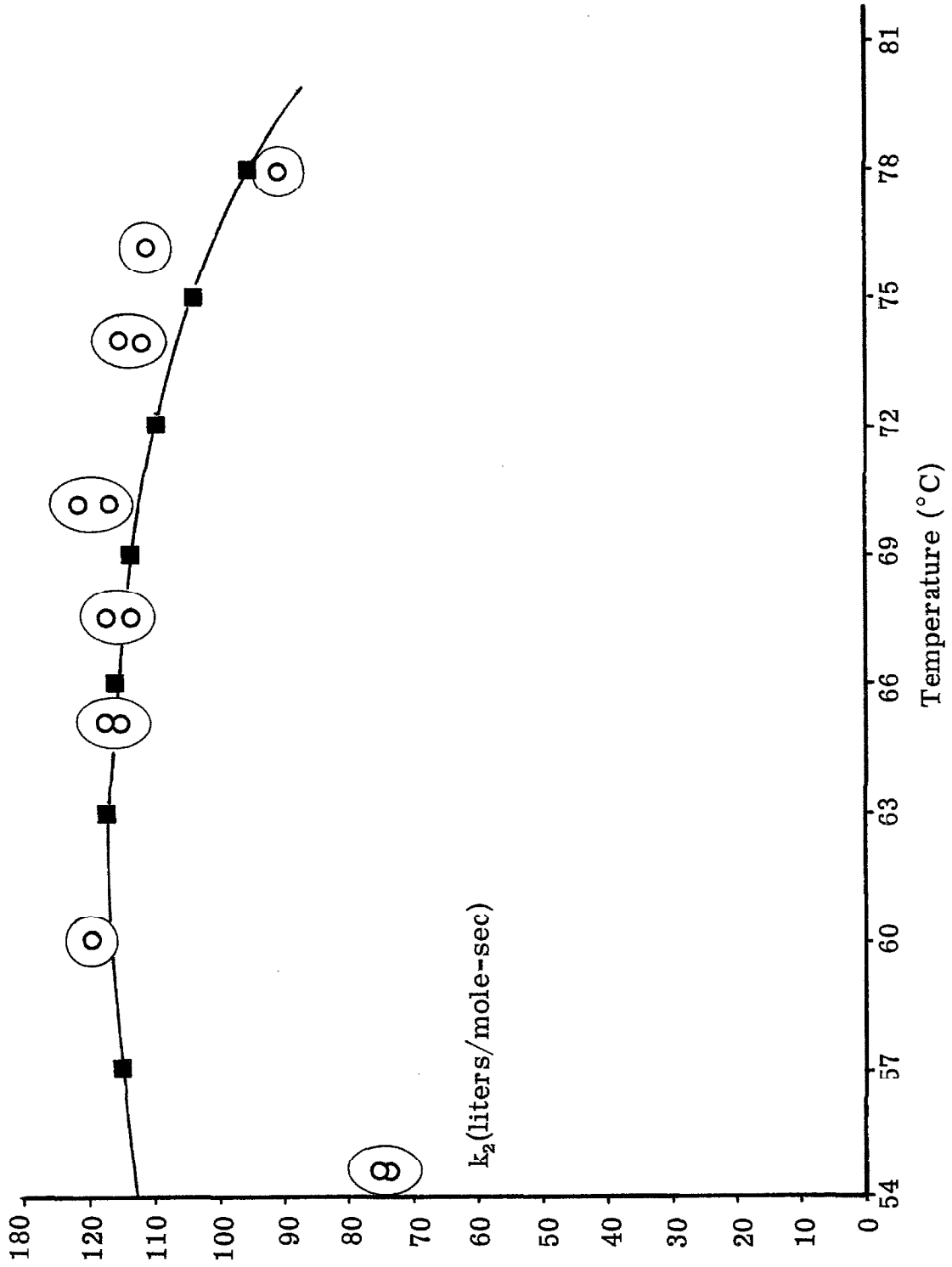

180
120
110
100
90
80
70
60
50
40
30
20
10
0
k_2(liters/mole-sec)
54
57
60
63
66
69
72
75
78
81
Temperature (°C)

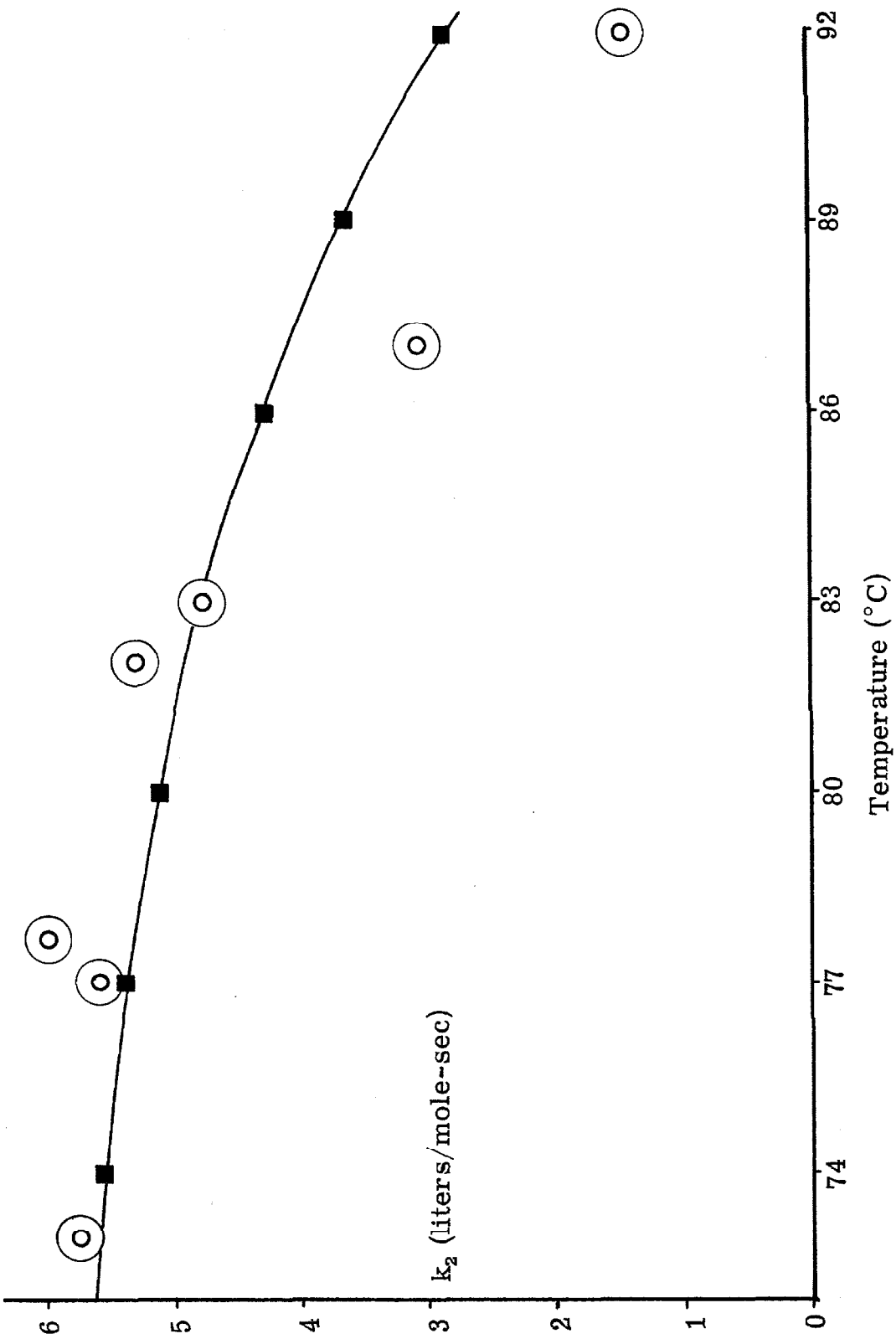
Figure 2-2

The Rate Constant for Renaturation of E coli
DNA as a Function of Temperature

O : experimental values

■: theoretical values - 49% GC
(Theoretical Rate Constant $\times$ 5.75/3.52 $\times 10^5$)

The disagreement between experiment and theory at 87 and 92° C. is discussed in the text.


6
5
4
3
2
1
0
k_2 (liters/mole-sec)
74
77
80
83
86
89
92
Temperature (°C)

The two highest temperature values for E coli DNA (figure 2-2) do not agree with theory. The theoretical prediction is that the rate should go to zero at T_m. Our experimental results, and the results of others (Ross and Sturtevant, 1962; Marmur and Doty, 1962) suggest that the rate goes to zero at a temperature about 5°C. below T_m. If sequences of runs of AT stop winding of the DNA long enough to make it necessary for a second nucleation to occur, so that a deviation from second order kinetics would be observed, the experimental rate constants would be expected to be diminished. Also, if f(i), the function used to describe relative difficulties of winding of the DNA at base pair i decreased with increasing i, then the theoretical curve would tend toward lower rate constants at elevated temperatures. We cannot distinguish between these alternatives with our data, but as our model for the renaturation process is not rigorous, it seems unwise to pursue this subject. Either of the two phenomena are the likely sources of the discrepancy.

The important point is that the model predicts, as did the simpler model of Saunders and Ross (1960), a plateau region in the renaturation process. Also, as we shall see in chapter 5, the more extended model employed here predicts something about the GC dependence of the kinetics of renaturation, a phenomenon which is experimentally observed.

We shall see in chapters 3 and 4 that the rate determining step of the process of renaturation may be diffusion limited. The dependence of the diffusion coefficient on temperature in water is equivalent to an activation energy of 4000-5000 cal/mole. A diffusion controlled pro-

cess may have only a slightly higher activation energy than this. The value of 7500 ± 2000 cal/mole for the forward rate, considering the possible error due to extraneous hyperchromicity, could be considered to be within experimental error of a possible activation energy for a diffusion controlled process. The activation energy with significant σ_2 effect is not in agreement. Although Eigen (1967) used a σ_2 in the theoretical fit of relaxation studies of short nucleotide sequence renaturation, we cannot justify the use of this parameter in the light of the data presented in this and future chapters.

The possible effect of intramolecular random base pairing was suggested to explain the decrease in the rate constant of renaturation at temperatures below the plateau region. Indeed, we are able to prevent renaturation entirely by keeping denatured DNA at low temperatures for periods of time somewhat longer than the times employed for following the renaturation reaction. It was necessary for this to be true to use the temperature jump system. A discussion of both inter and intra-molecular random aggregation is presented below.

6. Intermolecular random base pairing and aggregation.

In this section, we shall show that random sequence base pairing between segments on different strands should not be significant for DNA of $A_{260} = 1$ even at room temperature in molar sodium. Intermolecular random base pairing cannot exist under the conditions used for studying renaturation.

Given an N-mer, the probability of it having a given base se-

quence, where n = number of G + C bases, is

$$f_{GC}^{n} \ (1-f_{GC})^{N-n}/2^{N}$$

In an Lmer, $L \gg N$, the probability of finding an N-mer of a given base sequence is given by

$$L \cdot f_{GC}^{N} \ (1-f_{GC})^{N-n}/2^{N}$$

If P_T is the total phosphate concentration of the Lmers in solution, then the moles of a given sequence (moles of sequence and not phosphate) are given by

$$P_T \ f_{GC}^{N} \ (1-f_{GC})^{N-n}/2^{N}$$

e.g., OD 1.0, 50% GC, N = 2, the concentration of AGs would be $\frac{P_T}{16} = \frac{1.4 \times 10^{-4}}{16}$. The renaturation reaction may be written as

$A + A' \overset{K_{eq}}{\rightleftharpoons} B$ where $K_{eq} = [B] / [A] \cdot [A']$ and for 50% reaction, $[A] = [A'] = [B] / 2$ and $K_{eq} = \frac{2}{[A]}$ where $[A]$ is the free concentration of N (n) mer or its complement (equal concentration). The equilibrium constant for this system is defined by $0.3 \ \sigma \ S_{AT}^{N-n} \ S_{GC}^{n}$ liters/mole. Let r_b be the ratio of bound to free nucleotides, so that $r_b = K_{eq} [A]/2$ thus

$$r_b = \frac{0.3\,\sigma\, S_{AT}^{N-n}\, S_{GC}^{n}}{} \times \frac{P_T f_{GC}^{N} (1-f_{GC})^{N-n}}{2 \times 2^N}$$

Consider a 50% GC DNA with $P_T = 1.33 \times 10^{-4}$ moles/liter. With the parameters used for the previous rate calculations,

$$r_b = \frac{2 \times 10^{-5}\ \sigma\, S_{AT}^{N-n}\, S_{GC}^{n}}{4^N}$$

or with $\sigma\, S_{AT} \approx 5 \times 10^{-4}$ and $4\, S_{AT} \approx S_{GC}$, $r_b = \frac{10^{-8} S_{AT}^{N-1}}{4^{N-n}}$

At $s_{AT} = 1$, around the renaturation temperature normally employed, the ratio, $r_b = \frac{10^{-8}}{4^{N-n}}$, is much less than one for all cases. Unless there is duplication of information in a DNA on a rather large scale, one cannot consider intermolecular random base pairing to occur at the renaturation condition. We have neglected the possible effect of several short segments near to each other leading to aggregation, but as far as we can tell from observation of no scattering by the denatured DNA under standard conditions and from the experiments of Studier (1965) leading to sedimentation velocity data on denatured DNA at low temperatures in a wide range of salts, intermolecular random base pairing is not a factor in the study of renaturation under normal conditions.

At low temperature (near room temperature in molar sodium) where $s_{AT} = 4$, the ratio r_b is $10^{-8}\ 4^{n-1}$. This requires $n \geq 15$ for

stability. One can calculate that the probability of finding such a sequence in a moderately complex phage DNA is small. The total fraction of material paired by this phenomenon,

$$\sum_{N \geq n}' \quad \sum_{n \geq 15}' \quad \frac{N!}{(N-n)!\,(n)!} \qquad \frac{N}{4^N}$$

in terms of phosphate is also negligible, even at low temperatures.

The important point to note is that except at low temperatures, the probability of finding paired N-mers decreases with increasing N. This is due to the fact that increasing N leads to a decrease in concentration of available random N-mers. This effect is larger than the effect of increased stability due to increasing length.

7. Intramolecular random sequence base pairing and internal aggregation.

In this section, we shall show that random sequence base pairing between segments of the same strand should lead to collapse of denatured DNA molecules of moderate length above room temperature in molar sodium. This predicted effect is compared with experimental results obtained with the ultracentrifuge by Studier (1965). It is predicted that at $A_{260} = 1$, intramolecular effects should be greater than intermolecular effects. Nevertheless, under the normal conditions of renaturation, intramolecular aggregation is predicted to be negligible.

Given L N-mers of equal contents of all bases (50% GC), the

probability of finding a given sequence of N-mers is $L/4^N$. The probability of finding it twice is $(L/4^N)^2$. The total number of combinations of an N-mer is 4^N. Thus, the probability of finding any two complementary N-mers in a length of DNA, L, is $L^2/4^N$. Thus, identical N-mers should be expected for all N such that $L \geq 2^N$. If we take L as 10^4, the size equivalent to an alkaline S of 20 (see chapter 3), identical N-mers should be expected for all cases of $N \leq 13$. This is quite important, as 13 is a reasonably long sequence. For N greater than 13, the probability is less than one per N-mer, so such cases can be disregarded in dealing with intramolecular aggregation.

Except for $N \leq 5$, the probability of having more than two of one type of N-mer in L is less than one. Thus, we may assume that the duplicate N-mers, $6 \leq N \leq 13$, are randomly distributed in the chain. That is, the average contour distance between the complementary segments would be

$$\sum_{i=1}^{L} \left(\sum_{n=1}^{i} n + \sum_{n=1}^{L-i} n \right) \Bigg/ L^2 = \frac{L}{3} + \frac{1}{2} + \frac{1}{6L} \approx \frac{L}{3} \equiv L'$$

for all reasonable L. If we neglect the effect of spacing and assume that all the N-mers are at this average distance apart, we may treat this case similarly to that of the cyclization of λ DNA as treated by Wang and Davidson (1966a). Let b = b (native DNA)/4, L'(mean distance between N-mers) be 1 micron, and let the impact parameter remain 5 Å, then

$$r_b = K_{eq} = \frac{k_{-1,0}}{k_{0,-1}} \sigma S_{AT}^{N-n} S_{GC}^{n}$$

$$\frac{k_{-1,0}}{k_{0,-1}} = \frac{4/3 \pi \sigma_{AB}^{3}}{(2 \pi b L'/3)^{3/2}} = 7.7 \times 10^{-7}$$

$$K_{eq} = 3.85 \times 10^{-10} S_{AT}^{N-1} 4^{n} \text{ for } S_{AT} = \frac{S_{GC}}{4} \quad \text{and}$$

$$\sigma S_{AT} = S \times 10^{-4}$$

We have considered the entire group of all possible N-mers. The probability of each individual N-mer of a GC% 100n/N is given by

$$\frac{N!}{(N-n)!\, n!} \cdot \frac{1}{2^N}$$

We can say that a sequence would contribute as described in the probability calculation if it were stable for $N > n$ such that the fraction of stable contributions is given by

$$1 \approx \sum_{n_{\text{min stable}}} \frac{N}{(N-n)!\, n!} \quad \frac{1}{2^N}$$

Consider the case of the renaturation temperature where $S_{AT}=1$.

$$K_{eq} \approx 4 \times 10^{(3n/5-10)}$$

then $\frac{3n - 50 + 2}{5} \Longrightarrow n \geq 16$, required.

Thus no stable intramolecular aggregation would be expected in the renaturation region of the temperature profile, just as is the case for intermolecular aggregation.

Consider the next case with a temperature intermediate between the renaturation temperature and room temperature in molar sodium so that $S_{AT} = 2$. Then

$$K_{eq} \approx 4 \times 10^{(3N + 6n - 101)/10}$$

or $3N + 3n \geq 48$ or for the 13mer, $n \geq 3$ required. Stable aggregates would be expected in the case of 12, 11, 10,9 mers as well. Thus one should expect extensive intramolecular aggregation and accompanying collapse of the random coil of denatured DNA of moderate molecular weight at room temperature in molar salt, whereas under the same conditions, intermolecular aggregation is not expected to be a problem.

At subsequent lowering of T_m by lowering of the salt concentration to 0.01 M. sodium, the case $S_{AT} = 2$ would be moved to room temperature. One would predict that the collapsed molecules would expand due to loss of internal aggregation. This phenomenon is observed in sedimentation velocity experiments on denatured DNA in various salts an neutral pH at room temperature. (Studier, 1965). These calculations have not considered duplicated sequences of closer contour spacing than the average, but any effect at temperatures as high as those employed for renaturation would not be expected.

It might be noted as a comparison that the pairing of the ends of

$\lambda_{b_2 b_5 c}$ DNA (Wang and Davidson, 1966b) occurs at the same intramolecular and intermolecular extent at an A_{260} of $\lambda_{b_2 b_5 c}$ DNA of about 0.3. With random segments of denatured DNA, the intermolecular effect should be less than the intramolecular effect up to OD 250 for a 13mer with $L = 10^4$, the conditions considered above. Studier (1965) found no significant intermolecular aggregation problem in sedimentation velocity experiments under conditions of extensive intramolecular aggregation.

The lowest temperature experimental point for T4 DNA was taken near the predicted beginning of internal collapse. The extraneous hyperchromicity of denatured DNA begins to be more temperature-dependent at about this same temperature. It is not, therefore, possible to eliminate a small conformational change as the cause of the disagreement between experiment and theory at this lowest temperature case. In fact, it is probably because of the collapse of denatured DNA with decreasing temperature that the use of low temperature to prevent renaturation works at all.

CHAPTER 3

Molecular Weight Effects on the Rate of Renaturation of DNA

Contents: Pages:

A. Theoretical Expectations for Length and Complexity Effects on the Rate of Renaturation of DNA

We define complexity as the total number of DNA base pairs in non-repeating sequences of DNA in a virus, bacterium, or mammalian cell. In the case of a virus or bacterium, the complexity is the number of base pairs in the viral or bacterial chromosome, which is directly proportional to the molecular weight of the unfractured chromosome.

We define length as the average number of bases in the single strand of a DNA molecule in solution prior to renaturation.

For simplicity, assume that each base may act as a nucleation site for renaturation. If we were to assume, instead, that there were, on the average, a few bases per nucleation site, the results would be qualitatively the same.

Then if P is the DNA phosphate concentration, P/2N is the concentration of a single nucleation site, where N is the complexity. The rate, $v_{NUCLEATION}$, of nucleation for one nucleation site is given by

$$v_{NUCLEATION} = k_N (P/2N)^2$$

where k_N is the nucleation rate constant.

There are N different nucleation sites and N nucleation reactions.

$$v_{ALL\ NUCLEATIONS} = k_N (P/2N)^2 \cdot N$$

Following a successful nucleation, 2L bases will be paired if the DNA fragments are exactly complementary.

$$v_{\text{BASE PAIR FORMATION}} = k_N (P/2N)^2 \cdot N \cdot 2L$$

or

$$v_{\text{RENATURATION}} = k_N/2 \cdot (P)^2 \cdot L/N$$

We shall discuss the problem of random scission later in this chapter. It will be shown that the effect of random scission reduces the rate by a uniform factor of 2/3.

Experimentally, we calculate the second order rate constant, k_2, from

$$v_{\text{RENATURATION}} = k_2/2 \cdot (P)^2$$

Therefore, $k_2 = k_N L/N$.

Thus, for a given DNA, the rate constant for renaturation should be proportional to the length of the DNA strands in solution and inversely proportional to the complexity. In Chapter 4, we shall show that the inverse proportionality to complexity is actually observed. Therefore, k_N is not a function of complexity.

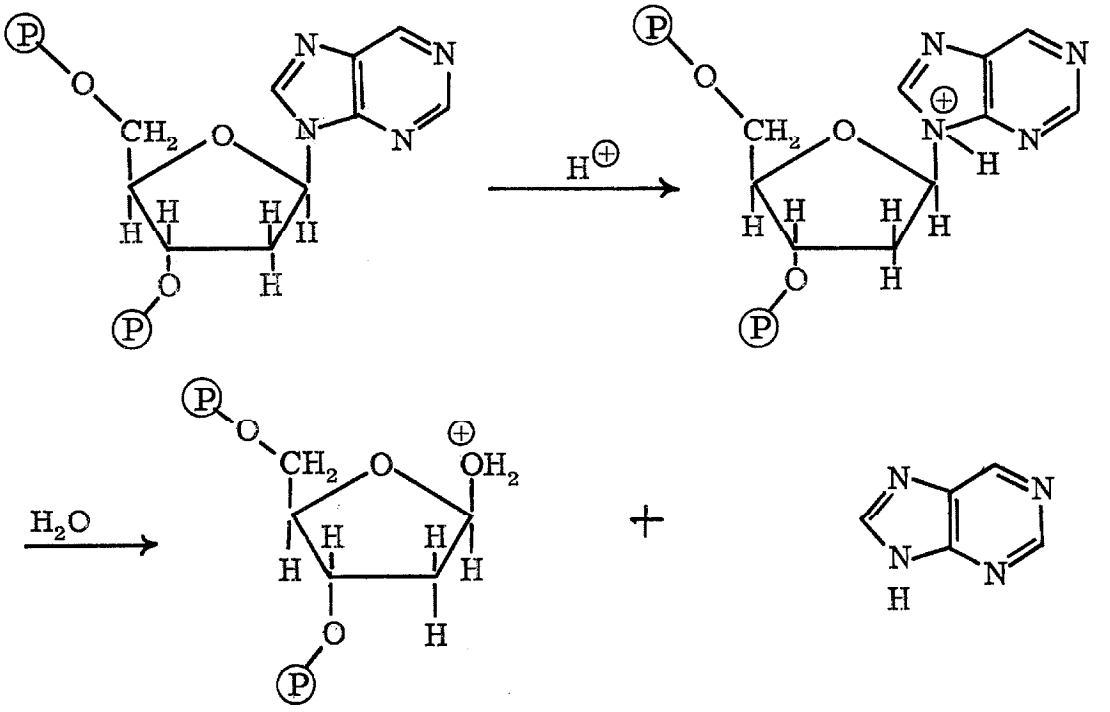
In this chapter, we shall show that k_N, the nucleation rate constant, is a function of the length of denatured DNA in solution. We shall assume the complexity results in Chapter 4 for the theoretical discussion at the end of this chapter.

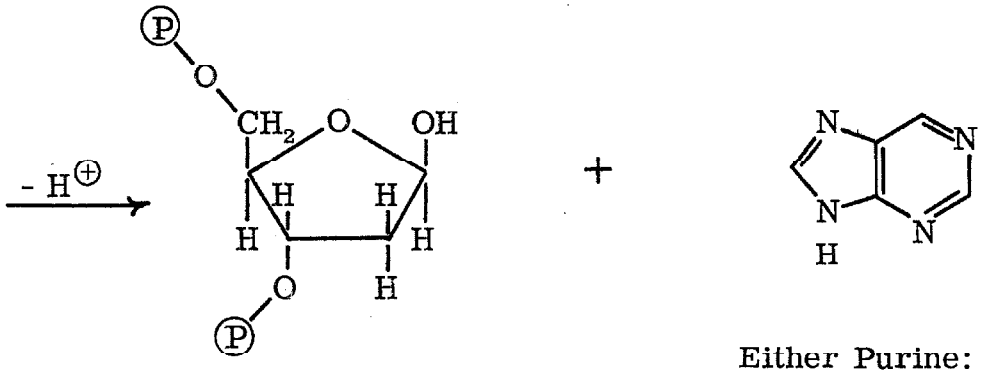
B. Degradation of DNA

We shall now discuss the methods used for degrading DNAs used in the study of the effect of length (molecular weight) on the rate of renaturation of DNA. The methods used resulted in random scission. A discussion of the effect of random scission on the rate of renaturation of DNA is included.

1. Hydrolysis as a method of DNA degradation.

The breaking of DNA phosphodiester bonds can be achieved by chemical, enzymatic, or mechanical means. The chemical process of hydrolysis has been described by several authors (Eigner, et al., 1961; Doty, et al., 1960; Greer and Zamenhof, 1962; Fiers and Sinsheimer, 1962). Chain scission is assumed to occur in two steps. The first step, depurination, occurs as follows:

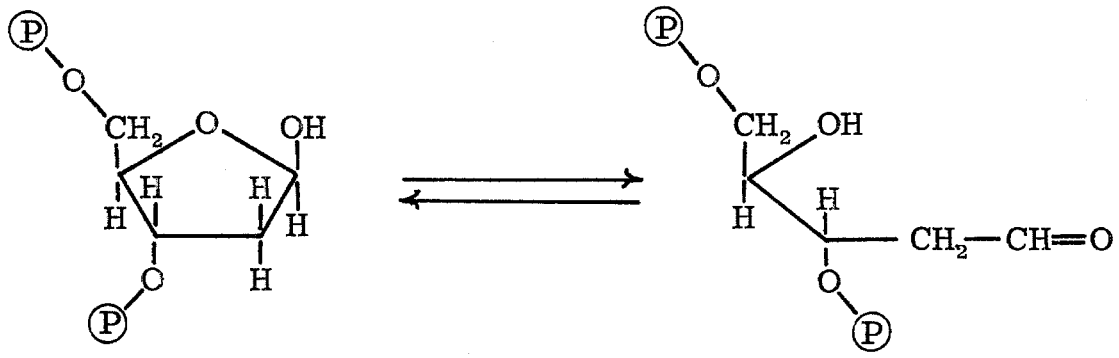


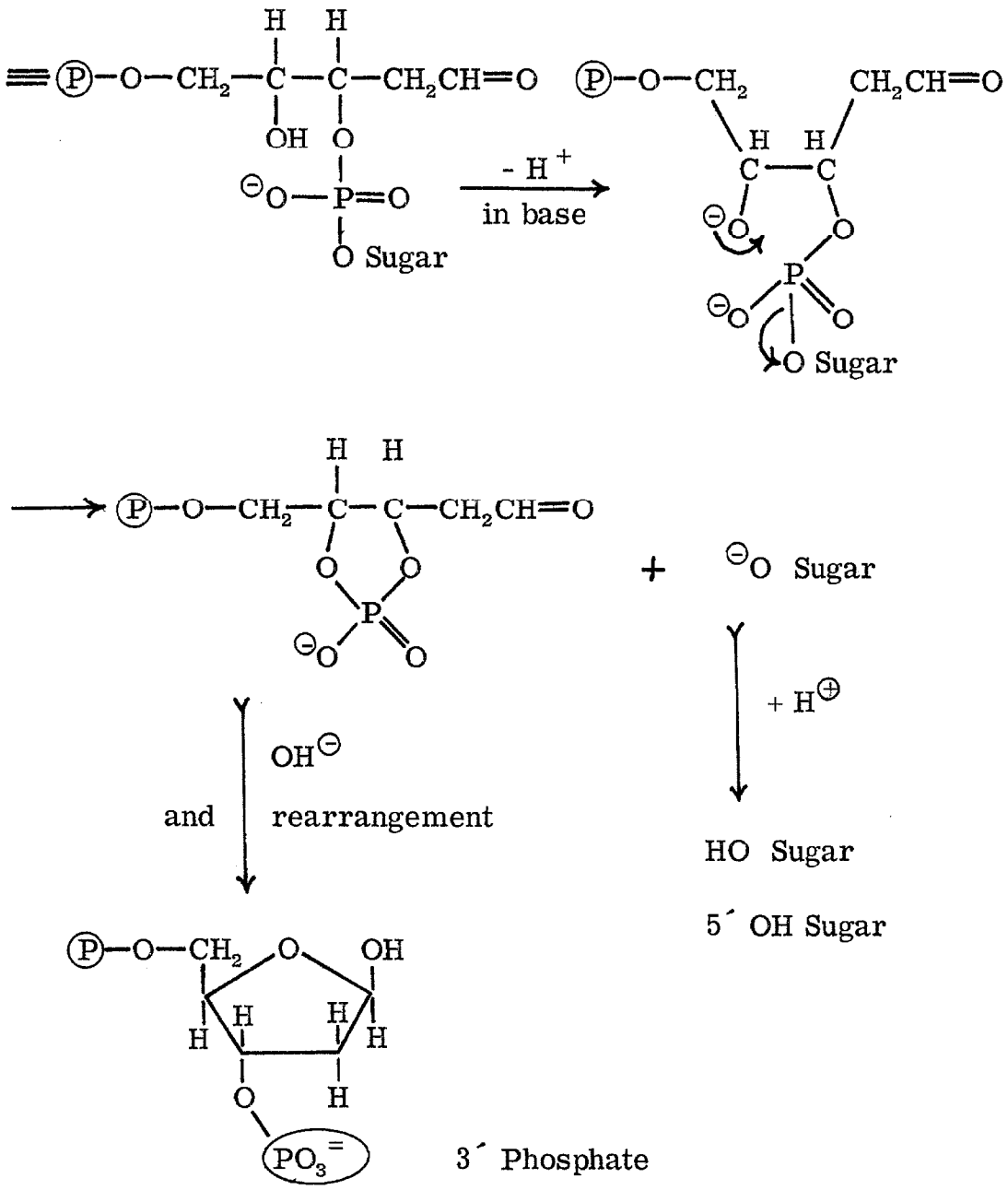


Either Purine:
Adenine or Guanine

All authors find, by measuring released purines (Greer and Zamenhof, 1962) or inactivation of biological activity (Fiers and Sinsheimer, 1962) that the reaction is acid catalyzed. It is also true that decreasing the salt concentration as well as increasing the temperature increases the rate of depurination. The reaction appears to have an activation energy of 30 ± 5 kcal/mole.

The second part of the chemical mechanism of degradation proceeds as follows:




- H+
in base
O Sugar
and
rearrangement
OH⊖
+ H⊕
⊖O Sugar
HO Sugar
5´ OH Sugar
3´ Phosphate

Thus in this mechanism scission following depurination involves a cyclic intermediate similar to the 2´-3´ cyclic intermediate in the hydrolysis of RNA in alkali.

Eigner, et al. (1961) found a half life for single stranded DNA of molecular weight 3.5×10^6 to be 15 min. at 100°C. and 3 hr. at 75° in 0.008 F. sodium ion, pH 6.8. Doty, et al. (1960) had quite analogous results. Fiers and Sinsheimer (1962) found 50% loss of biological activity, perhaps without chain scission, at 78° for 10 min. with øx 174 DNA (1.5×10^6 MWU) in 0.03 sodium ion at pH 7.5. Increasing pH caused decreasing rates of inactivation. Inactivation appears to be faster than chain scission and to occur to an appreciable extent at renaturation temperatures for small DNAs in moderate salt. We found that if melting preceded renaturation reproducible renaturation results in 0.4 - 1.0 F. sodium ion, pH 6.8 could only be obtained with DNAs of a single strand molecular weight up to 3×10^6 Daltons. Larger DNAs were degraded to this size under our practical method of heat denaturation. If a sample of T4 DNA were heated at 100°C. for 15 min. in 0.0008 F. sodium ion, the DNA was degraded to an $S_{20,w}$ at pH 13 of about 2, corresponding to a molecular weight of less than 30,000, indicating that, as stated above, greater degradation occurs at lower salt concentrations than in the high salt concentrations used in the experiments described here. In several renaturation experiments, performed on highly polymerized DNA, alkaline sedimentation velocity experiments were performed before and after the renaturation reaction was observed. The DNAs were never subjected to

temperatures above the renaturation temperature. In these test cases, identical velocities were obtained both before and after renaturation.

We have found that with denatured DNA, heated to cause depurination and subsequently treated with alkali for both molecular weight analysis and as the first step in the renaturation experiment, reproducible results are the rule for both rate constant and molecular weight determinations. Renaturation results with DNAs degraded in this manner were consistent with results obtained with DNAs degraded by mechanical means.

A possible form of hydrolysis, attack of phosphate by base to displace O-sugar$^-$ was not encountered in this work on renaturation kinetics. Enzymatic methods of degradation were not employed.

Random degradation, as for depurination followed by hydrolysis, is treated in Tanford (1961) pp. 611-618. Let k be equal to the rate constant for chain scission, and n be the nucleotide complement of unbroken DNA strands, then

$$\bar{x}_\omega = \frac{n\alpha^2 + 2(1-\alpha)\left[(1-\alpha)^n + n\alpha - 1\right]}{n\alpha^2}$$

where nkt = breaks/strand $\gg 1$, $n \ggg 1$ such that $kt \ll 1$,

$$1 - e^{-kt} \approx kt \text{ and } e^{-kt} \approx 1.$$

$$\bar{x}_\omega = 1 + \frac{2e^{-kt}\left\{e^{-nkt} + nkt - 1\right\}}{n(kt)^2}$$

$$\bar{x}_\omega \approx 1 + 2 \cdot 1\{0 + nkt - 1\}/n(kt)^2$$

$$\bar{x}_\omega \approx 2/kt \; ; \quad \text{likewise } \bar{x}_N = \frac{1}{kt}$$

This result is the same as is achieved starting with a randomly polymerized polymer rather than a homologous polymer solution in terms of molecular weight.

The molecular weights of degraded DNAs were determined by alkaline band velocity sedimentation experiments. We shall now discuss the types of band profiles obtained with DNAs prepared by random scission.

Over a centrifuge cell, within 5%, $\frac{D_1}{D_2} \approx \frac{S_1}{S_2}$

where D is the distance a segment of a band has travelled and S is the sedimentation coefficient of that band segment. A plot of O. D. versus x, the degree of polymerization, may be transformed into a plot of O. D. versus S or D using the transformation

$$dS = kx^{-0.6}\,dx$$

since the sedimentation coefficient is related to molecular weight by

$$S = k' M^{0.4} = k'' x^{0.4} \qquad \text{(Studier, 1965)}$$

No account is taken of radial dilution due to sector shape or to diffusion, which is assumed to be negligible for large DNAs.

Thus the new plot of absorbance versus S is given by a plot of

$$kx(S)^{1.6}[M_x] \quad \text{versus} \quad S(x)$$

For $x \ll n$, $[M_x] = Ce^{-kxt}$. The maximum of the absorbance versus S plot is obtained when $x(S) = 1.6/kt$. That is, the use of the sedimentation velocity of the maximum is in error compared to the weight average sedimentation velocity by

$$S(\overline{M}_\omega) = (1.25)^{0.4} S(\text{Max})$$

or $$\Delta \log S = 0.04$$

The error on sedimentation velocity is about 9%. The kinetics results are not sensitive to such a small error in S. The error is in such a direction as to partially mask the effect of entering the first random scission on the rate of renaturation, an effect discussed later on in this chapter.

Normalized absorbance (normalized to the maximum absorbance) versus normalized sedimentation coefficient (normalized to the sedimentation coefficient at the maximum absorbance) is plotted theoretically in Figure 3-1 along with the experimental trace of one photograph from a sedimentation run in alkali on E coli DNA (also a normalized plot). The DNA had been heated in 1.0 F. NaCl, pH 7.0 for 2 hours at 100°C. Note that the curves, which are superimposable, appear nearly symmetric to the eye. This fact makes the determination of the maximum a relatively easy chore. It is also important to note that the distribution plotted is independent of sedimentation velocity. Thus if sufficient breaks (more than 10 per whole molecule) are made in a DNA, subsequent degradation is subject to the same type of distribution.

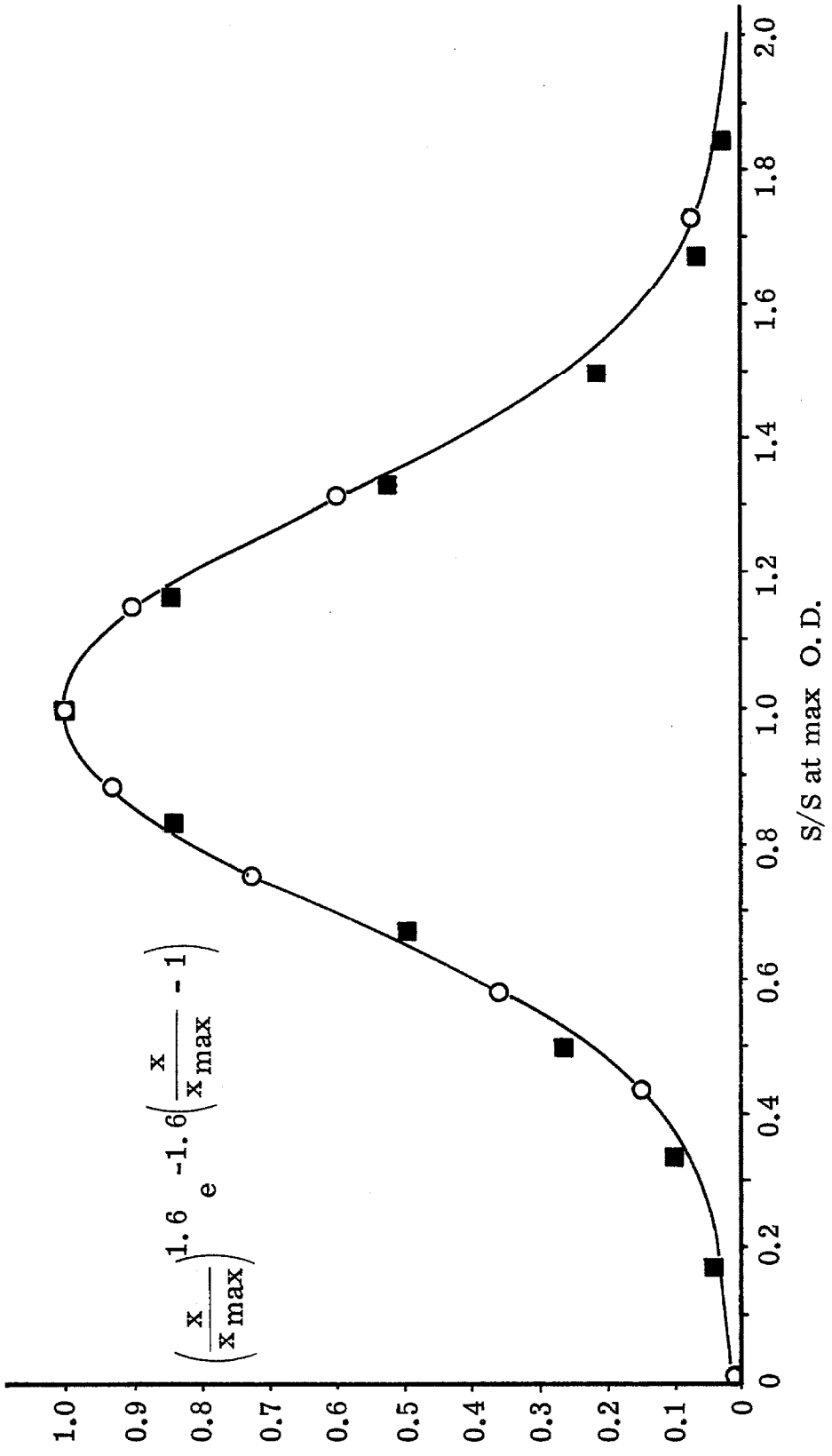
Figure 3-1

The Experimental and Theoretical Distribution of Heat Plus Alkali Degraded Material in a Centrifuge Cell

O: theoretical points

■: experimental points

E coli DNA. $S^{pH\ 13}_{20,w}$ = 12.3. Degraded by heating 2 hrs. at 100°C. in 1.0 Na^+, pH 6.8.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
S/S at max O.D.
$\left(\frac{x}{x_{max}}\right)^{1.6} e^{-1.6\left(\frac{x}{x_{max}} - 1\right)}$

The procedure of heating followed by alkaline treatment was used for producing some DNA solutions in the general range of sedimentation velocities of 12.3 to 28.5 S. Mechanical methods were used to produce sizes corresponding to S values of 3.75 - 20 S.

2. Mechanical processes of degradation

Mechanical means of degradation were applied to native DNA although denatured DNA in alkali may likewise be sheared (Davison and Felsenfeld, 1966). Shear can be accomplished by stirring or flow through a capillary (Zimm and Harrington, 1965) as well as by sonic means (Pritchard, et al., 1966). Both methods were employed in this work. Passage of a DNA solution through a No. 27 gauge syringe needle 15 to 20 times with maximum pressure applied by hand quite reproducibly produced DNAs with alkaline sedimentation velocities of 20 ± 1.5 S. The spread of DNA sizes is not significantly greater than that achieved with more sophisticated apparati.

Sonication was used to degrade DNAs to molecular weights corresponding to alkaline S values of 3.75 - 9.6 S. A Branson Sonic Power Model S125 Sonifier was employed. With a solid tiphorn, irradiation at power levels 6 - 8 for pulsed exposures of 30 seconds, adding up to 2 - 4 minutes, of water cooled solutions of DNA saturated with and covered with helium, the DNA was degraded to a size corresponding to S (pH 13) of around 4. A tapped end tiphorn was used with ice cooled DNA solutions for similar application times at power level 2 in an atmosphere of air. Sizes were produced corresponding to about 8 S. The former procedure will be termed heavy

sonication and the latter light sonication. In all cases the tips were cleaned by sonication in salt water and distilled water until no absorbance was introduced into the solution as observed in the U. V.

The molecular size distributions produced by sonic degradation were comparable to those produced by shear degradation and hydrolysis. Band velocity sedimentation experiments performed in alkali showed nearly symmetric bands.

3. Effects of random scissions or circularly permuted strands on the rate of renaturation of DNA

All of the methods used to degrade DNA led to random degradation products in which the complementary chains would not be expected to be perfectly matched. We shall now consider the effect of this mismatching on the rate of renaturation of DNA.

Consider the rate of base pair formation to be proportional to the number of nucleotides contained in a given molecule.

As an example, consider two strands of 32 segments (or nucleation sites).

$$a_1\, a_2\, a_3\, a_4 \cdots\cdots a_{10}\, a_{11}\, a_{12}\, a_{13} \cdots\cdots a_{30}\, a_{31}\, a_{32}$$

$$b_1\, b_2\, b_3\, b_4 \cdots\cdots b_{10}\, b_{11}\, b_{12}\, b_{13} \cdots\cdots b_{30}\, b_{31}\, b_{32}$$

Let the rate of base pair formation be proportional to 32^2. Break these molecules exactly in half. Then the rate of each half is proportional to 16^2 or the overall rate 2×16^2. For fourths, the rate would then be 4×8^2 or rate max/number of parts. These examples would

illustrate proportionality between rate of renaturation and length of molecules. Consider now the case where the breaks are entered randomly but the lengths are all comparable. Consider the possible segments which could react with the 4-mer, $a_{10}\, a_{11}\, a_{12}\, a_{13}$. The sequence $b_{10}\, b_{11}\, b_{12}\, b_{13}$ will be produced $\frac{1}{4}$th of the time by random scission of the complementary b strand; the sequences b_{9-12} and b_{13-16} will be produced $\frac{1}{4}$th of the time, and similarly for b_{7-10} plus b_{11-14} and b_{8-11} plus b_{12-15}. The respective rates of base pair formation will be proportional to the square of the number of overlapping segments. Thus,

$$\text{rate} \sim \tfrac{1}{4}(4^2 + 2\cdot 3^2 + 2\cdot 2^2 + 2\cdot 1^2) = 11$$

$$v/v_{\text{matched}} = 11/16$$

For the general case, $\text{rate}/\text{rate}_{\text{matched}}$ may be seen to be

$$\left\{ 2 \sum_{n=1}^{L-1} n^2 + L^2 \right\} / L^3 .$$

In the case we have just observed, $L = 4$,

$$2 \sum_{n=1}^{L-1} n^2 = 2(L-1)(L)(2L-1)/6 = 28$$

$$(16 + 28)/64 = 44/64 = 11/16$$

Expanding the above expression

$$\{L^2 + \frac{2}{6}(L - 1)(L)(2L - 1)\}/L^3$$

$$= \frac{1}{L^3}(L^2 + \frac{4L^3}{6} - \frac{6L^2}{6} + \frac{2L}{6})$$

$$= \frac{2}{3} + \frac{1}{3L^2} = \frac{2}{3} + \frac{1}{48} = \frac{32}{48} + \frac{1}{48} = \frac{33}{48} = \frac{11}{16}$$

As soon as random scission produces random segments,

$$v/v_{matched} = \frac{2}{3} + \frac{1}{3L^2}$$

For any reasonable length of L, the segment length,

$$v/v_{matched} = \frac{2}{3}$$

These arguments are valid no matter what is the dependence of nucleation rate constant on size, since they were derived to compare randomly and matched degraded samples of DNA of the same size. The factor of 2/3 times the maximum rate should apply for all but whole non circularly permuted DNA molecules such as T7 whole molecules. For any circularly permuted molecules such as T4, the rate of renaturation of whole molecules would be like T7 whole molecules only if the DNA cyclized faster than polymerization took place. This concentration dependent effect was not observed because T4 DNA molecules without breaks were not studied. For any circular molecule, such as SV40, renaturation could only be studied after placement of breaks in the strands. Only for the case of broken single strands renaturing with single strand circles should the faster rate as with

whole genetically linear molecules be observed.

We did not observe a very large, if any, unexpected increase in the rate of whole T7 molecules. The failure to observe any effect may be due to the presence of some trailing material in a band velocity run representing broken single strands. Another masking effect could be the slight disagreement between the weight average molecular weight and band sedimentation maximum molecular weight as previously mentioned. At most, the effect would be one that would displace log k_2 by only 0.176, which would be barely above experimental limitations of error. See Figures 1-7 and 1-8.

C. Determination of the Molecular Weights of Denatured DNA

The length of single stranded DNA in alkaline sedimentation has been correlated with the sedimentation coefficient by Studier (1965) and Abelson and Thomas (1966). The equation relating length to alkaline sedimentation coefficient has been found to be $S_{20,w}^{pH\,13} = KM^{\alpha}$ where

α (Studier, 1965) = 0.40

α (Abelson and Thomas, 1966) = 0.38

We have used the average of the relations of Studier and Abelson and Thomas in considering the relation of the rate of renaturation of DNA and molecular weight. The value $\alpha = 0.39 \pm 0.02$ should encompass all possible errors.

The molecular weights of samples of T4 and T7 DNAs were determined by alkaline sedimentation velocity experiments. Band sedimentation velocity in the analytical ultracentrifuge (Vinograd, Bruner, Kent and Weigle, 1963) or boundary velocity sedimentation in the ultracentrifuge were used. In the former case, 50 microliters of $A_{260} = 0.2$ were used in 30 mm path length cells containing 3.0 F. CsCl, 0.1 F. NaOH, density 1.37. In the latter case, the DNA, $A_{260} = 0.4$ was sedimented in 1.0 F. sodium ion at pH 13 in 12 mm path length cells. In CsCl, pH 13, the correction factors for Cs vs. Na DNA, viscosity and buoyancy were 1.22 for a 50% GC DNA and 1.24 for T4 DNA. In 1.0 F. sodium ion, the correction factors were 1.16 and 1.18 respectively. All sedimentation velocities stated are

good to 5%. Examples of samples of T7 DNA run by both band and boundary sedimentation are given in Table 3-1.

TABLE 3-1

Alkaline Sedimentation Velocity Determinations

DNA	Method	$S_{20,w}^{pH\ 13}$
T7 DNA	Boundary	34.1 ± 0.7
T7 DNA	Band	33.7 ± 0.7
Sheared T7 DNA	Boundary	18.8 ± 0.4
Sheared T7 DNA	Band	18.2 ± 0.4

Of the two methods, band velocity sedimentation is preferable and was used for most of the S value determinations used in this work.

In the determinations made on degraded DNAs, no matter by what method, symmetric bands and boundaries were obtained.

D. Experiments Relating the Rate of Renaturation of T4 DNA and T7 DNA with Alkaline Sedimentation Coefficients, and hence with Molecular Weight

The rate of renaturation of T4 DNA was determined at 67.5°C. (T_m - 24.5°C.) and at 73°C. (T_m - 19°C.) in a few cases over a large range of molecular weights. Table 3-2 lists rate constants for renaturation of T4 DNA, versus alkaline sedimentation velocity, at a DNA concentration of 70 micrograms/ml (A_{260} = 1.4).

TABLE 3-2

$S_{20,w}^{pH\ 13}$	Prep. Method	k_2 (liters/mole-sec)
41	As obtained	270
28.5	Hydrolysis **	200 *
23.7	Hydrolysis **	138
20.5	Shear	116 *
15.5	Hydrolysis **	94
8.0	Light sonication	40.6 *
4.9	Heavy sonication	21
4.2	Heavy sonication	16.2
3.75	Heavy sonication	15

* Checked to be similar at 73°C.

** Hydrolysis times in 0.875 NaCl, 0.01 phosphate buffer, pH 6.8 at 100°C. were 5 minutes, 10 minutes and 20 minutes to produce molecular weights corresponding to $S_{20,w}^{pH\ 13}$ = 28.5, 23.7 and 15.5 S respectively. These results demonstrate the fact that use of melting as a method of denaturation is only satisfactory for small DNAs.

Results shown in Table 3-3 are for T4 DNA at 7 micrograms/ml, or 10 times more dilute solutions of DNA than the other solutions used for experiments reported in Table 3-2.

TABLE 3-3

$S^{pH\,13}_{20,w}$	Prep. Method	k_2 (liters/mole-sec)
30	Hydrolysis (5', 100°)	230
20	Mechanical shear	120
7.7	Light sonication	36

Results obtained for T7 DNA at 70 micrograms/milliliter are given in Table 3-4.

TABLE 3-4

$S^{pH\,13}_{20,w}$	Prep. Method	k_2 (liters/mole-sec)
34.7	As obtained	1880
20.5	Mechanical shear	980
14.2	Hydrolysis (60', 100°, pH 7.0)	640
9.6	Light sonication	324
5.15	Heavy sonication	158

Results obtained for T7 DNA at 7 micrograms/milliliter are given in

Table 3-5.

TABLE 3-5

$S_{20,w}^{pH\ 13}$	Prep. Method	k_2 (liters/mole-sec)
30	As obtained	1700
19	Mechanical shear	1040
7.5	Light sonication	340

The results of Tables 3-4 and 3-5 are plotted in Figure 3-3, which is a duplicate of Figure 1-8. The results of Tables 3-2 and 3-3 are plotted in Figure 3-2, which is a duplicate of Figure 1-7.

The straight lines in Figures 3-2 and 3-3 are drawn with a slope of 0.78 or

$$\text{Log}_{10}\ S_{20,w}^{pH\ 13} = 0.78\ \log_{10}\ k_2 + \text{Constant}$$

then as

$$\text{Log}_{10}\ S_{20,w}^{pH\ 13} = 0.39\ \log_{10}\ M + \text{Constant}$$

then $k_2 = \text{Constant} \cdot M^{0.50}$

The important conclusion is that the rates of renaturation of T4 and T7 DNAs at two concentrations (and at two temperatures) obey the rule that the rate constant of renaturation of any DNA broken into pieces of various size depends on the square root of the size. The different absolute values for T4 and T7 DNAs of comparable molecular

Figure 3-2

The Rate Constant for Renaturation of T4 DNA
as a Function of Alkaline Sedimentation Velocity

O: 70 μg/ul

■: 7 μg/ul

The theoretical straight line fits the equation

$$\text{Log}_{10}\ S_{20,w}^{pH\ 13} = 0.78\ \text{Log}_{10}\ k_2 + \text{Constant}$$

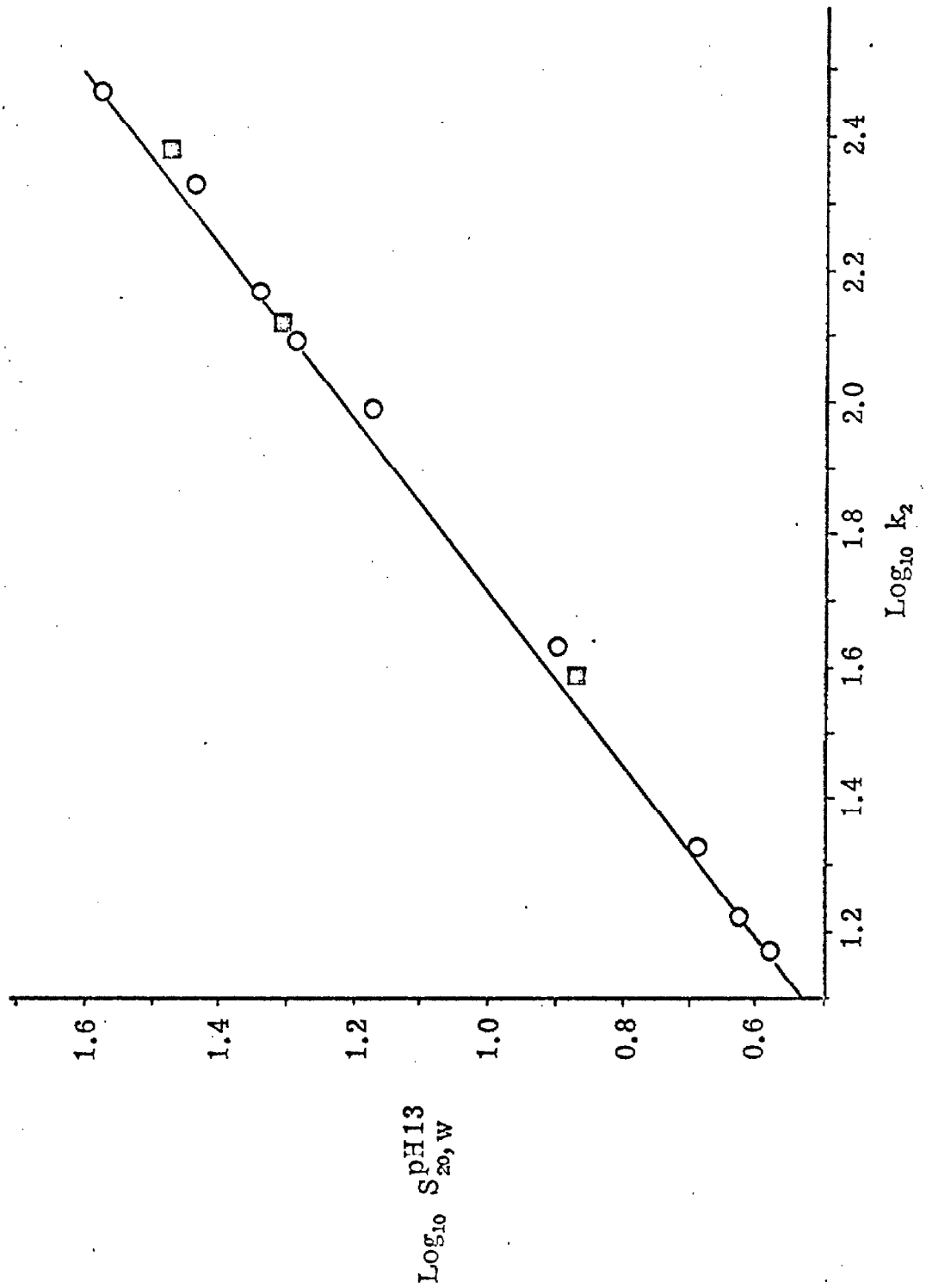

1.6
1.4
1.2
1.0
0.8
0.6
Log10 S20,w pH13
1.2
1.4
1.6
1.8
2.0
2.2
2.4
Log10 k2

Figure 3-3

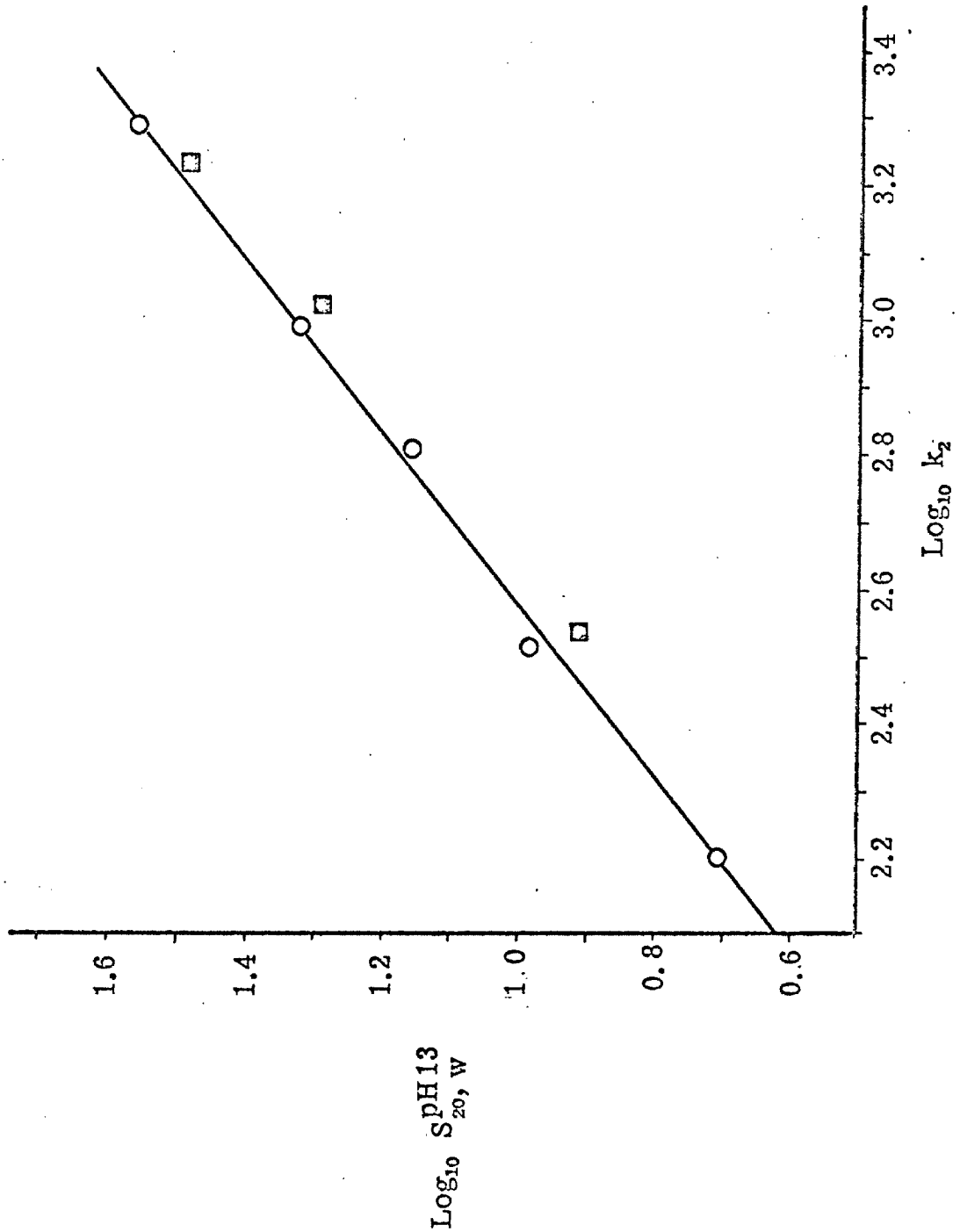
The Rate Constant for Renaturation of T7 DNA
as a Function of Alkaline Sedimentation Velocity

O: 70 μg/ul

■: 7 μg/ul

The theoretical straight line fits the equation

$$\text{Log}_{10}\ S_{20,w}^{pH\ 13} = 0.78\ \text{Log}_{10}\ k_2 + \text{Constant}$$


1.6
1.4
1.2
1.0
0.8
0.6
2.2
2.4
2.6
2.8
3.0
3.2
3.4
$Log_{10}\ S^{pH13}_{20,w}$
$Log_{10}\ k_2$

weight are related to the complexities of the DNAs before shear. This problem is discussed in Chapter 4.

We have previously shown that the measured rate constant, k_2, is related to the rate constant for nucleation by the equation

$$k_2 = k_N L/N$$

Assuming the result that the experimental rate constant is inversely proportional to complexity, as will be proved in the next chapter, we find k_N, the nucleation rate constant, to be inversely proportional to the square root of the length.

$$k_N = \text{Constant}/L^{\frac{1}{2}}$$

E. Theoretical Discussion of the Dependence of the Rate of Renaturation on Molecular Weight of Single Stranded DNA

1. Consideration of a diffusion controlled mechanism (translational).

The results giving a dependence of the rate constant for nucleation on the inverse of the square root of the molecular weight are in agreement with a diffusion controlled mechanism depending on the translational diffusion constant of the DNA strands which is itself inversely proportional to the square root of the molecular weight of the strands. The translational diffusion coefficient is also proportional to T/η.

$$\text{Translational Diffusion Coefficient} = D = \text{Constant } T/\eta L^{\frac{1}{2}}$$

The viscosity and temperature dependence of the experimental rate constant for renaturation is shown in Chapter 6 to be exactly in agreement with a translational diffusion controlled rate limiting step.

The diffusion coefficient of a DNA nucleotide may be estimated to be about 5×10^{-6} cm^2/sec. The rate of diffusive encounters is given by $4\pi D \sigma_{AB} N_A N_B$ for $A + B \underset{k_2}{\overset{k_1}{\rightleftharpoons}} AB$ (Wang and Davidson, 1966b). That is, $k_1 = \dfrac{4\pi D \sigma_{AB} N_0}{1000}$ liters/mole-sec

$$k_1 \ (\sigma_{AB} = 5\text{Å}) \approx 4 \times 10^{14} D$$

for $D = 5 \times 10^{-6}$, $k_1 \approx 2 \times 10^9$ liters/mole-sec. This is the rate observed for diffusion controlled reactions cited in the literature for

relatively small molecules (Benson, p. 503, 1960). For large polymers, diffusion controlled chain termination reactions of polymerization are slower, as is expected from the molecular weight dependence of the diffusion coefficient.

The rate of renaturation of whole T7 DNA under optimum conditions is about 2000 liters/mole-sec. The length and complexity are equal to about 4×10^4. Thus k_2 may be established as

$$k_2 = 4 \times 10^5 \, L^{\frac{1}{2}}/N \text{ liters/mole-second}$$

and, therefore,

$$k_N = 4 \times 10^5/L^{\frac{1}{2}} \text{ liters/mole-second.}$$

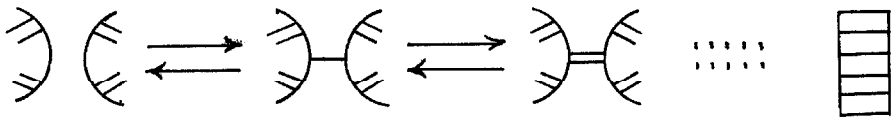
For a complexity of 1 and a length of 1, we find a value of 4×10^5 liters/mole-sec, whereas by consideration of a translational diffusion mechanism, we predicted a rate of 2×10^9 liters/mole-sec for base pair interactions. We find, then, that the real rate is about 5000 times too slow to be accounted for by a simple translational diffusion mechanism.

This value of 4×10^5 liters/mole-sec for length and complexity 1 was used in Chapter 2 for the determination of the forward rate constant for sequential base pair formation, k_f. Note that we predict from the equation above a rate constant for poly A plus poly U of 8×10^6 liters/mole-sec under the conditions employed in our experiments and with a length of 400. Ross and Sturtevant (1962) find a rate of 1.5×10^6 liters/mole-sec at 34°C. in 0.5 F. NaCl. If we correct for salt, temperature and viscosity, as described in Chapter 6, we

predict, from experimental data, a rate of 6×10^6 liters/mole-sec, which is in good agreement with the theoretical result with DNAs. As the homopolymer investigations were done on RNAs, it is surprising that such good agreement is obtained. Even if we consider charge effects (small due to high salt), or steric effects, we cannot account for this slow rate if the reaction is diffusion controlled. In Chapter 6, the problem of the dependence of the rate of renaturation on salt is discussed. The data compared so far were obtained in a sodium ion concentration of 1 M., where a DNA strand should see negligible charge effects when approaching a second DNA strand. Over a range of 0.4 M. to 4.0 M. sodium ion, there is only a small dependence of the rate of renaturation on salt concentration -- not enough to correct for the factor of 5000 between predicted diffusion controlled rates and experimental rates in high salt. Furthermore, one might expect the diffusion controlled rate to be even faster because of segment diffusion. A discussion of segment diffusion is given in Wang and Davidson, 1966b.

Thus, we need to take the sequential model presented in Chapter 2.

$$A + A' \underset{k_{0,-1}}{\overset{k_{-1,0}}{\rightleftharpoons}} C_0 \underset{k_{1\,0}}{\overset{k_{0\,1}}{\rightleftharpoons}} C_1 \underset{k_{2\,1}}{\overset{k_{1\,2}}{\rightleftharpoons}} C_2 \;\cdots\cdots\; C_N$$



In order to observe an effect of decreasing rate with increasing temperature at a temperature significantly below T_m, it is necessary for the rate constants of base pair formation to play a part. The backward rate from the completely native state is zero in this temperature range.

We find, by fitting the theoretical expression to the experimental data extrapolated to complexity and length 1, a forward rate constant for the segmental model to be 3×10^9 sec^{-1}. There is a stacking parameter, σ, of the order of 0.001 in the ratio $k_{01}/k_{10} = \sigma s$. This can effect either k_{01} or k_{10}, and we write

$$k_{01} = k_f \sigma_f, \quad k_{10} = k_f/s\,\sigma_b, \quad \text{with } \sigma_f \sigma_b = \sigma .$$

If we set $\sigma_f = 1$, that is, the stacking effects only the dissociation rate, then a pre-equilibrium exists before the formation of the second base pair as k_{12} is then much greater than k_{10}. The forward rate constant calculated above, 3×10^9 sec^{-1}, is of the same order of magnitude as $k_{0,-1}$, the rate constant for diffusion out of the impact volume. If, instead of changing the backward rate constant for the first base pair formation step, the stacking effect alters the forward rate constant ($\sigma_b = 1$), a pre-equilibrium exists before the formation of the first base pair, as k_{01} is much greater than $k_{0,-1}$. In either case, the reaction is not controlled by translational diffusion. However, the forward rate constant for base-pairing is fast enough to be considered as a diffusion controlled step of a different kind. We shall consider this possibility after consideration and exclusion of the possibility of

significant excluded volume effects accounting for either the molecular weight dependence or the deviation from predicted diffusion controlled rates.

2. The excluded volume problem

For a polymer with a radius of gyration R_G, with r as the distance from the center of mass in solution, the probability of finding a reacting segment for a random coil is given by

$$P(r) = \left(\frac{3}{2\pi R_G^2}\right)^{3/2} e^{-3r^2/2R_G^2}$$

The probability of finding it anywhere in space is the integral over all space which is equal to 1 for this normalized function. The probability of finding a reacting segment of a second random coil in a volume element at a distance r from the center of mass of the first random coil, where the center of mass of the second random coil is displaced a distance d from the center of mass of the first random coil, is given by

$$P(r,d) = \left(\frac{3}{2\pi R_G^2}\right)^{3/2} e^{-\frac{3}{2R_G^2}(r^2+d^2-2rd\cos\phi)}$$

The rate of reaction as a function of d is proportional to the product of the probabilities integrated over all space:

$$V(d) = \left(\frac{3}{2\pi R_G^2}\right)^3 \int_0^{2\pi} d\theta_1 \int_0^{\pi/2} \text{Sin}\ \phi_1\, d\phi_1 \int_{-\infty}^{\infty} e^{-\frac{3}{2R_G^2}(2r^2 + d^2 - 2rd\,\text{Cos}\,\phi)}\, r^2\, dr$$

If we now allow the polymers to completely interpenetrate, integrating over all d we find that the rate does not depend on any of the properties of the polymer.

$$V(d) = \left(\frac{3}{2\pi R_G^2}\right)^3 \cdot 2\pi\, e^{-\frac{3d^2}{2R_G^2}} \int_0^{\pi/2} \sqrt{\frac{\pi R_G^2}{3}} \left(\frac{R_G^2}{6} + \frac{d^2\,\text{Cos}^2\phi}{4}\right) e^{\frac{3}{R_G^2}\frac{d^2\,\text{Cos}^2\phi}{4}} \text{Sin}\ \phi\, d\phi$$

$$V(d) = \left(\frac{3}{2\pi R_G^2}\right)^3 \cdot 2\pi\, e^{-\frac{3d^2}{2R_G^2}} \frac{R_G^3}{6}\sqrt{\frac{\pi}{3}}\, e^{\frac{3d^2}{4R_G^2}}$$

$$V(d) = \frac{9}{8\pi^2 R_G^3}\sqrt{\frac{\pi}{3}}\, e^{-\frac{3d^2}{4R_G^2}}$$

$$V_{\text{all d}} = \frac{9}{8\pi^2 R_G^3}\sqrt{\frac{\pi}{3}}\int_0^{2\pi} d\theta_2 \int_0^{\pi} \text{Sin}\,\phi_2\, d\phi_2 \int_0^{\infty} e^{-\frac{3d^2}{4R_G^2}}\, d^2\, dd$$

$$V_{\text{all d}} = \frac{9}{2\pi R_G^3}\sqrt{\frac{\pi}{3}}\;\frac{R_G^2}{3}\times\sqrt{\frac{4R_G^2\pi}{3}} = 1$$

For the case of an excluded volume to exclude d less than d_0, we calculate

$$V_{\substack{\text{excl vol}\\ \text{to } d_0}} = \frac{9}{2\pi R_G^3}\sqrt{\frac{\pi}{3}}\int_{d_0}^{\infty} e^{-\frac{3d^2}{4R_G^2}}\, d^2\, dd$$

$$V_{\substack{\text{excl vol}\\ \text{to } d_0}} = \frac{4}{\sqrt{\pi}}\int_{\frac{3d_0^2}{4R_G^2}}^{\infty} e^{-u^2}\, u^2\, du$$

$$V_{\substack{\text{excl vol} \\ \text{to } d_0}} = 1 - \frac{4}{\pi} \int_0^t e^{-u^2} u^2 \, du$$

$$= 1 + \frac{2te^{-t^2}}{\pi} - \frac{2}{\pi} \int_0^t e^{-t^2} \, dt$$

where $t = \sqrt{\frac{3d_0^2}{4R_G^2}}$

$$V_{\substack{\text{excl vol} \\ \text{to } d_0}} = 1 + \left(\frac{3}{\pi}\right)^{\frac{1}{2}} \frac{d_0}{R_G} e^{-\frac{3d_0^2}{4R_G^2}} - 2\phi\left(\frac{3d_0^2}{4R_G^2}\right)$$

where $\phi(t) = \frac{1}{\sqrt{2\pi}} \int_0^t e^{-t^2/2} \, dt$

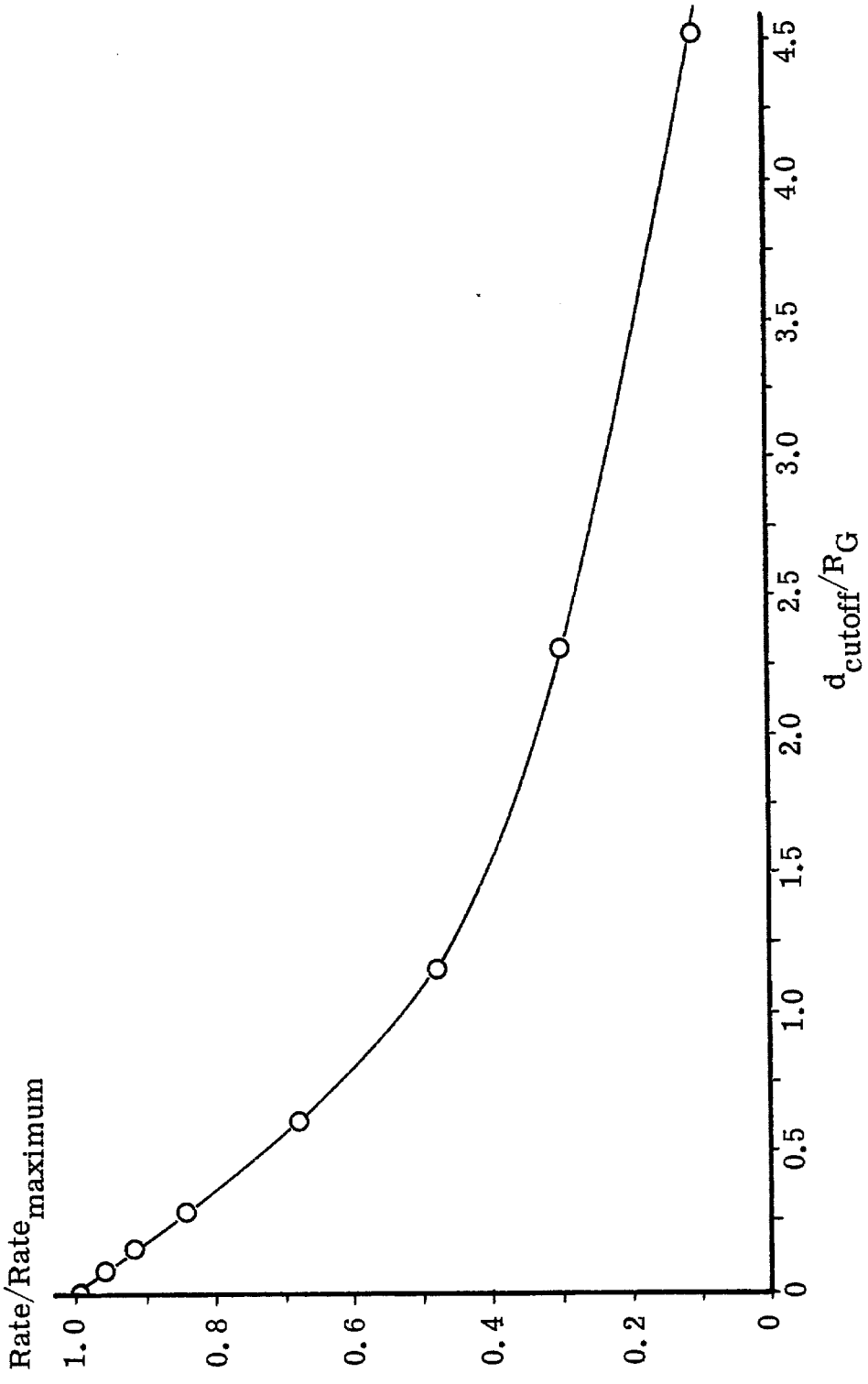
We may look up the area of $\phi(t)$ in any table of Gaussian distribution and subsequently calculate the rate/rate-maximum = $V_{\text{excl vol to } d_0}$. A plot of the relative rate as a function of the minimum impact diameter in terms of R_G is given in Figure 3-4. Any value of d_0 greater than two times the radius of gyration would be unreasonable. We see in Figure 3 that if this maximum value is taken, the rate would be decreased by only a factor of 3. Over the range of molecular sizes studied, the square root of the molecular weight effect amounted to a

Figure 3-4

The Rate of Renaturation of DNA with Limited Penetration Relative to the Rate of Renaturation without a Penetration Limit

d_{cutoff} = minimum distance of approach of the centers of mass

R_G = radius of gyration


Rate/Rate$_{maximum}$
1.0
0.8
0.6
0.4
0.2
0
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
d_{cutoff}/R_G

factor of 20. Alteration of the excluded volume could account for only a small part of this change. Also, the disagreement between the theoretically expected rates for a diffusion controlled reaction and the observed values, a difference of 5000, could not arise at all from an excluded volume effect. We conclude that if any excluded volume effect is present, it would be too small to be detected.

3. Concerning the possibility of diffusion controlled winding

We have seen that a preequilibrium must exist prior to complete base-pairing to account for the temperature profile and the absolute values of the renaturation rate constants.

We have seen that excluded volume and aggregation effects should not be important. In the latter case, intermolecular aggregation would be concentration dependent, whereas we have seen in Chapter 1 that the rate constants are essentially independent of concentration. Intramolecular aggregation should be negligible for the smallest DNAs, which DNAs still follow the same general rate equation.

The dependence of the rate of renaturation on viscosity (Chapter 6) is in agreement with a hydrodynamically controlled rate limiting step. The dependence of the rate of renaturation on molecular weight is unexplained.

In Chapter 2, we presented a sequential model for the renaturation reaction. When the experimental data are extrapolated to complexity and length of unity, there is a rate constant for renaturation of 4×10^5 liters/mole-second. The model then gives for k_f, the forward rate constant for base pair formation, a value of

3×10^9 sec^{-1}. We propose, more or less ad hoc, to interpret the observed dependence of nucleation rate on size and viscosity as being due to a variation of k_f with size and viscosity. That is, we view the rate controlling step in the k_f process as being the diffusion of the two bases, adjacent to the helical section, to each other. The rate constant of 3×10^9 sec^{-1} may be related to the time to diffuse a mean distance of 5 Å by

$$\overline{x}^2 = 2Dt \qquad \text{with } 1/t = k_f$$

Then $D = 3.75 \times 10^{-6}$ $cm^2/sec.$

The corresponding diffusion coefficient for the base in the length 1 case is in agreement with the value expected for the diffusion coefficient of a monomer. If the diffusion coefficient is proportional to T/η and to the reciprocal of the square root of the molecular weight, then all experimental results in molar sodium would be explained.

It is easy to imagine the diffusion control of the forward rate constant being dependent on size up to sizes of DNA of a statistical segment length. Why the diffusion coefficient governing the base pair formation reaction is not the segmental diffusion coefficient (Wang and Davidson, 1966b) for larger segments is not clear. Nevertheless, without any information to contradict this assumption, and with all other considerations of mechanism mentioned above, we must accept that something like this mechanism governs the renaturation of DNA.

CHAPTER 4

The Effect of DNA Complexity on the Rate of Renaturation

Contents: Page:

A. The Effect of Complexity on the Rate of Renaturation of DNA

The effect of complexity on the rate of renaturation of DNA was first noted by Marmur and Doty (1961), who found that phage DNA renatured faster than bacterial DNA, which in turn renatured faster than animal DNAs. In their work, an animal DNA, calf thymus DNA, did not renature at all on their time scale. One would expect that for DNAs sheared to the same size, the rate of renaturation should be proportional to the square of the concentration of nucleation sites (inversely proportional to the square of the molecular complexity) summed over all nucleation sites (directly proportional to the molecular complexity). That is, the rates of renaturation should be inversely proportional to the complexities of two DNAs being studied. For example, at a given molecular size obtained by similar fragmentation procedures, T7 DNA should renature $130 \times 10^6/25 \times 10^6$ times as fast, where the numbers in the ratio are the molecular weights (complexities) of the unbroken DNA molecles. The only assumption involved is that the number of nucleation sites per whole molecule is proportional to the length of the whole molecule. If we look at the data presented in Chapter 3, we will find that this result is approximately obtained with T4 and T7 DNAs. Bolton, et al. (1965) and Waring and Britten (1966) and Britten and Kohne (1967) have shown this inverse proportionality to be true for a variety of DNAs broken to the same molecular size by shear in a French Pressure device (McCarthy and Bolton, 1964).

We have verified these results for DNAs over a range of complexities of about 10^6. Table 4-1 gives the results for a variety of DNAs. The relation $6.0 \times 10^8 \ (S_{20,w}^{pH\ 13})^{1.25}/k_2$ is used to calculate approximate complexities in terms of molecular weight of the unique genetic complement of the organisms. The relation takes into account the molecular weight dependence discussed in Chapter 3.

TABLE 4-1

DNA	A_{260}	$S_{20,w}^{pH\ 13}$	k_2	$6.0 \times 10^8 \ S^{1.25}/k_2$
Ascites tumor	9.4	6.8	0.00276	2.4×10^{12}
E coli	3.5	12.3	5.2	2.6×10^9
E coli	1.75	12.3	4.92	2.8×10^9
E coli	0.23	12.3	5.7	2.4×10^9
T4	1.4	28.5	200	2.0×10^8
T4	1.4	8.0	40.6	2.3×10^8
T4	0.14	30	230	1.8×10^8
T4	0.14	7.7	36	2.1×10^8
N1	0.14	30	1570	2.7×10^7
N1	0.14	7.9	350	2.3×10^7
T7	1.4	34.7	1880	2.7×10^7
T7	1.4	5.15	158	2.9×10^7
T7	0.14	30	1700	2.5×10^7
T7	0.14	7.5	340	2.2×10^7
SV40	0.16	6.6	1120	5.7×10^6

Average values of the calculated complexities of the final column of Table 4-1 are given in Table 4-2 along with known complexity values and other pertinent data.

Complexities of DNAs were obtained from the following sources: SV40 DNA: Crawford and Black, 1964. T7 DNA: Abelson and Thomas, 1966 and Freifelder and Kleinschmidt, 1965. E coli DNA: Cairns, 1962; Cairns, 1963: Watson, 1965, p. 99. N1 DNA: Chapter 8 of this thesis. T4 DNA: Rosenbloom and Cox (1966) and Abelson and Thomas, 1966. Ascites tumor DNA: Watson, 1965, p. 415. GC contents of DNAs are described in Chapter 5.

TABLE 4-2

Complexity Effects Retabulated

DNA	Complexity	GC%	Calculated Complexity	Complexity/ Calculated Complexity
Ascites tumor	$2.5\pm0.5 \times 10^{12}$	45	2.4×10^{12}	0.80 - 1.25
E coli	$2.5\pm0.5 \times 10^{9}$	50	2.6×10^{9}	0.77 - 1.15
T4	$1.3\pm0.1 \times 10^{8}$	34	2.05×10^{8}	0.59 - 0.68
N1	$3.3\pm0.15 \times 10^{7}$	64	2.5×10^{7}	1.26 - 1.36
T7	$2.5\pm0.1 \times 10^{7}$	49	2.6×10^{7}	0.92 - 1.00
SV40	$3.3\pm0.1 \times 10^{6}$	41	5.7×10^{6}	0.56 - 0.60

Over a factor of 10^6 in molecular size (complexity), no error as large as a factor of two is observed. At a glance at Table 4-2, it is obvious that the complexity ratio complexity/calculated complexity

is too high for N1 DNA, a DNA of high GC%, and too low for T4 and SV40 DNAs, DNAs of lower GC% than the others of nearer to 50% GC. The GC dependence of the rate of renaturation will be discussed in Chapter 5.

The results shown in Table 4-1 and 4-2 are in agreement with the rule that the rates of renaturation of any two DNAs of the same molecular size are inversely proportional to the complexities of the two DNAs (within an error of a factor of 2 due to GC dependence). This is a restatement of the fact that the rate of renaturation obeys the law of mass action in terms of nucleation concentrations under defined conditions of comparison.

B. Renaturation of DNAs of Higher Organisms

It has been suggested that the DNAs of higher organisms cannot be renatured due to a competition reaction of mismatched segments with the reaction at the nucleation sites (Wada and Yamagami, 1964). Nevertheless, some success has been obtained in renaturation of animal DNAs by the agar-gel method (Britten and Kohne, 1966). We have renatured DNA extracted from the nuclei of ascites tumor cells (from a single cell line) using high A_{260} and long times to overcome the expected slow rate. It would be expected that the rate of renaturation of the DNA from mammalian cells would be about 1000 times slower than that obtained from bacterial sources (Watson, 1965). Thus to achieve the small amount of renaturation achieved with E coli DNA at $A_{260} = 1.0$ for 15 minutes, it would be necessary to renature a mammalian DNA for 25 hours at $A_{260} = 10$. Such an experiment was performed.

A sample of $A_{260} = 9.5$ was placed in a 2 mm path length cell. The balance was set, with a filter, so that the A_{260} corresponded to 0.86 on the chart paper. The DNA was then melted. The maximum achieved was 1.58. This corresponded to a hyperchromicity of 38%. The sample was cooled and a small fraction removed for electron microscopy. The sample was then allowed to heat to the renaturation temperature. The data are presented in Table 4-3.

There was, after renaturation, a melting curve corresponding to the gradual melting of the remaining denatured DNA and the sharp melting of the renatured material. The melting curve was as

TABLE 4-3

A_{260} chart	Time (min.)	$(A_{260} - A_{260, \infty})$ chart	$A_{260} - A_{260, \infty}$	Time (sec.)
1.45	16	0.59	2.95	960
1.44	52	0.58	2.90	3120
1.43	104	0.57	2.85	6240
1.42	152	0.56	2.80	9120
1.41	260	0.55	2.75	15600
1.40	408	0.54	2.70	24480
1.39	600	0.53	2.65	36000
1.38	828	0.52	2.60	49680
1.37	1020	0.51	2.55	61200

sharp as far less complex DNAs. A second sample was obtained for electron microscopy prior to the second melting. Uranyl stained Kleinschmidt photographs of the DNAs obtained before and after renaturation appear in Plates 4-1 and 4-2. The highly aggregated material in Plate 4-1 and to some extent in Plate 4-2 corresponds to denatured DNA in compact form in high salt at room temperature. Only after renaturation did a significant amount of apparently native material appear.

Figure 4-1 shows a rate plot for ascites tumor DNA. There is a minor fast renaturing component and a major slow renaturing component. The fast renaturing component could not be due to cooling resulting in residual hyperchromicity, as the DNA was below T_r at

the beginning of the experiment and kept at T_r throughout the course of the experiment. $T_r = T_m - 21°C.$ was used where $T_m = 95°C.$ Two rates are observed in Figure 4-1. The first rate constant was calculated after elimination of the slow rate was performed as indicated in Table 4-4. Also see Figure 4-2.

TABLE 4-4

$A_{260} - A_{260}$, corrected min.	Time (sec.)	$1/(A - A_\infty)$
2.95 - 2.81 = 0.14	960	7.15
2.90 - 2.80 = 0.10	3120	10.0
2.85 - 2.78 = 0.07	6240	14.3
2.80 - 2.77 = 0.03	9120	----

The faster renaturation has a rate constant of about 6.7 liters/mole-second, which is comparable to that of bacterial DNAs. The observed reaction took place over a range $A_{260} - A_{260,\infty} = 0.15$. That is, the faster reacting material was about 5% of the total reacting material. The slow rate was followed for only 8-9% of the total possible reaction due to limitations of time. The purpose of this experiment was primarily to show that a mammalian DNA could, in fact, be renatured and a rate of renaturation determined. The fast component of ascites tumor DNA was not studied any further. A study of the rate of renaturation of all nuclear DNA components (Waring and

Figure 4-1

The Second Order Rate Plot for Renaturation of Ascites Tumor DNA

The initial rate is calculated in Figure 4-2. The final rate -- that presumed to represent non-repeated sequences of ascites tumor DNA -- is calculated to be 2.76×10^{-3} liters/mole-sec.

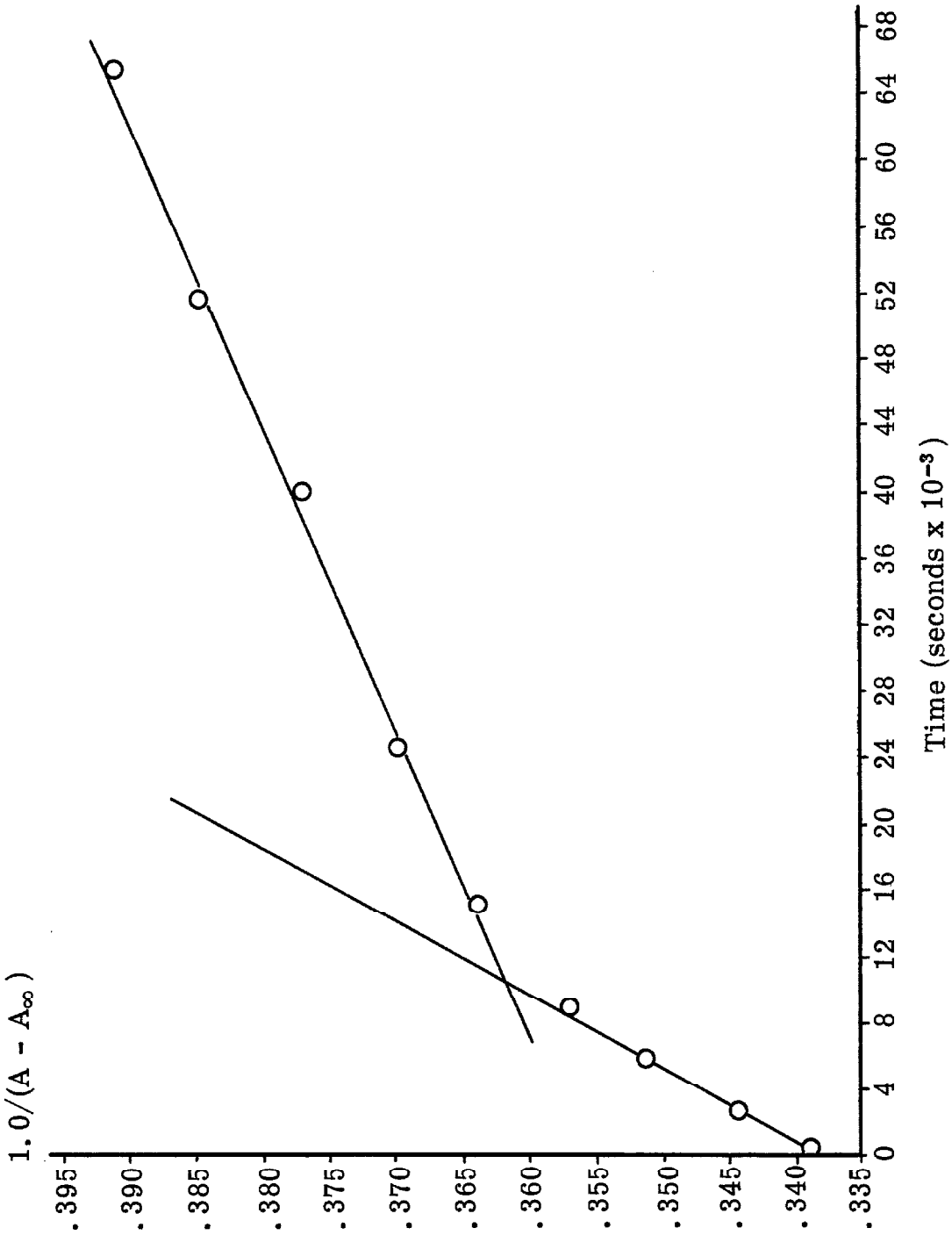

1.0/(A - A∞)
.395
.390
.385
.380
.375
.370
.365
.360
.355
.350
.345
.340
.335
0
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
68
Time (seconds x 10⁻³)

Figure 4-2

A Second Order Rate Plot for Fast Renaturing Ascites Tumor DNA

The rate constant calculated is 6.7 liters/mole-sec.

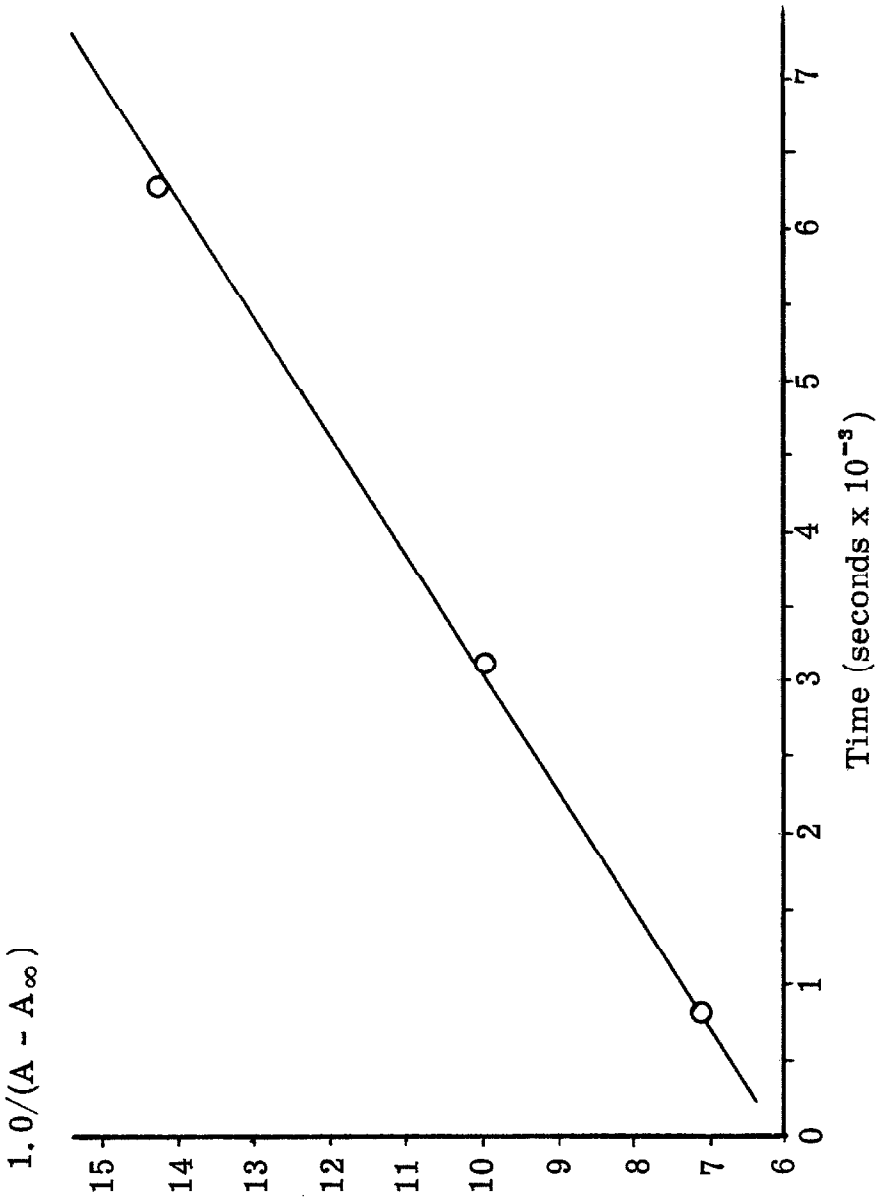
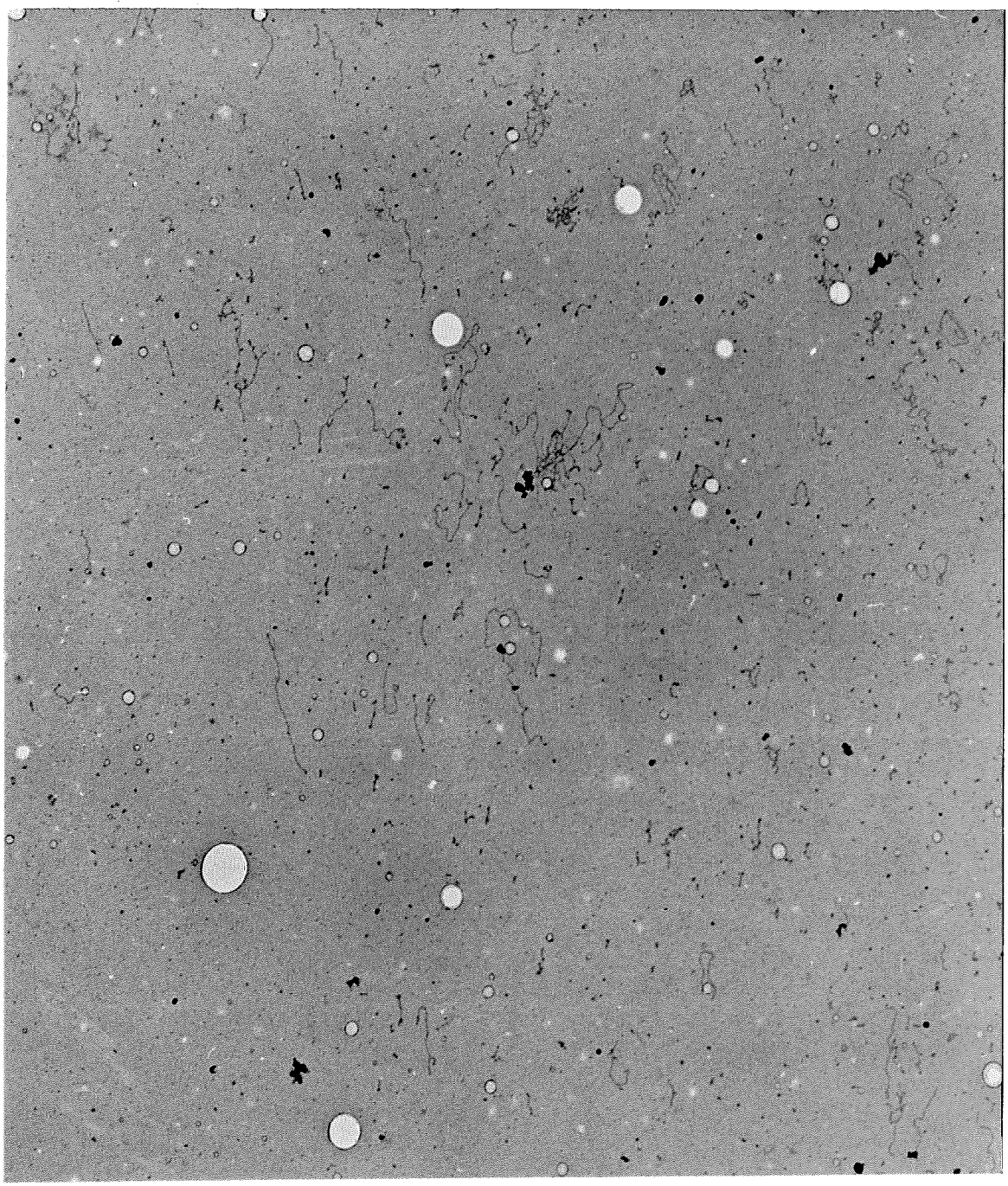

1.0/(A - A∞)
15
14
13
12
11
10
9
8
7
6
0
1
2
3
4
5
6
7
Time (seconds x 10⁻³)

PLATE 4-1

Denatured ascites tumor DNA at $S_{20,w}^{pH\ 13} = 6.8$.
Uranyl stained Kleinschmidt. x 16,000.

PLATE 4-2

Renatured ascites tumor DNA at $S^{pH\ 13}_{20,w} = 6.8$. Uranyl stained Kleinschmidt. Renatured for 20 hours at T_m - 21°C. x 16,000.



Britten, 1966; Britten and Kohne, 1967) has previously demonstrated fast renaturing nuclear DNA.

CHAPTER 5

The Effect of GC Content on the Rate of Renaturation of DNA

Contents: Page:

A. Density Gradient Sedimentation: Determination of GC Content

The banding of T4, T7, N1 and M. lysodeikticus DNAs in CsCl, density 1.704, 0.01 Tris OH plus HCl, pH 7.8 was accomplished at 44,770 rpm in a Spinco model E analytical ultracentrifuge as described by Vinograd and Hearst, (1962). N1 DNA banded with T4 and T7 DNAs alone served to identify the N1 band in the presence of all the other DNAs. The tracing of the four bands in the analytical centrifuge cell appears in Figure 5-1. An interpolation between M. lyso. DNA and T7 DNA was used to determine the GC content of N1 DNA. The interpolation appears in Figure 5-2. The linear relation between buoyant density and GC content of DNA was established by Schildkraut, Marmur and Doty (1962). The densities obtained for T4, T7 (like E coli DNA) and M. lyso. DNAs are within 0.001 g/ml of the values obtained by Vinograd, Morris, Davidson and Dove (1963). The values with their GC contents appear in Table 5-1.

TABLE 5-1

The Buoyant Densities of Several DNAs

DNA	Buoyant Density	GC%
T4	1.692	(34-35)
T7	1.703	49
N1	1.718	64
M. lyso.	1.727	72

Figure 5-1

A Microdensitometer Tracing of the Equilibrium Banding Pattern of T4, T7, N1 and M. lysodoikticus DNAs in CsCl.

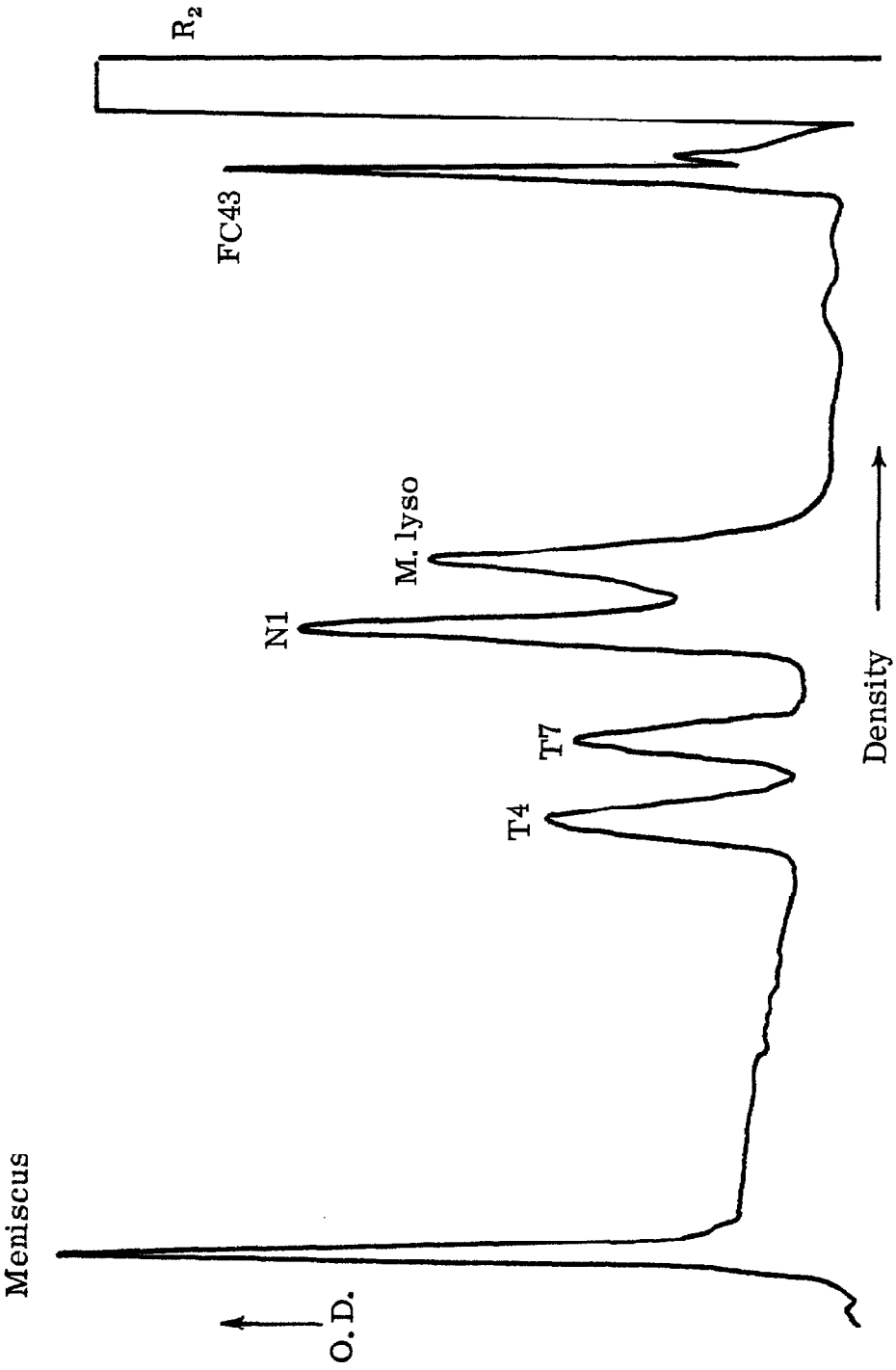
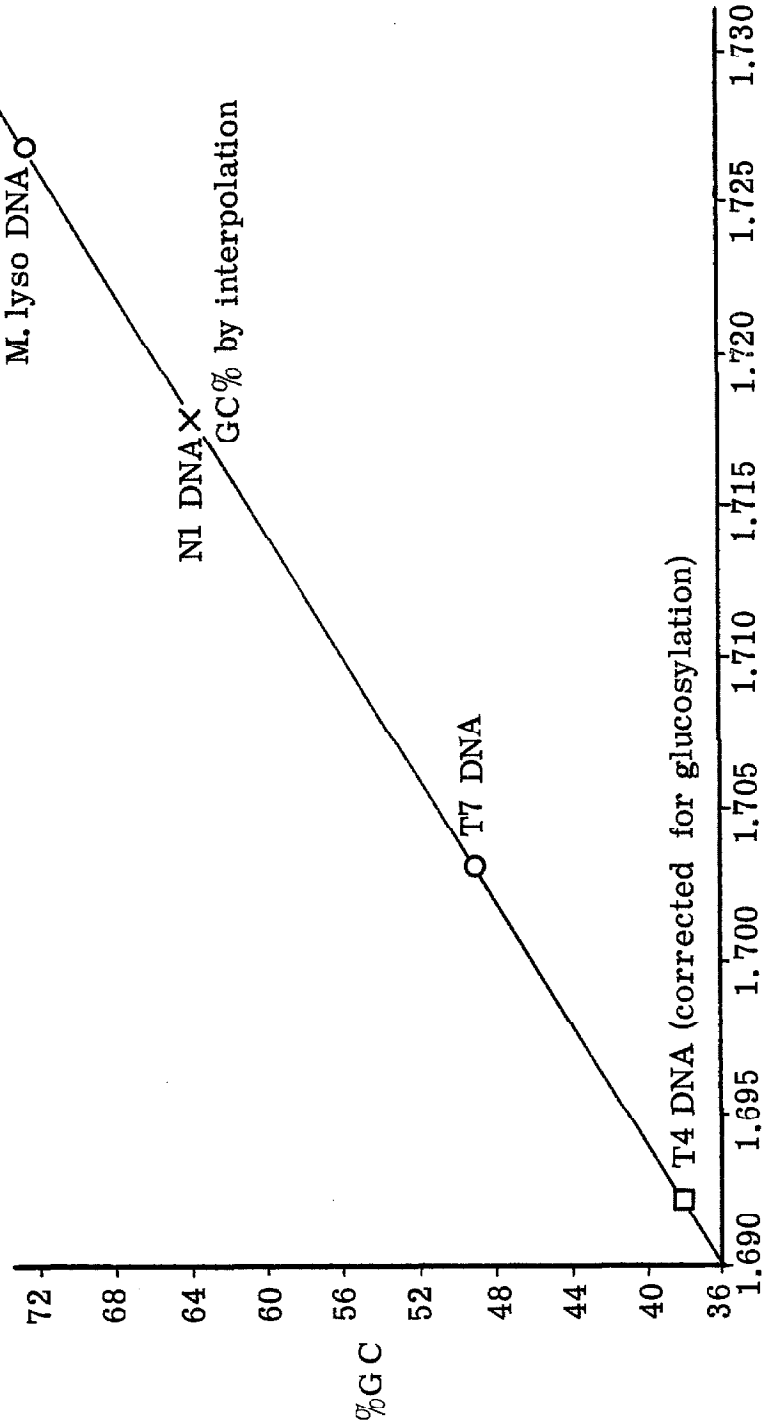

R2
FC43
M. lyso
N1
T7
T4
Meniscus
O.D.
Density

Figure 5-2

Evaluation of the GC Content of N1 DNA by Interpolation with DNAs of Different GC Contents and Buoyant Densities


M. lyso DNA
N1 DNA
GC% by interpolation
T7 DNA
T4 DNA (corrected for glucosylation)
%G C
72
68
64
60
56
52
48
44
40
36
1.690
1.695
1.700
1.705
1.710
1.715
1.720
1.725
1.730
Buoyant density

The GC content of ascites tumor DNA was determined by C. S. Lee (1966). The GC content of SV40 DNA was determined by Crawford and Black (1964). The high GC content measured for N1 DNA is in agreement with the base composition analysis of Scaletti and Naylor (1959). The buoyant density of T4 DNA is artificially elevated above the value expected for its GC content due to glucosylation.

The molecular weights of T4, T7 and N1 DNAs are known within 5% to be (see Chapters 4, 8) T4 DNA: 130×10^6; T7 DNA: 25×10^6; N1 DNA: 33×10^6.

B. The effect of GC Content on the Rate of Renaturation of DNAs of Known Lengths

The ratio of complexity/calculated complexity for T4, N1, and T7 DNAs can be corrected to fit the expected value of 1 if they are divided by the following factors:

N1 (64% GC)	$f = 1.31 \pm 0.05$
T7 (49% GC)	$f = 0.96 \pm 0.04$
T4 (34% GC)	$f = 0.635 \pm 0.064$

Although these factors are small, there is a variation of a factor of 2 between the DNA of highest and that of lowest GC content. We have excluded SV40 DNA from the list of known complexities due to anomalous results which will be discussed below.

C. Theoretical Interpretations of GC Content Effects

The computer program described in Chapter 2 was used to determine the rate of renaturation of DNAs, corrected for complexity

and size, of varying GC contents. The following parameters were specified:

1. $\sigma(2) = 1.0$ (There is no decreased stacking for the second base pair)
2. $H_f = 7500$ cal/mole (The enthalpy of activation for the propagation step, k_f)
3. %GC = 10, 34, 41, 49, 55, 60, 64, 90

The results of these calculations, as a function of temperature, are plotted in Figure 5-3. The maximum rate vs. GC% is plotted in Figure 5-4. The results are in agreement with increasing rate with increasing GC%. The absolute maximum rates and corresponding correction factors (f) are listed in Table 5-2. The factors obtained for the three specified DNAs are f = 0.69 for T4 DNA, 0.96 for T7 DNA and 1.27 for N1 DNA. These results are in good agreement with the factors predicted from experimental results.

The temperature of maximum rate vs. %GC is plotted in Figure 5-5. The dependence of maximum rate on GC content was determined by Marmur and Doty (1961). These results predict the same and expected trend found in their results. The maximum predicted rates occur at T_m - 26 to T_m - 33°C. in agreement with experiment (see Chapter 2). The temperature of maximum rate decreases almost exactly as the melting temperature decreases with decreasing GC% (Marmur and Doty, 1959; Marmur and Doty, 1962).

The corrected ratios of actual complexity/calculated complexity are given in Table 5-3.

Figure 5-3

Theoretical Rate Constants for Renaturation of DNA
as a Function of Temperature for Selected GC Contents

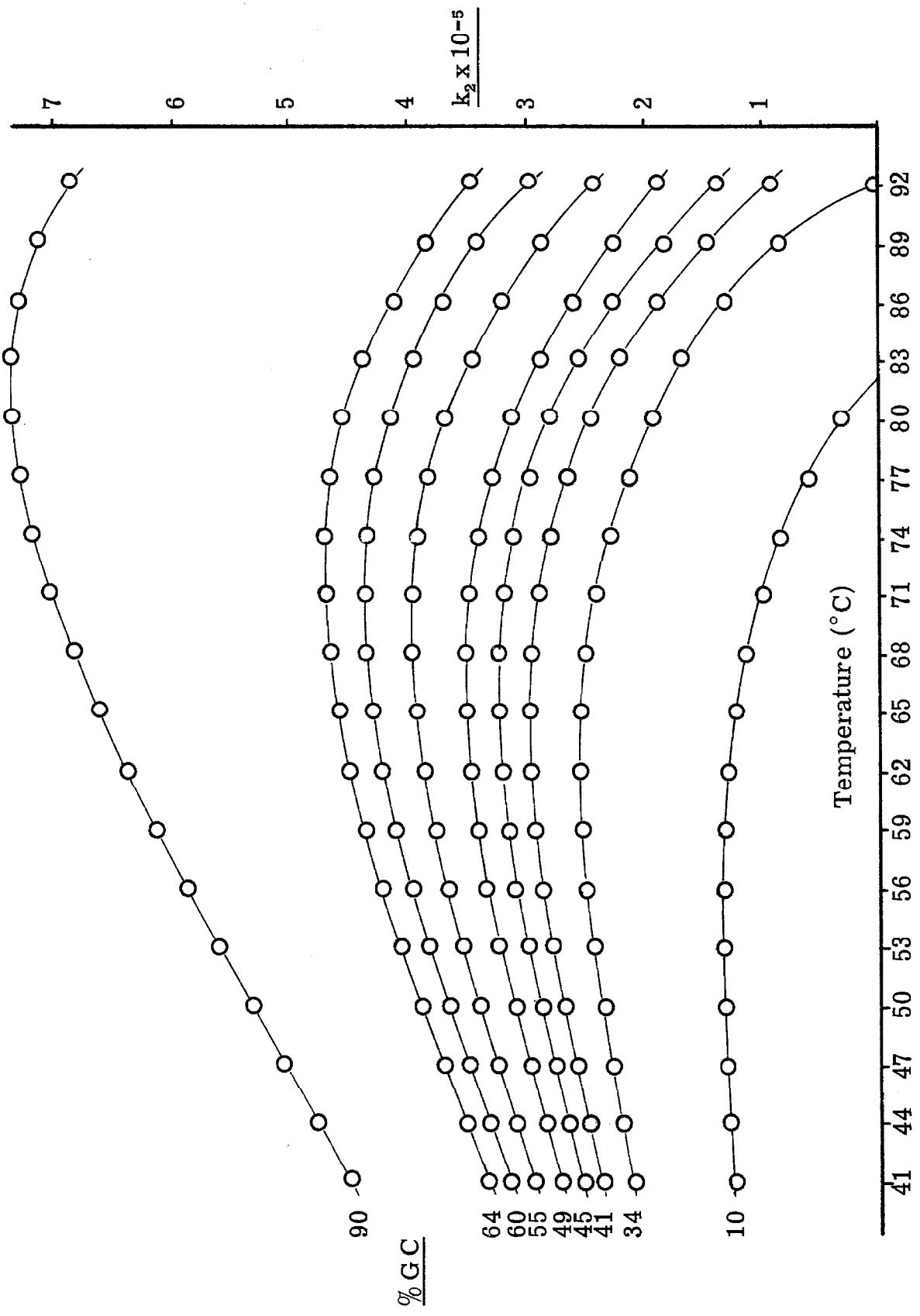

$k_2 \times 10^{-5}$
7
6
5
4
3
2
1
Temperature (°C)
41 44 47 50 53 56 59 62 65 68 71 74 77 80 83 86 89 92
%GC
90
64
60
55
49
45
41
34
10

Figure 5-4

Theoretical Rate Constants for Renaturation of DNA at the Temperature of Maximum Rate as a Function of GC Content

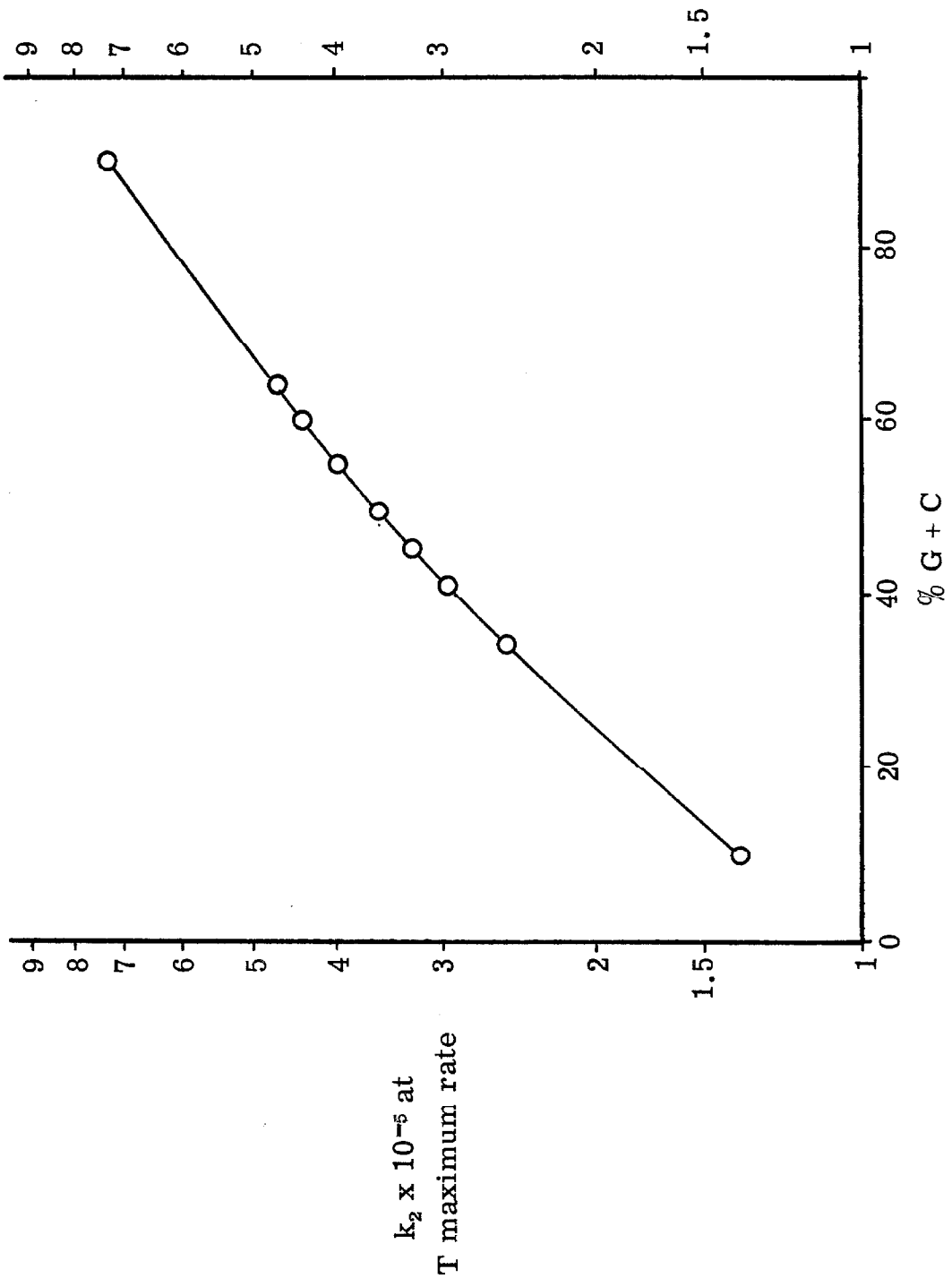

9
8
7
6
5
4
3
2
1.5
1
0
20
40
60
80
% G + C
k_2 x 10^{-5} at
T maximum rate

Figure 5-5

Theoretical Temperatures of Maximum Renaturation Rate as a Function of GC Content

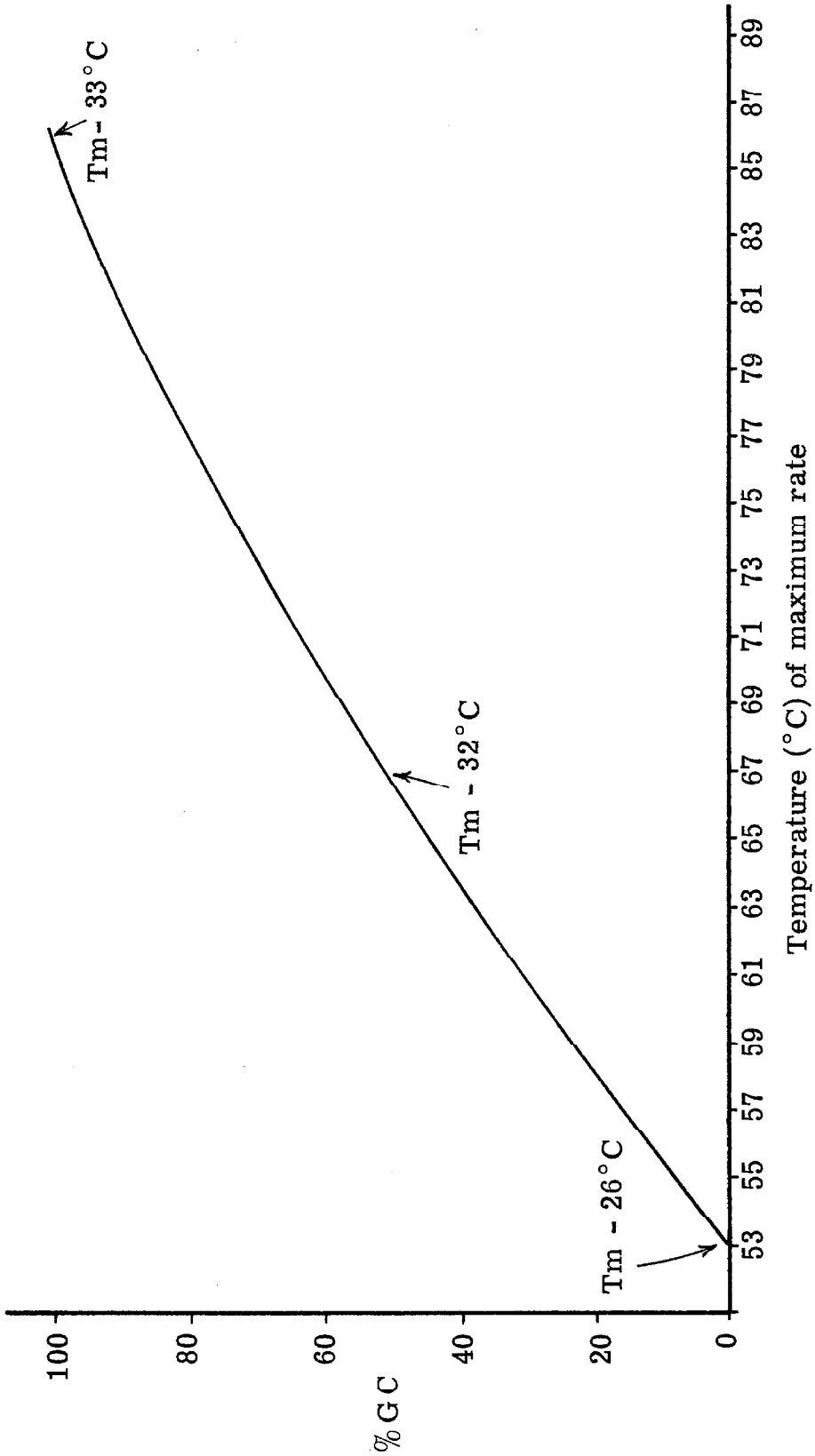

Tm - 33°C
Tm - 32°C
Tm - 26°C
%GC
100
80
60
40
20
0
53
55
57
59
61
63
65
67
69
71
73
75
77
79
81
83
85
87
89
Temperature (°C) of maximum rate

TABLE 5-2

Theoretical Prediction of GC Effects

% GC	Max. Rate $\times 10^{-5}$	Max. Rate/3.7×10^{5}
10	1.36	0.37
34	2.56	0.69
41	2.98	0.81
45	3.24	0.88
49	3.52	0.96
55	3.96	1.07
60	4.36	1.18
64	4.69	1.27
90	7.37	2.00

TABLE 5-3

The Effect of Complexity on the Rate of Renaturation of DNA

DNA	% GC	Complexity Ratio	f (Theory)	Corrected Complexity Ratio
Ascites tumor	45	0.80 - 1.25	0.88	0.91 - 1.42
E coli	50	0.77 - 1.15	0.98	0.78 - 1.17
T4	34	0.59 - 0.68	0.69	0.86 - 0.99
N1	64	1.26 - 1.36	1.27	0.99 - 1.07
T7	49	0.92 - 1.00	0.96	0.96 - 1.04
SV40	41	0.56 - 0.60	0.81	0.69 - 0.74

Except for SV40 DNA, the results of the corrected complexities are very close to 1.0. The improvement of the fit of experimental and expected data by use of computed rates is remarkably good considering the inexact nature of the theoretical model which was programmed and the fact that the constants used in the program are all subject to some error.

The SV40 DNA was a gift of Mr. W. Bauer and was obtained from the extract of the whole virus pellet of a preparation of SV40 virus. Such an extract might contain more than one similar virus or one virus of varying genetic forms. The error of 25-30% is just outside possible experimental errors, and it is not, therefore, possible to state that this discrepancy has a genetic origin. The important point to note is that this error is the largest error over the wide range of complexities investigated.

CHAPTER 6

The Effect of Solvent on the Rate of Renaturation of DNA

Contents: Page:

A. The Effect of pH on the Rate of Renaturation of T4 DNA

Measurements of the rate of renaturation of T4 DNA were accomplished on mechanically sheared DNA ($S_{20,w}^{pH13} = 20$) using the Gilford type system. With buffers of pH values below 7.94, the DNA was first denatured with alkali, neutralized, diluted into buffer solution in the cold and added to the Gilford cell which was already at temperature. For buffers with pH values greater than or equal to pH 7.94, the DNA was denatured by melting. In both cases melting curves were obtained after renaturation. The buffers used were such as to produce a sodium ion concentration of 0.1 M. except for Tris OH plus HCl, which was present in a concentration of 0.02 F. The total sodium in all samples was 0.40 M. The results of renaturation experiments appear in Table 6-1. In all cases pH measurements were made before and after the experiments and found to be in agreement.

The range of k_2 values from Tris OH plus HCl to Acetate buffer pH (7.94 - 5.0) is small and, within experimental error, there appears to be little or no dependence of the rate of renaturation on pH in this region. In this same pH region, there is little variation in the melting temperature of DNA.

In the higher pH region, with the appearance of a decrease in the melting temperature comes a decrease in the rate of renaturation of DNA. If this change in T_m is due to titration of some of the bases in the DNA, then a corresponding effect should be seen in the rate of renaturation of DNA due to electrostatic repulsion effects similar to the effect of lowering the salt concentration, a subject discussed later

in this Chapter. The important practical fact in that within the pH range

$$pH = 7.0 \pm 2.0$$

there is no effect of pH on the rate of renaturation of T4 DNA in the presence of 0.40 F. sodium ion.

TABLE 6-1

The Effect of pH on the Rate of Renaturation of T4 DNA

T_m	pH	T_r	$T_m - T_r$	k_2	Buffer
68	9.85	48	20	14.9	carbonate
81	9.15	52	29	48.8	borate
88.5	7.94	65	23.5	68.6	Tris HCl
87	6.95	60	27	75.2	phosphate
88	5.8	60	28	61.0	phosphate
87.5	5.0	60	27.5	59.2	acetate

B. The Effect of Viscosity on the Rate of Renaturation of T4 DNA

If the rate determining step of a reaction is hydrodynamically controlled, as in a reaction controlled by translational diffusion, rather than chemically controlled, then the product of the rate constant and the viscosity divided by the absolute temperature ($k\eta/T$) should be a constant.

1. Measurements of viscosity

Viscosities of ethylene glycol, sucrose and glycerol solutions used in this work were measured at temperatures at or very near the renaturation temperatures used in this work. The viscosities were measured on solutions containing the appropriate salt relative to the same salt solutions. An Oswald type viscometer in a thermostated bath was used. The temperature control was good to $\pm 0.1°C$.

The viscosity of $NaClO_4$ solutions relative to water at 20°C. was obtained by S. Mickel and J. Vinograd (1966). The determination of viscosities at elevated temperatures was done in analogy to the temperature dependence of the viscosity of water. This provides only a first order approximation to the viscosity of $NaClO_4$ solutions at elevated temperatures, but probably a good enough approximation for the rate constant range achieved in view of the small (a factor of 2 at the most except for the highest concentration result) viscosity change at 20°C. observed for the majority of the $NaClO_4$ concentrations employed.

2. Effects of sucrose (and glycerol) on the rate of renaturation of T4 DNA

The rate of renaturation of DNA has been observed to be viscosity dependent by Subirana and Doty (1966) and by Thrower and Peacocke (1966).

T4 DNA (mechanically sheared to $S_{20,w}^{pH13} = 20$) in 1.0 F. sodium ion in phosphate buffer, pH 7.0, after denaturation and neutralization, was added to sucrose (or glycerol) containing 1.0 F. NaCl to bring the concentration of sucrose (or glycerol) up to the reported value. The solution was prepared weight/volume before dilution. These measurements were carried out with the modified temperature jump system and were followed by melting. The maximum temperature to which the DNA was subjected before and during the renaturation process is referred to as T_{max} in the following tables. The quantity (η/T) rel is the value of η/T for the actual solvent at the temperature of renaturation divided by η/T for the equivalent aqueous salt solution at the temperature specified in each table.

The best fit for the results for (T_m (0% sucrose) - T_m) versus % sucrose is 1.67° C./10% sucrose added with 1.0 M. sodium ion. Within experimental error, the rate of renaturation × relative viscosity/relative temperature is seen to be a constant. This is the case for diffusion controlled reactions. The change in T_m could reflect an effect which anomalously makes the reaction appear to be diffusion controlled. However, as we shall see below in cases of greater and lesser effect of solvent on T_m, the viscosity dependence continues to hold.

TABLE 6-2*

The Effect of Sucrose (or Glycerol) in k_2 in 1.0 F. Sodium Ion

%	T_m	T_r	$T_m - T_r$	$T_m^{0\%} - T_m$	T_{max}	k_2	η rel/T rel	$k_2 \eta$ rel/T rel
0%	92	67	25	0	67	110	1.0	110
30% sucrose	87	62	25	5	62	37.4	2.54	95
40% sucrose	86	62	24	6	62	24.6	3.6	89
53% sucrose	82	62	20	10	62	13.8	6.8	94
36% glycerol	86	67	19	6	67	53.2	2.3	122

* Relative viscosity taken relative to the viscosity of the salt solution at 67°C.

Mechanically sheared T4 DNA in 0.4 F. sodium ion (chloride plus phosphate) pH 7.0, was examined using the Gilford system. The results appear in Table 6-3.

Again, $k_2 \times$ relative viscosity/relative temperature is approximately a constant. This time, however, the decrease in T_m on adding sucrose is only about 5/6° C./10% sucrose, or about 1/2 the effect obtained in molar sodium ion.

3. The effect of ethylene glycol on the rate of renaturation of T4 DNA

Results were obtained in the same manner as those in 0.4 F. sodium ion, sucrose. The results appear in Table 6-4.

Results in 40% ethylene glycol with various sodium concentrations appear in Table 6-5. Results at various salt concentrations in 72% ethylene glycol appear in Table 6-6.

These results show that there may be a deviation from viscosity limitation in low salt. This effect could, however, be due to increased binding of cations by DNA in the presence of a more organic solvent in salt concentrations where limited cation binding occurs in a salt-water solution. The relatively elevated T_m in lower salt solutions implies that this may be the case.

The dependence of T_m on % ethylene glycol in 0.4 M. sodium ion is determined in Figure 6-1a to be 3.6° C./10% ethylene glycol. The effect is increased to 3.9° C./10% ethylene glycol in 1.0 M. sodium ion and decreased to 2.6 - 3.1° C./10% ethylene glycol in low salt in the range 40 - 72% ethylene glycol.

TABLE 6-3*

The Effect of Sucrose on k_2 in 0.4 F. Sodium Ion

% Sucrose	T_m	T_r	$T_m - T_r$	$T_m^{0\%} - T_m$	T_{max}	k_2	η rel/T rel	$k_2 \eta$ rel/T rel
0	88.5	65	23.5	0	91	70.6	0.85	60
0	88.5	55	33.5	0	91	76.6	1.0	76.6
20	87	55	32	1.5	91	45.6	1.67	76
30	86.5	65	21.5	2	91	22.6	1.95	44
35	85	55	30	3.5	91	28.0	2.74	77
40	86	65	21	2.5	91	22.2	2.8	62
45	85.5	55	30.5	3.0	91	20.8	4.15	86
50	83.5	55	28.5	5.0	91	14.6	5.4	79
58	84	55	29	4.5	91	11.2	7.7	86

*Relative viscosity taken relative to the salt solution at 55°C.

TABLE 6-4

The Effect of Ethylene Glycol on k_2 in 0.4 F. Sodium Ion*

% Ethylene glycol	T_m	T_r	$T_m - T_r$	$T_m^{0\%} - T_m$	T_{max}	k_2	η rel/T rel	$k_2 \eta$ rel/T rel
0	88.5	65	23.5	0	91	70.6	0.85	60
0	88.5	55	33.5	0	91	76.6	1.0	76.6
10	84.5	60	24.5	4	91	56	1.12	62.5
20	82.5	60	22.5	6	91	50	1.38	69
30	79.5	60	19.5	9	91	34	1.74	59
40	74	55	19.0	14.5	91	32.4	2.5	81
50	71	45	26.0	17.5	80	29	4.0	116
60	67	45	22.0	21.5	80	14.9	5.7	85
70	63	45	18.0	25.5	80	9	7.7	69

* Relative viscosities taken relative to the salt solution at 55°C.

TABLE 6-5

The Effect of Salt on Renaturation Rate in 40% Ethylene Glycol

Na^+	T_m	T_r	$T_m - T_r$	$T_m^{0\%} - T_m$	T_{max}	k_2	η rel/T rel	$k_2 \eta$ rel/T rel	k_2 $(H_2O)^*$
1.0	76	52.5	23.5	16	84	69.2	2.6	180	150
0.4	74	55	21	14.5	92	34.4	2.5	86	76
0.2	73.2	45	27.8	10.8	84	18.2	3.0	55	27
0.1	69	45	24	10.5	84	4.6	2.5	11.5	5.3

* $k_2(H_2O)$ is the rate constant for renaturation without ethylene glycol. We obtained k_2 from salt dependence of the rate of renaturation and the rate obtained in 0.4 F. sodium ion for this DNA. Salt dependence is discussed later in this Chapter. The relative viscosity is taken relative to the salt solution under renaturation conditions used in the study of salt dependence.

TABLE 6-6

The Effect of Salt on Renaturation Rate in 72% Ethylene Glycol

Na^+	T_m	T_r	$T_m - T_r$	$T_m^{0\%} - T_m$	T_{max}	k_2	η rel/T rel	$k_2 \eta$ rel/T rel	$k_2(H_2O)$
0.84	63	45	18	27.5	69	12.4	8.3	103	112
0.4	63	45	18	25.5	80	9.0	7.5	68	76
0.18	61	35	26	22	69	2.54	9.25	23.4	21.2
0.09	58	35	22	22.5	69	1.18	8.5	10.0	4.3

The deviation of T_m without glycol is greater than the deviation in the 1.0 F. sodium ion-sucrose solution. In all cases, in 0.4 M. and 1.0 M. sodium ion, the renaturation reaction appears to follow the rules for diffusion controlled reactions.

4. The effect of $NaClO_4$ on the rate of renaturation of DNA

In the following experiments, 0.002 M. Tris OH plus HCl was used in all sodium perchlorate solutions at pH 7.65. The Gilford system was used to measure the rate of renaturation of T4 DNA in various concentrations of sodium perchlorate. The use of chaotropic agents to lower the melting temperature of DNA has been described by Hamaguchi and Geiduschek (1962) and theoretically treated by Robinson and Grant (1966). The effect of the concentration of sodium perchlorate on T_m is given in Figure 6-1b. The results of the renaturation experiments appear in Table 6-7.

Over a range of 1.0 F. to 5.2 F. sodium perchlorate, the rate of renaturation obeys the diffusion controlled rate law. There appears to be a very small activation energy amounting to less than 4 kcal/mole, which is of unknown origin.

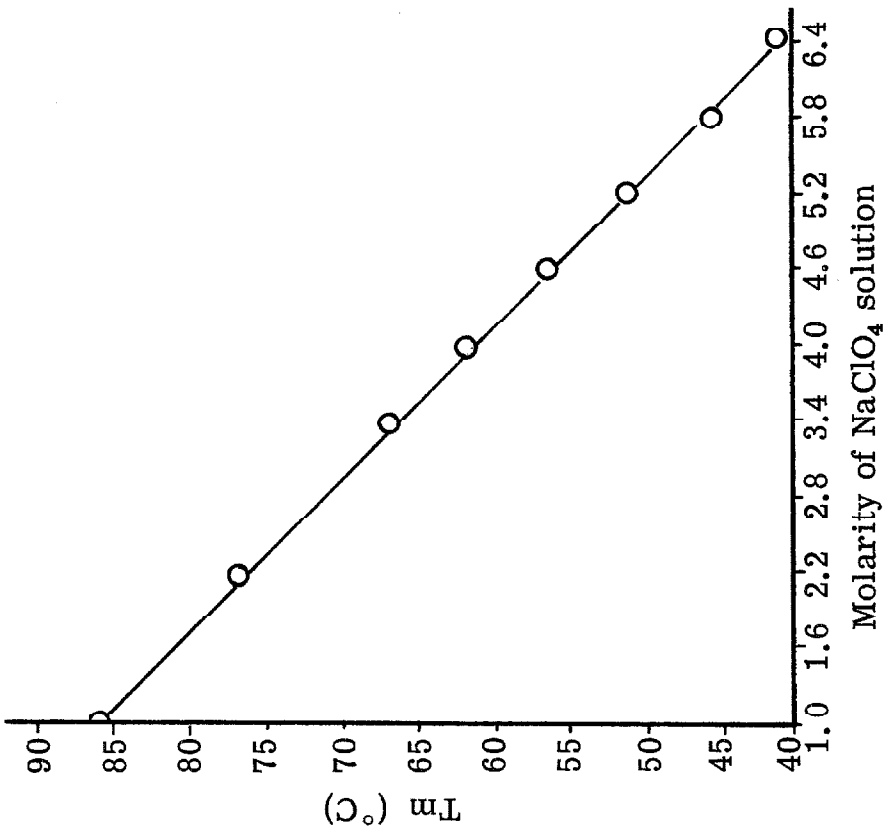
In addition to a possible activation energy, there is the error inherent in the evaluation of viscosities of $NaClO_4$ solutions previously described. Also, the rate for the same DNA in sodium chloride plus phosphate is 150 liters/mole sec and the value of 184 may be exaggerated. Also, as the GC dependence of melting in perchlorate is not the same as that in chloride, the results are not quite comparable. Nevertheless, within experimental and other errors, the diffusion

Figure 6-1

Solvent Effects on the Melting Temperature of T4 DNA

a. The dependence of T_m on % ethylene glycol in 0.4 M. Na^+.

b. The dependence of T_m on molarity of $NaClO_4$ at pH 7.65.


b.
Tm (°C)
90
85
80
75
70
65
60
55
50
45
40
1.0 1.6 2.2 2.8 3.4 4.0 4.6 5.2 5.8 6.4
Molarity of NaClO4 solution

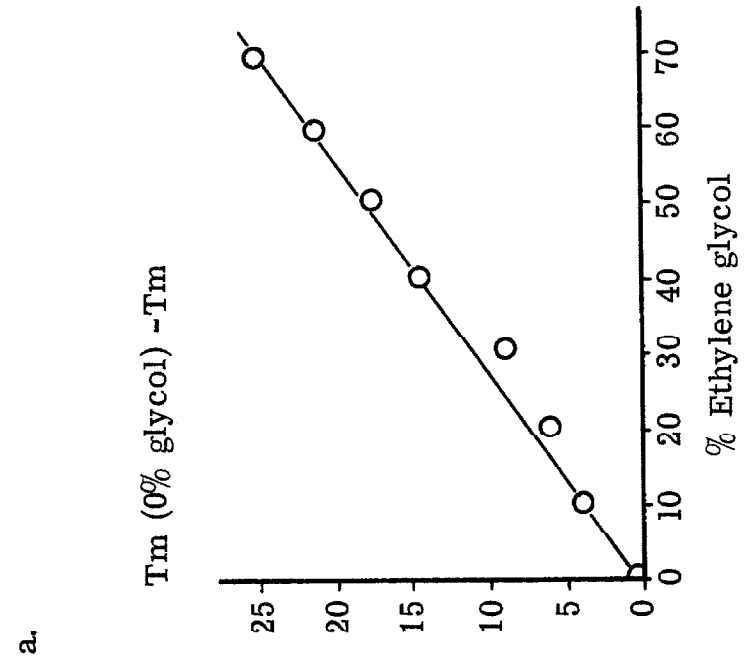

a.
Tm (0% glycol) -Tm
25
20
15
10
5
0
0 10 20 30 40 50 60 70
% Ethylene glycol

TABLE 6-7

The Effect of $NaClO_4$ on the Rate of Renaturation of T4 DNA*

F. $NaClO_4$	T_m	T_r	$T_m - T_r$	T_{max}	k_2	η rel	η rel/T rel	$k_2 \eta$ rel/T rel
1.0	86	67	19	95	176	1.05	1.05	184
2.2	77	60	17	95	109	1.4	1.43	156
3.4	67	50	17	75	64	2.1	2.2	142
4.0	62	40	22	75	54	2.8	3.05	166
4.6	56.5	35	23.5	75	37.2	3.4	3.8	141
5.2	51	26	25	58	24.4	4.5	5.1	135
5.8	45.5	--	--	--	--	--	--	--
6.4	41	21	20	58	11.6	6.9	8.0	95

* Relative viscosities taken relative to water at 67°C.

control situation with saturating amounts of cation appears to hold.

E coli DNA, $S_{20,w}^{pH13} = 12.3$, was renatured in 5 F. $NaClO_4$ with $T_m = 66°C.$, η rel to 1.0 F. sodium ion at standard T_r in chloride and phosphate is 2.85 at 44°C. η rel/T rel = 3.1. The following data were obtained.

TABLE 6-8

The Effect of $NaClO_4$ on the Rate of Renaturation of E coli DNA

T_m	T_r	k_2	$k_2 \eta$ rel/T rel	Solvent
66	38	2.24	7.0	5.0 F. $NaClO_4$, TrisHCl, pH 7.65
66	44	2.22	6.9	5.0 F. $NaClO_4$, TrisHCl, pH 7.65
66	49	2.16	6.7	5.0 F. $NaClO_4$, TrisHCl, pH 7.65
98	74	5.2	5.2	1.0 F. NaCl, phosphate, pH 7.0
98	74	4.92	4.92	1.0 F. NaCl, phosphate, pH 7.0
98	74	5.7	5.7	1.0 F. NaCl, phosphate, pH 7.0

The greater value of $k_2 \eta$ rel/T rel in high perchlorate is within experimental error of the value in molar sodium ion with chloride plus phosphate, considering all the previously related possibilities for error.

This last solvent system, sodium perchlorate, may be used to compare results at low renaturation temperatures with those expected at higher temperatures required in a chloride system if relative viscosities and temperatures are taken into account. This system

provides the greatest decrease in melting temperature of any of the systems studied.

All the systems studied in this section in the presence of 0.4 M. sodium ion or higher appear to follow the requirements for a diffusion limited rate determining step for the renaturation reaction. Further discussion of the consequences of these measurements was given in the theoretical section at the end of Chapter 3.

C. The Effect of Salt Concentration on the Rate of Renaturation of T4 DNA

The effect of ionic strength on the rate of renaturation of DNA has been studied by Marmur and Lane (1960). Just as is the case with the melting temperature of DNA, above 0.4 F. sodium ion in a chloride system, there is only a small change with increasing salt concentration. There is a large effect of salt on melting temperature (Dove and Davidson, 1962) and on the rate of renaturation at lower salt concentrations (Marmur and Lane, 1960; Britten and Kohne, 1967). The effects are likely comparable in origin, reflecting changes in the electrostatic medium of the DNA polymer in solution. The significance of these results will be discussed in the next section.

The low salt renaturation runs were corrected for molecular weight changes due to degradation by measurement of alkaline band or boundary sedimentation velocity after renaturation experiments. The initial value was $S_{20,w}^{pH13} = 20$. The maximum change of k_2 by such corrections was an increase of about 50%. That is, in 0.06 to 0.25 sodium ion, there was degradation at T_{max} prior to renaturation sufficient to alter the rate by 2/3 times that achieved for the sheared DNA of $S_{20,w}^{pH13} = 20$.

The results for the effect of salt concentration on the rate of renaturation of T4 DNA at pH 7.0 (phosphate buffer) are listed in Table 6-9.

TABLE 6-9

The Effect of Salt on the Rate of Renaturation of T4 DNA

Sodium Ion	T_{max}	T_r	T_m	k_2 (corrected for alkaline velocity)
0.06	88	40.5	75.5	0.54
0.06	88	49	75.5	0.80
0.06	91	60	75.5	0.40
0.10	88	49	79.5	4.4
0.15	91	60	82	9.6
0.25	97.5	61	85.8	30.2
0.40	91	65	88.5	68.0
0.40	97.5	61	88.3	61.0
0.40	91	65	88.5	70.6
0.40	91	55	88.5	76.6
0.70	97.5	61	90.8	97.0
1.00	70	68	92	130 (Temperature jump)
1.00	98	70	92	126.6
1.00	98	70	92	119
1.5	98	70	93	146.8
1.5	98	70	93	148.4
1.85	98	70	93.3	148
1.85	98	70	93.3	148
3.2	95	70	91	216
3.2	95	70	91	208

D. Discussion of Salt Effects

The effect of cation concentration on the melting temperature of DNA has been investigated by Dove and Davidson (1962) over a large range of cation concentrations. Theoretical treatments of the effects have been given by Kotin (1963) and Walker (1964) and Schildkraut and Lifson (1965). The equilibrium between the native and partially denatured forms of DNA shifts towards the denatured state at low salt concentration. This implies that electrostatic interaction between phosphate groups of complementary strands increases with decreasing salt concentration. Our data in Table 6-9 show this decrease in the melting temperature of DNA with decreasing salt concentration.

If these phosphate interactions affect the equilibrium, they might also be expected to affect the nucleation step of the renaturation reaction. In fact, decreasing the salt concentration from 0.25 F. to 0.06 F. sodium leads to a drop in the rate of renaturation of about 40 times.

In high salt, the melting temperature of DNA is not greatly altered by changes in the cation concentration. If this reflects a saturation of the electrostatic shielding effect of cations on the phosphate interactions, as predicted in the two theoretical treatments of the subject, then one might also predict that the rate of renaturation of DNA should also become insensitive to the salt concentration in high salt. Over the range 0.4 - 3.2 F. sodium, twice the range considered previously, the rate of renaturation is seen to increase by only a factor of 3. This is a decrease in the effect of salt by a factor of 25.

In high salt, it is easy to compare results at various salt concentrations as the salt dependence is so small. In principal, it should be possible to compare results at all salt concentrations, but the large salt dependence of the rate of renaturation makes this task very difficult.

We were able to demonstrate a dependence of the rate of renaturation on viscosity in 0.4 M. or 1.0 M. sodium with sucrose or ethylene glycol or in high perchlorate solutions. This dependence of rate on viscosity does not appear to hold at lower salt concentrations. This result may be due to increased shielding of phosphate groups in lower salt in the more organic solvent with its lower dielectric constant. It is possible that the salt effect could anomalously cancel the viscosity effect. Thus it is not clear whether the increased effect of electrostatic repulsion results in a change in the mechanism of renaturation. The dependence of the rate of renaturation on the square root of the molecular weight was proved well only at 1.0 F. sodium, but was also seen to hold at 0.4 F. sodium. We cannot say that either the molecular weight or viscosity effects hold below the high salt region.

As the dependence of the rate of renaturation on salt concentration is small in high salt, this implies that the electrostatic term in the free energy related to the equilibrium of the base pairs has become independent of salt concentration. This is analogous to previous work dealing with the dependence of melting temperature on salt. If this is true, it is reasonable to suppose that the forward rate

constant for base pair formation is independent of an electrostatic contribution. If this is so, the discrepancy of 5000 between the predicted rate controlled by translational diffusion and the observed rate could not be due to an electrostatic effect.

E. Summary of Renaturation Kinetics Results

Chapter 1: The renaturation reaction, in 1.0M. sodium, pH 7, was shown to be second order in DNA phosphate concentration. The method of calculation of rate constants was outlined. The data were shown to be reproducible to within 10%. The products of renaturation of highly polymerized denatured DNA at moderately high DNA phosphate concentration were shown by electron microscopy to be highly polymerized native DNA with no unusual forms.

Chapter 2: The renaturation reaction was shown to be almost entirely temperature independent for T4 and E coli DNAs in the temperature range of 20 - 30 degrees below the melting temperatures; thus some latitude is permitted in the choice of renaturation temperature to still get the maximum rate. A model was proposed to account for the temperature dependence of the rate of renaturation. The model agrees with experimental data, at least qualitatively, above 35 degrees below the melting temperature. The problems of intra- and inter-molecular aggregation were discussed. It was shown that neither inter- or intra-molecular aggregation should effect the denatured DNA in solution at the renaturation temperature. It was shown that at usual DNA phosphate concentrations, the intra-molecular effect should become important at low temperatures. Inter-molecular aggregation, on the other hand, should not be important even at room temperature in high salt.

Chapter 3: It was demonstrated that for any given DNA, the rate of renaturation is proportional to the square root of the molecular

weight of the denatured DNA undergoing reaction. The molecular weights were determined by sedimentation velocity experiments in alkali. Excluded volume effects were excluded as the explanation of the molecular weight dependence. The absolute rates, corrected for complexity (Chapter 4) and size, were too slow to be accounted for by a simple translational diffusion control. A discussion of the rate of the forward step of base pair formation leads to a possible explanation of this effect.

Chapter 4: The dependence of the rate of renaturation on the inverse of the complexity of the DNA was demonstrated for DNAs of complexity varying over a range of 10^6. All rate constants were corrected for molecular weight effects before comparison.

Chapter 5: The calculated complexities were found to be corrected to the known complexities of several DNAs if a GC effect was introduced. The effect is small, resulting in an increase in the rate of renaturation by about a factor of 2 over the GC% range 34-64%. The model outlined in Chapter 2 correctly predicted the GC dependence to within experimental error of the measurement of the known complexities. Thus it is possible, by measuring the GC content of a DNA (the buoyant density), the molecular weight of its denatured form (alkaline sedimentation velocity), and the rate of renaturation, to determine the complexity of the DNA to within an experimental error of generally less than 15%. These results are true in 1.0 F. sodium ion at neutral pH.

Chapter 6: The rate of renaturation of DNA was demonstrated to be almost independent of pH in the pH range 5-9. Thus, the choice of buffer systems for studying renaturation kinetics is quite varied. The difficulty of maintaining material at constant size, however, increases with decreasing pH. The best range of pH in which to work is 7 - 8. The dependence of the rate of renaturation on $T(^{\circ}K.)/\eta$ was demonstrated in high sodium perchlorate solutions as well as in 0.4 and 1.0 F. sodium salt solutions containing varied amounts of sucrose or ethylene glycol. In all these cases, the melting temperature of the DNA was altered to some degree when the solvent was changed. Although the melting temperature was found to be changed by very different amounts in these solvent systems, the rate of renaturation was shown to obey a dependence on T/η, a requirement for a reaction in which the rate determining step is a hydrodynamically controlled step. The viscosity results were assumed in the discussion of mechnism in Chapter 3. The dependence of the rate of renaturation on salt concentration was demonstrated to be sufficiently small in the salt range above 0.4 F. to make the problem of comparing results at different salt concentrations very simple. The rate increases 3 times when the salt is increased from 0.4 F. to 3.2 F. At lower salt concentrations, however, the rate of renaturation is much more salt dependent. The salt dependence increases 25 times when the same ratio of salt concentrations is observed in a salt region below 0.4 F. The demonstration of the dependence of the rate of renaturation on viscosity and the square root of the molecular weight has only been

proved in the salt range 0.4 F. or higher. The large salt effect may in practice increase the experimental error of renaturation measurements, though in principle this need not be so. For simplicity, it is best to use a high enough salt concentration to exclude electrostatic effects from the important variables of the renaturation process.

CHAPTER 7

Electron Microscopic Observations of DNA

Contents:

A. A Review of DNA Mounting Procedures

The problem of mounting DNA, either native or denatured, has been solved in three ways. We shall compare these procedures. The process of direct application of DNA to a surface was first used by Hall and Litt (1958). The process of mounting a DNA-protein mixed film was developed by Kleinschmidt and Zahn (1959). A streaking technique has been subsequently developed by Beer (1961).

The process of direct mounting can be subdivided into three forms:

1. Spraying the DNA onto the surface with an atomizer.
2. Mounting of a drop and subsequent drying.
3. Mounting of a drop and freeze drying.

In Table 7-1, all of the mounting procedures are compared.

TABLE 7-1

Potential	Mounting Procedures: Spray	Drop	Drop and freeze dry	Streak	Mixed film
1. Long DNAs can be used:	No	Yes	Yes	Yes	Yes
2. Aggregation suppressed:	Some	No	Some	Some	Yes
3. Pseudo-replica unnecessary:	Yes	Yes	No	Yes	Yes
4. Reproducible:	Yes	No*	Yes	No	Yes
5. No DNA distortion:	No	No	Yes	No	Yes
6. High resolution possible:	Yes	Yes	Yes	Yes	No

*Not sure due to ever present aggregation phenomenon.

The drop mounting procedure is always unacceptable due to lateral aggregation of DNA molecules on drying. The mixed film technique is always preferred unless high resolution is desired. If high resolution is desired, one of the remaining three techniques must be employed. We shall assume below that high resolution is required.

Spraying is the easiest technique for short DNA molecules if their configuration as twisted circularity (Vinograd, et al., 1965) may be distorted. This was the first technique ever employed (Hall and Litt, 1958). It was used in conjunction with a pseudo-replica technique, but this added labor is not necessary. Complete elimination of aggregation is possible if the sprayed DNA and receiving grid are kept at elevated temperatures (Bartl and Boublik, 1965).

If the length of the DNA must be maintained, only the streaking or the drop and freeze dry systems may be used. The difficulty with the streaking technique is lack of reproducibility. The concentration of DNA on the surface (and hence the degree of aggregation) depends on the cleanliness of the supporting film, the concentration of DNA in solution (much more than expected), the time of streaking, the method of drying, etc. With many trials, some success can be obtained. If the pseudo-replica technique can not be employed, as is the case for positive staining of DNA bases (Beer and Maudriankis, 1962; Maudriankis and Beer, 1965; Bartl, Erickson, and Beer, 1967), the streaking technique is the only method of mounting long DNA molecules. The use of elevated temperatures for mounting might be acceptable for elimination of aggregation in the streaking procedure.

If the pseudo-replica technique can be employed, the drop plus freeze drying technique is preferable for long DNAs. In this case, it is not necessary to suppress aggregation. If the configuration of the DNA is to be kept unchanged, as with twisted circular molecules (Vinograd, et al., 1965), this technique is the only possible method for high resolution. The requirement of making a pseudo-replica with a freeze drying technique is imposed by the fact that electron microscope support films break when frozen. An example of a use for this technique is the observation of protein molecules bound to DNA. With the pseudo-replica technique, shadow casting is the only contrasting option. The use of this technique circumvents the aggregation problem which otherwise may only be completely eliminated by using elevated temperatures. High temperatures would disrupt labile protein-DNA or DNA plus short DNA or RNA.

There is no way to maintain configuration, achieve high resolution and positive stain.

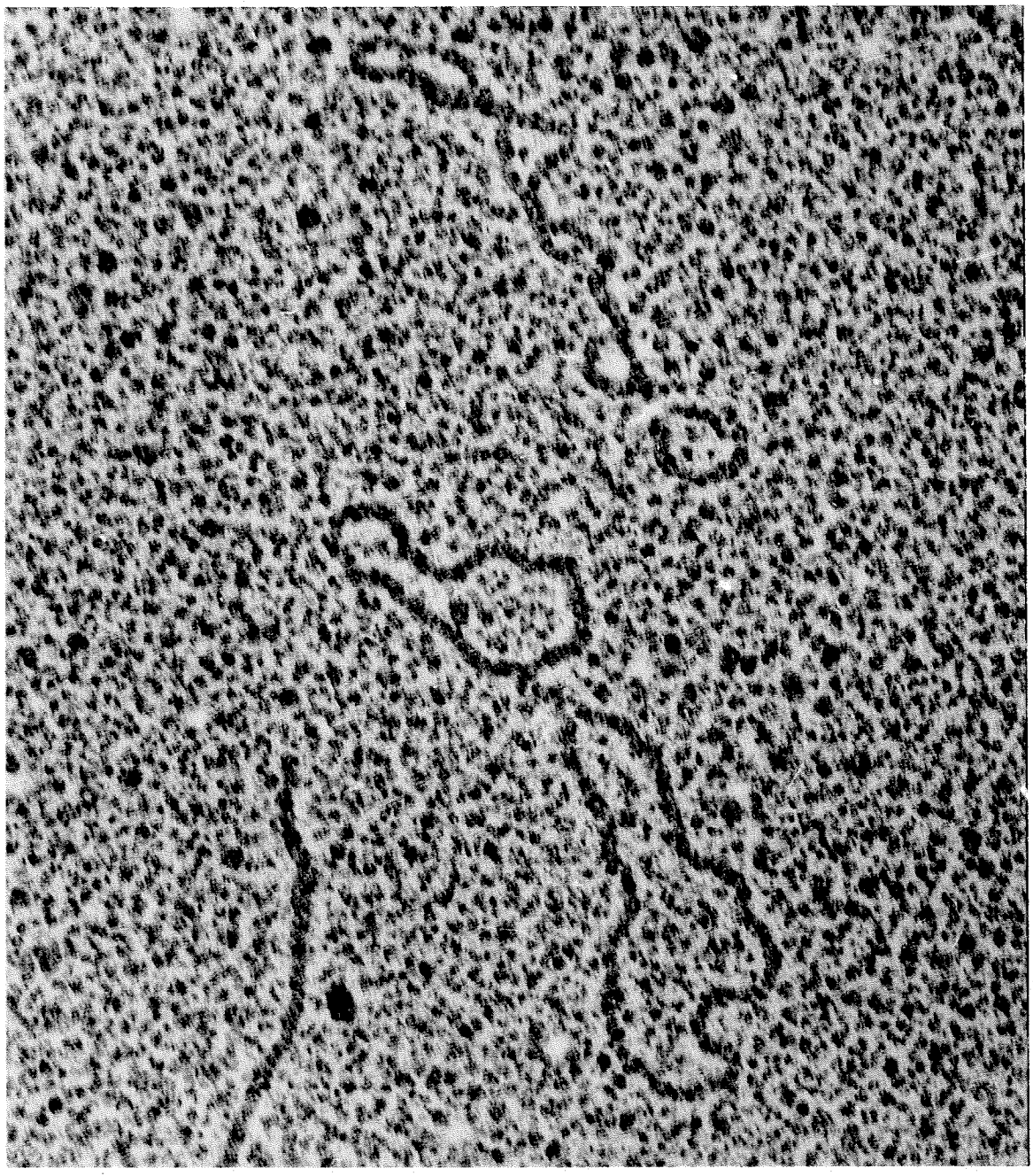
The methodology for freeze-drying is well established in the electron microscopy literature (see Kay, 1965). Its only limitation for high resolution is the requirement for shadow casting. Unless positive staining is desired, it is the method of choice for high resolution work except where the limitations of the spraying technique are unimportant. The only advantage then for the spraying technique is a great saving of time. Freeze drying is accomplished on freshly cleaved mica. A drop of DNA at 0.1 - 1.0 μg/ml is spread over the surface. A volatile buffer is employed. The entire mica sheet is

plunged into liquid nitrogen and removed to a cold block in a desiccator. The desiccator is evacuated with a mechanical pump which is separated from the desiccator by a liquid nitrogen trap. About half a day is required to remove all of the ice and volatile buffer.

An example of the results obtained with this technique appears in Plate 7-1. Polyoma I (Vinograd, et al., 1965) DNA was prepared by the pseudo-replica technique. The mica was placed on a rotating table and shadowed with tungsten (Hart, 1963; Hart, 1965) at 7 cm at an angle of 10/1. The 17.5 mil tungsten wire was heated with 26.5 amps for 10 minutes. The wire spanned about 3 cm between the holders. A large deposit of tungsten was used in preparing this replica, as can be seen from the width of the DNA and the contrast on the print. It is not necessary to use such a large deposit of heavy metal. After normal shadowing with carbon, the mica was placed in 100% humidity at 37° C. for 1 hour and stripped. Note that the twisted circular structure of Polyoma I is not disturbed by using this procedure.

PLATE 7-1

Polyoma I DNA freeze dried on mica.
Pseudo-replica with tungsten shadowing. x 195,000.



B. Notes About Shadow Casting and Contrast

If a DNA molecule is shadowed with a small amount of heavy metal such that the dimensions of the molecule are not altered, the thickness of material deposited on the DNA compared to the background thickness is approximately given by $\frac{2}{\pi}$ Cotan θ + 1, where θ is the angle between the plane of the mica and the line to the source. Thus for an angle of 7/1, the ratio of deposit on DNA to deposit on background is 5.5.

About 3 Å of density 20 material produces a 2% change in transmittance at 60-80 Kv with moderate aperture size (Hall, 1966). Such a change in transmittance is enough to be observed on a fluorescent screen with difficulty. The slope of the curve relating photographic film density to exposure is 2.5 for Kodak Fine Grain Positive film normally employed in electron microscopy. After similar contrasting when printing from the photographic film, 3 Å of density 20 material produces a 15% decrease in reflectance. If larger deposits are used, the contrast falls off more rapidly for material less elevated above the mica surface. For small deposits, the only way of telling native from denatured DNA structured would be by width. For large deposits, the only way would be by contrast. The resolution limit imposed by shadow casting is the grain size of the particles making up the deposit (on a perfectly flat surface). The grain size increases with increasing deposit size. The best way to achieve high resolution of short nucleotides would be to use the smallest visible deposit.

C. A Procedure for Positive Staining DNA-protein Mixed Films

The procedure of Kleinschmidt and Zahn (1959) is the best possible DNA mounting procedure for low resolution work. If a grid is prepared under normal conditions except that a parlodion (only) mounting film is used, the standard grid drying procedure can be used as a staining procedure. Positive staining was previously tried by Stoeckenius (1961) with very limited success. The problem apparently was the use of too high a concentration of stain and an unsatisfactory solvent for the stain.

A concentrated staining solution is made up with 5×10^{-2} F. HCl, 5×10^{-3} F. UO_2Ac_2 in 95% ethanol. Such a solution has been kept for almost a year without any sign of alteration. This solution is diluted 100 times into the 90-95% ethanol drying solution into which the grid is dipped for 30 seconds. The grid is then washed for 10 seconds in isopentane. Plate 7-2 contains electron micrographs of DNAs stained in this manner. Other electron micrographs in Chapters 1, 4, and 8 were prepared in a similar manner. The use of acid in the staining solution is to prevent uranyl polymerization.

DNAs stained by this procedure were photographed at 80 Kv with film of density/exposure determined to be 2.5. The negatives were traced with a Joyce-Lobel Microdensitometer. Densitometer tracings gave the following results:

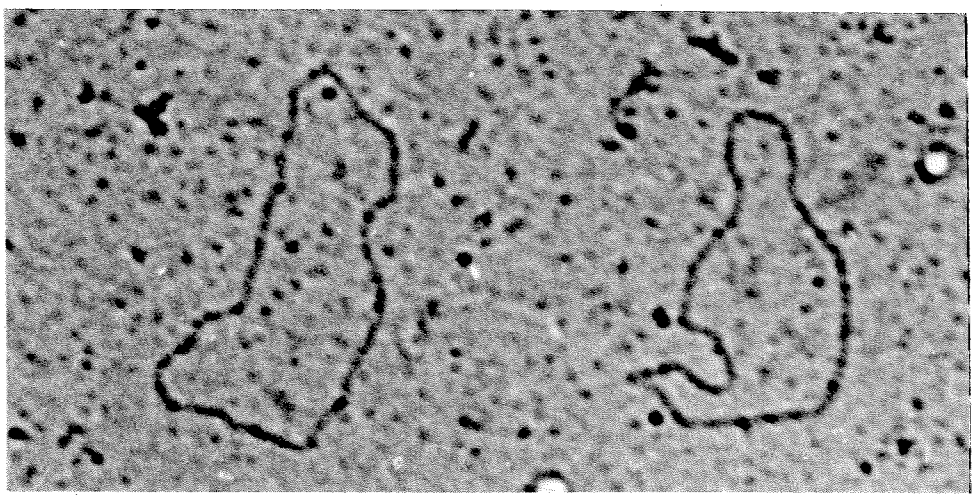
D (hole in film) - D (background) = 4 ± 0.3 cm.

D (background) - D (DNA) = 3.8 ± 0.3 cm.

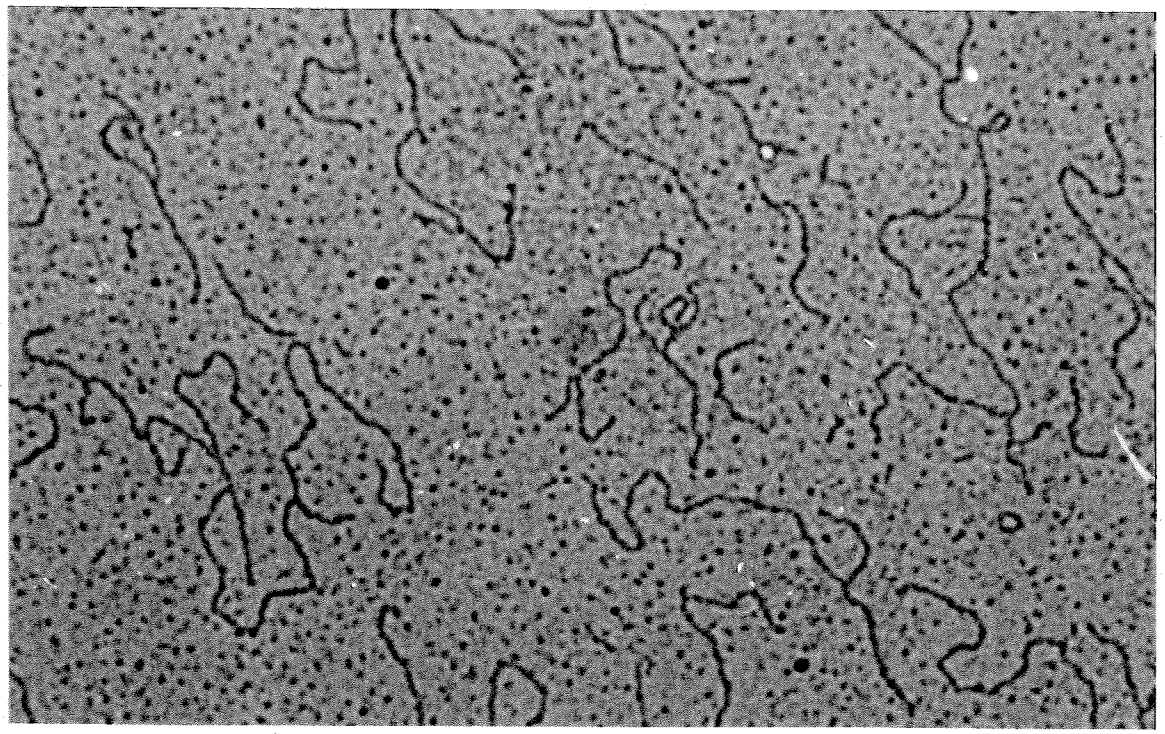
where the slope of the wedge was 0.043 D/cm.

PLATE 7-2

A. Uranyl stained Kleinschmidt of Polyoma II DNA. x 82,000.



B. Uranyl stained Kleinschmidt of synthetic dAT. x 60,000.



Data taken at 40 Kv and 80 Kv are listed below:

Density	through hole:	80 Kv - 1.4	40 Kv - 1.4
	film:	1.235	1.07
	DNA:	1.07	0.74

These data are in agreement with the inverse dependence of the scattering cross section on potential (Hall, 1966).

Thus at 80 Kv, $D_{film\text{-}hole} = D_{film\text{-}DNA} = 0.18$ which is equivalent to a density difference on the fluorescent screen of 0.07. This is equivalent to about 36 Å of density 20 material (Hall, 1966). The DNA-protein film is 100 - 200 Å wide. This contrast determination is equivalent to an overall density of DNA-protein-stain of about 5. That is, the protein must be saturated with uranyl ions and/or uranyl polymers. The stain is not specific for DNA. This staining technique gives contrast results comparable to well shadowed DNA preparations.

CHAPTER 8

The Physical Properties of the DNA of Bacteriophage N1: A DNA with Reversible Circularity

Contents: Page:

A. The Physical Properties of N1 DNA

The DNA of N1 bacteriophage (Naylor and Burgi, 1956), like that of its host, Micrococcus lysodeikticus, has a high guanine plus cytosine content (Scaletti and Naylor, 1959). The determination of the GC content as 64 ± 1% was described in Chapter 5.

N1 and T7 DNAs were prepared as described in Chapter 1 except that they were dialyzed into 0.1 F. NaCl, 0.01 F. EDTA, pH 7.8 and exhaustively against 0.1 F. NaCl, 0.01 F. Tris-OH plus HCl, pH 7.8. λ_{b2b5c} DNA was a gift of Professor J. Wang.

Band velocity sedimentation experiments (Vinograd, Kent, Bruner and Weigle, 1963) were performed with 30-50 microliters of A_{260} = 0.1 - 0.15 DNA in the pocket and with a 30 mm light path. Bulk solutions were 3.0 F. CsCl (density 1.37) with 0.01 F. Tris OH plus HCl, pH 7.8 or, for analysis of single stranded DNA, with 0.1 F. NaOH.

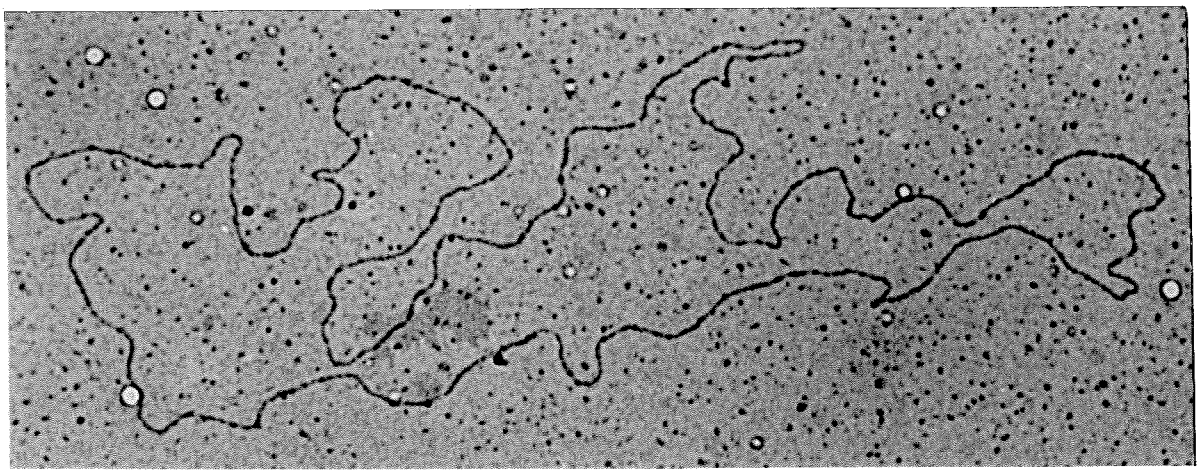
DNA electron microscopy was performed by the modified basic protein film technique described in Chapter 7.

N1 phage were prepared for electron microscopy by staining with 4% phosphotungstic acid, pH 7 and mounting on ionized carbon grids. The grids were washed once with distilled water.

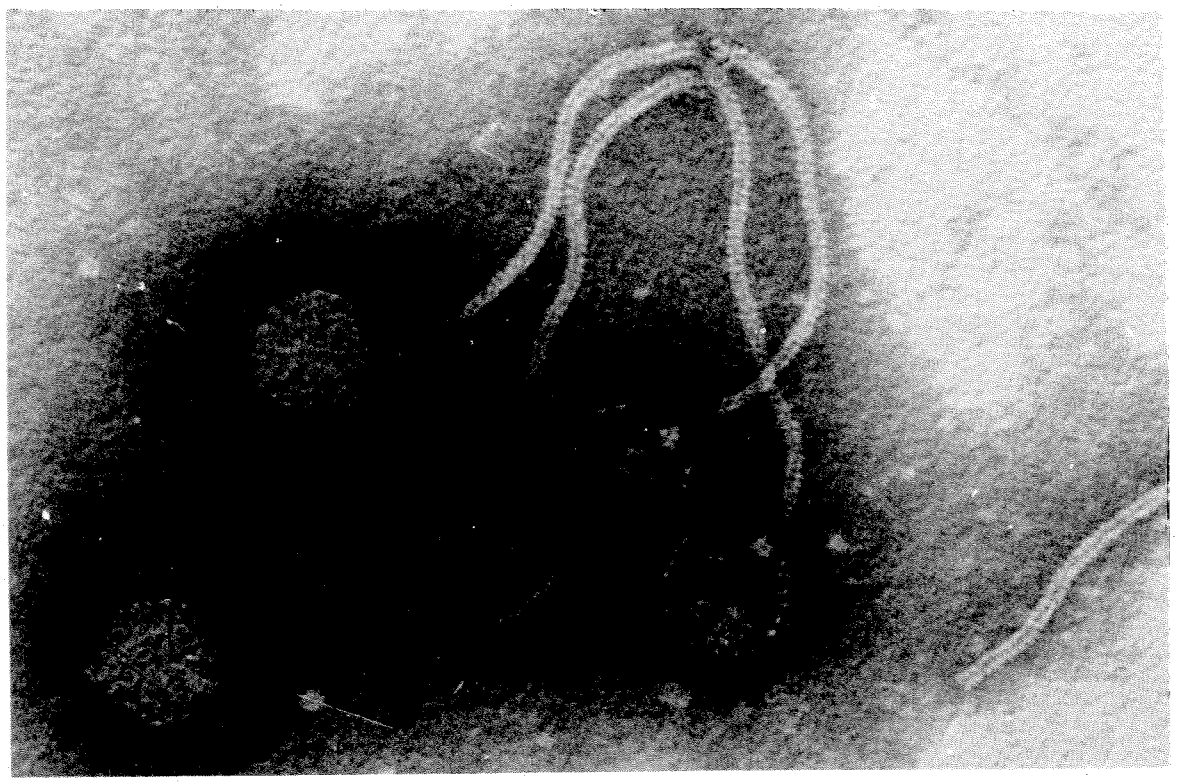
A sample of N1 DNA was heated in 1.5 M. NH_4Ac, 0.001 F. EDTA, pH 7 to 70° C. and cooled slowly. By electron microscopy, the sample was seen to contain more circular than linear molecules. Plate 8-1 shows a circular N1 DNA molecule as well as a group of N1 phage plus parts. The tails are longer (2000 Å vs.

PLATE 8-1

Cyclized N1 DNA. x 41,000. Uranyl stained Kleinschmidt preparation.



N1 Phage. x 300,000. Negative stained with phosphotungstic acid.



1500 Å) in N1 phage than λ phage. A core is clearly visible inside the tail of N1 phage. Otherwise the phage look alike.

All further annealing experiments were performed in the salt medium specified plus 0.01 F. EDTA, pH 8.0. Failure to add EDTA resulted in 100% irreversible conversion to linear molecules. Band velocity sedimentation experiments on a sample which by electron microscopy contained both linear and circular molecules showed two bands with relative S values of circular/linear = 1.105. This agrees with the ratio observed for other DNAs (Vinograd, et al., 1965; Hershey, Burgi and Ingraham, 1963). Thus, band velocity sedimentation may be used to measure the relative amounts of linear and circular molecules.

A sample of N1 DNA at A_{260} = 0.25 had a circular/linear (C/L) ratio of 0.7 with, in addition, a clearly visible dimer peak. After annealing for six hours at 25° C. in 2.0 F. NaCl at A_{260} = 0.1, the sample was almost completely cyclized (C/L = 4.0). Heating for one hour at 80° in 0.1 F. NaCl and quenching caused almost complete conversion to linear molecules. Subsequent annealing of this same sample at 30° in 2.0 F. NaCl for 12 hours resulted in C/L = 3.8, demonstrating the reversibility of the process. Thus N1 DNA resembles λ DNA (Hershey, Burgi, and Ingraham, 1963) in its ability to reversibly form circles and multimers.

The rate of cyclization seems to be faster for the case of N1 than for λ_{b2b5c} DNA (Wang and Davidson, 1966a). The melting temperature for the cohesive ends appears to be slightly lower for the

N1 case. Annealing experiments were carried out in 0.09 F. NaCl, 0.01 F. EDTA, pH 8.0 with N1 DNA at A_{260} = 0.15. The starting sample contained 60 ± 5% linear molecules. Results appear in Table 8-1.

It is, therefore, seen that the melting temperature of the ends of N1 DNA lies between 40.6 and 44.7° C. For comparison, the melting temperature of the ends of λ_{b2b5c} DNA in the same salt (Wang and Davidson, 1966a) would be about 49.6°C.

TABLE 8-1

Experiment	Time Annealed	Temperature(°C. 0.5°)	% Linears
1	44 hours	30.0°C.	43±6%
2	16 hours	30.5°C.	33±5%
3	44 hours	40.6°C.	42±6%
4	16 hours	44.7°C.	61±5%
5	24 hours	47.2°C.	75±5%
6	24 hours	57.5°C.	88±5%

Annealing of the cohesive ends of N1 DNA in 0.09 F. NaCl, 0.01 F. EDTA, pH 8 with N1 DNA at 7.5 micrograms per milliliter.

Experiment number one has obviously not yet reached equilibrium. Experiment number two may reflect some deviation from equilibrium. The minimum circular content achieved (annealed in 2.0 F NaCl) was 18%. This result may be due to inability of a fraction of the molecules to cyclize. The maximum linear content achieved

was 88%. This result appears to be due to cyclization occurring at 20° C. in the 3.0 F. CsCl bulk solution used for sedimentation. This result implies that the rate of cyclization for N1 DNA is much faster than for λ_{b2b5c} DNA (Wang and Davidson, 1966a).

The lower melting temperature of the cohesive ends of N1 DNA could be due to a lower GC content, to shorter end-segment length or to damage to the ends. The last possibility seems extremely unlikely as those molecules found cyclized after extraction and dialysis had the same characteristics as those found in linear form.

The parameters related to the length of the ends could be measured in a similar way to those measurements on the ends of λ_{b2b5c} DNA (Wang and Davidson, 1966a). A better system would be annealing in 0.3 F. NaCl plus buffer and analysis in 0.3 F. NaCl in D_2O to facilitate dumping of band centerpieces. In this system, a conveniently fast rate should apply in the region of melting of the cohesive ends and a sufficiently slow rate at 20° C. where analyses are performed.

The relative sedimentation velocities of linear N1 and T7 DNAs were 1.14 at pH 7.8 and 1.12 at pH 13 after correction for buoyancy factors. Accepting a value of 25×10^6 for the molecular weight of T7 DNA, these ratios imply (Crothers and Zimm, 1965; Studier, 1965) a molecular weight of 33×10^6 for N1 DNA. The runs at pH 13 showed less than 20% single strand breaks in either DNA. Length histograms of λ_{b2b5c} and N1 circular molecules gave relative lengths of 0.87, which agreed almost exactly (Caro, 1965; MacHattie and Thomas,

1964) with that expected from the sedimentation determination of the molecular weight of N1 DNA.

B. A Discussion of Lysogenic Phage Systems

The two series of phage DNAs with cohesive ends found to infect E coli are derived from or are the DNAs of lysogenic phage. The two groups are U. V. inducible and non-U. V. inducible (Jacob and Wollman, 1956; Jacob and Wollman, 1961). The U. V. inducible and non-U. V. inducible phage recombine with their own group but not with the other group. If the recombinants are to be able to form circular molecules, their cohesive ends must be similar. Baldwin, et al. (1966) have shown that DNA of phages 424, 21 and 434 can form oligomers with λ DNA. Yamagishi, Nakamura and Ozeki (1965) have shown that the DNA of phages 80 and 81 can form oligomers with λ DNA. All of these phage belong to the U. V. inducible group. Baldwin, et al. (1966) have also shown that the DNAs of phages 186 and 299, of the non-U. V. inducible group, do not form oligomers with λ DNA.

Campbell (1962) suggested that the cohesive sites are a method of producing circular molecules, which are in turn necessary for the recombination process leading to lysogeny. P22, a lysogenic phage, does not contain cohesive ends, but contains a terminal redundancy. It is easy to see how a terminal redundancy can lead to circular molecules.

The various U. V. inducible phage map all over the E coli chromosome. Also, the immunity markers are different. It is possible that a family of lysogenic phage, as the U. V. inducible E coli group, started out as one phage and changed into a large group due to various mutational processes. If recombination is selected for, as

one would suppose, any alteration of the cohesive ends would be selected against. It might not be surprising, therefore, if the cohesive ends of the group were all identical. On the other hand, these phage may have arisen from more than one source, and those phage with similar functional sequences and cohesive ends selected. The most likely hypothesis is that the phage have a common source. If this hypothesis is true, it is likely that the non-UV inducible group arose from a second source. A study of the homology between phage DNAs of the two groups could lead to increased certainty that the groups are unrelated.

The presence of cohesive ends in N1 DNA suggests that the phage N1 is part of a family of lysogenic phage. M. lysodeikticus phage N1 - N6 have been partially characterized (Naylor and Burgi, 1956). N5 is lysogenic and U. V. inducible. The use of oligomer formation could be used to classify the phage N1 - N6 just as oligomer formation is common for the lambdoid group. The fact that both the lambdoid phage and N5 are U. V. inducible suggests that the U. V. inducible mechanism may be common to many unrelated lysogenic phage systems. Although N5 has not been shown to be unrelated to the lambdoid phage group, such a relation is highly unlikely.

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PROPOSITION 1

The Fate of Parental RNA in Rous Sarcoma Virus Infection

ABSTRACT

It is proposed that the fate of parental P^{32} labelled Rous Sarcoma Virus RNA be studied to determine the structure of the replicative intermediate produced in the process of infection or transformation. A system is devised which would lead to isolation and identification of double-stranded or partially double-stranded RNA-RNA or RNA-DNA intermediates into which the parental RNA is incorporated. Such a study might help to clear up the questionable existence of a DNA provirus in Rous Sarcoma infected or transformed cells.

There is a controversy as to whether, contrary to all precedent and current dogma about RNA viruses, the in vivo replication of the RNA of Rous Sarcoma Virus involves an intermediate formation of a DNA strand which is complementary to the viral RNA.

Rous Sarcoma Virus (RSV) of the Bryan high titer strain, along with the helper virus of this strain, Rous Associated Virus (RAV), and the related Avian Myeloblastosis Virus and the Rauscher Mouse Leukemia Virus, has been shown to contain a single stranded RNA of molecular weight about 10×10^6 Daltons (Robinson, Pitkanen, and Rubin, 1965; Robinson and Baluda, 1965; Duesberg and Robinson, 1966). The infection process of RSV in chick embryo cells begins with the disappearance of active virus. From one hour after infection until 12-14 hours after infection, the level of active virus remains at less than 5% of the input activity (Vogt, 1965). Following infection by RSV, no RNA-dependent RNA-polymerase can be detected (Wilson and Bader, 1965) for either RSV or RAV. A transient requirement for DNA synthesis occurs during the eclipse period with RSV or RAV infection or RSV transformation (Tamin, 1963; Bader, 1964; Vigner and Golde, 1964; Temin, 1964a; Bader, 1965a; Bader, 1965b).

An indirect experiment by Bader (1966) concerning the course of RSV infection seems to show that the DNA synthetic phase proceeds in the absence of significant protein synthesis. He states that this means that the RSV intermediate requirement for DNA synthesis relies on the host cell enzymes. It seems highly unlikely that cells of higher organ-

isms should have a system for initiating their own transformation by RNA. In this experiment, the level of enzyme synthesis was decreased by 90% in the presence of puromycin. If the rate determining step in transformation is not protein synthesis, then the results are meaningless. Bader's work is mentioned to show that anomolous conclusions can easily be drawn by performing experiments on whole cells with drugs whose action is interpreted by using a presupposed scheme for the steps involved in the infection or transformation processes. It is quite possible that the experiments regarding a transcient requirement forDNA synthesis, which rely on chemicals added to cells, could be incorrectly interpreted. In this case, all possible actions of the drugs cannot be known, and their mechanism of action is preconceived.

One hypothesis to explain the DNA requirement results is that the single stranded parental RNA is converted into a DNA-RNA duplex and subsequently into a DNA provirus. The DNA provirus could then produce RSV RNA as a messenger RNA. After the transient requirement for DNA synthesis, blockage of DNA synthesis does not alter virus production, whereas blockage of RNA synthesis leads almost immediately to cessation of virus production.

The only direct experiment relating to the provirus is a hybridization experiment of Temin (1964b). If the results are believed, then a DNA provirus has been demonstrated. However, as the data were not above what should be considered a noise level, there is good reason to doubt these experiments.

We propose that the question of the non-occurrence or occurence of a DNA intermediate in RSV replication be resolved by following the

fate of P^{32} labelled infecting RSV RNA during the course of infection and transformation. P^{32} labelled infecting RSV could be prepared by the method of Robinson, Pitkanen and Rubin (1965). With 500 milicuries per milimole of phosphate in the medium used for growing the virus, a label of 5×10^{-5} cpm/viral RNA molecule should be achieved. The rate of killing of virus via P^{32} decay should be sufficiently small if the labelled virus is used within three days. Transformed cells producing a regular daily titer of virus could be selected for P^{32} labelling.

A maximum of 10% of a plate of cells can be infected (Temin and Rubin, 1958). Crawford and Crawford (1961) have estimated that 750 virus obtained from a density gradient sedimentation experiment are needed to produce one focus forming unit. To measure 10% transformation of 10^9 cells (about 100 plates), 10^{12} virus would be needed. Robinson and Baluda (1965) describe production of 60 times this much virus.

After infection of plates of chick embryo cells with P^{32} labelled virus for 30 minutes, plates could be washed with anti-RSV serum to remove excess virus. After a defined length of time, the cells could be cooled, broken, and centrifuged to remove cell debris and coated virus remaining. The total nucleic acid could be extracted and fractions counted for ethanol precipitable P^{32} counts. If the RNA in each potentially transformed cell has not been degraded, 5000 cpm should be precipitable. The portion of precipitable counts will represent

single stranded RSV (and RAV) RNA and any intermediate double-stranded or partially double-stranded forms. After washing the precipitate thoroughly and resuspension, pre-boiled RNase could be added to degrade single-stranded RSV RNA. After precipitation again, the supernatant could be counted to determine the amount of RNA degraded. This procedure could be repeated until no label is found in the supernatant.

If 1% of the cells undergoing transformation contain parental RNA protected from RNase, 50 cpm would be obtained in the final precipitate. Thus isolation of 1% of the parental RNA in an intermediate state could lead to detection. The experiment could be repeated at different times after infection to determine the percentage of RNase resistant parental RNA as well as the percentage of in vivo degraded RSV RNA.

If the infection process were not at all synchronous, the half-life of an RNase resistant intermediate would need to be 6 minutes for detection. It seems likely, considering that the process is somewhat synchronous and that 6 minutes is a small portion of the eclipse time and that progeny RNA synthesis immediately precedes virus production, that an intermediate should be trapped. We shall now consider identification of RNase resistant material as an intermediate in the infection process, rather than a possible noise level of RNase resistant single-stranded RSV RNA.

The DNA content of a chicken cell is 2.4 picograms (Chargaff and Davidson, 1955). 10^9 cells would contain 2.4 mg of DNA or

50 A_{260} units. By sedimentation in $CsSO_4$ (Hearst and Vinograd, 1961), it should be possible to band any RNA containing species well away from the bulk of the DNA in about 30 ml of solution. The difficulty of the high concentration of DNA could be overcome by choosing the $CsSO_4$ concentration such that DNA moves as a boundary toward the top of the centrifuge tube. If the radioactivity were found at a position expected for a DNA-RNA hybrid, then an intermediate leading to the hypothetical provirus would be demonstrated. Its molecular weight could be determined by sedimentation a sucrose gradient. If the RNA were found to band near the banding position of the RNA of a single-stranded RNA virus like TMV or double-stranded RNA virus like rheovirus, then the degree of double-strandedness could be estimated. The sedimentation velocity and banding position should be determined both before and after denaturation. A definitely double-stranded RNA with label would identify an RNA-RNA intermediate in the infection process. If, after denaturation, two RNA sizes were found, annealing with cold RSV RNA and sedimentation could lead to a decision as to whether a partially double-stranded replicative form was involved.

The label could be found with a DNA band if the RNA were non-specifically stuck to DNA or if RNA were integrated into DNA. In both cases, the RNA could be made to band separately from DNA if the DNA were sheared before banding. As incorporation of a provirus into a chromosome requires a region of homology between the chromosomal DNA and the provirus and as RSV infects chickens, hamsters, dogs and human tissue culture cells, etc. (Ahlström, Kato and Levan,

1964; Rabotti, Anderson and Sellers, 1965; Jensen, Girardi, Gilden and Koprowski, 1964; Rabotti, Grove, Sellers and Anderson, 1966) and as it is unlikely that similar homology regions exist in all these species, a DNA-RNA hybrid banding with DNA is unlikely.

The rate of breakdown of infecting RNA should be noted, as P^{32} could be transferred into the metabolic pool and then into cellular nucleic acid. This complication is particularly important if the RNA counts appear in an RNA banding position.

If the hybrid is observed as a function of time, then from experiments stopped at different times after infection, some information about the time course of the infection could be learned in addition to information about the mechanism of infection.

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PROPOSITION 2

Studies of the Proteins produced by the Resistance Transfer Factor Episome.

ABSTRACT

A procedure is devised to make the proteins synthesized by the resistance transfer factor account for the majority of cellular proteins. A possible method is devised for identification of specific proteins related to resistance markers. Consideration is given of the mechanism of operation of these proteins.

The simultaneous acquisition by bacteria producing bacillus dysentary of resistance to streptomycin, chloramphenicol, tetracycline and sulfonamide was discovered in Japan several years ago. The resistance to these four drugs has been attributed to a bacterial episome called the R factor or resistance transfer factor. A review of information obtained before 1963 was written by Watanabe (1963). The episome has been mapped as far as the transfer factor and four immunity markers are concerned. It has been found to segregate or become incorporated into the host chromosome in some bacteria. The most recent article concerning the genetics of the R factor is Watanabe and Ogata (1966). Another R factor, R_6, has subsequently been isolated by Lebek (1963) and characterized by Watanabe, Ogata and Sato (1964). This R factor contains an additional gene producing resistance to neomycin, kanomycin, framycetine, paromoycin and aminodisine, all related antibiotics.

The mechanism of acquired resistance seems to be a decreased permeability of the bacterial cell walls to the drugs (Watanabe, 1963; Izaki, Kinchi and Arima, 1966). Experiments in spheroplast systems show no drug resistance. Also, labelled antibiotics are found at lower levels in growing cells if the cells contain the R factor.

The cells could be made resistant by either altering the present cell wall or by altering newly synthesized cell walls. In the latter case, a cell with a newly acquired episome rapidly becomes immune to the antibiotics. We can conceive that the protein synthesized by

the episome alters the cross-linking of the membrane of the cell, plugs up holes in the cell wall, or interacts specifically with the antibiotic at the cell surface. Although massive enzymatic degradation of drugs in the presence of the R factor is not reported, the last possibility of interaction at the cell surface is not ruled out. Thus, it would be interesting to isolate the proteins coded by the resistance markers in order to learn more about the mechanism of their action.

Rownd, Nakaya and Nakamura (1966) have examined the R factor physicochemically. They have found that in Serratia or E coli, only one replication of the episome takes place each cell generation. With buoyant density sedimentation data on R factor and host DNA and other older data, they conclude that the R factor contains $25\text{-}30 \times 10^6$ Daltons of DNA. In Proteus mirabalis (40% GC), the replication of episome and host chromosome has been uncoupled. There are 12 R factor molecules per host chromosome, or 12% of the DNA is episomal DNA. The R factor has segments of 52% and 58% after shear degradation.

We propose that it should be possible to isolate specific proteins associated with the R factor. It should be possible to produce specific mutations in the Tc, Sm, Su and Cm markers by mutagenesis and replica plating in media with and without the drugs. The mutant episomes should produce an altered protein coded by the mutant resistance marker, Ptashne (1967) used this comparison technique to identify the C1 protein of phage λ. The mutagenesis would have to be

done in a bacterium which contains only one copy of the episome. It would be necessary to show that the bacterium had not been "cured" of the entire episome. This could be done by testing the other drug resistances. The tetracycline resistance marker can be segregated from the other markers (Watanabe, 1963). There is a known segregant related to the fertility of the R factor (Watanabe, Mishida, Ogata, Arai, Sata, 1964). It would be necessary to use bacteria which did not segregate for the resistance factors with a frequency near to the mutagenesis frequency.

It has been shown that the R factor is randomly replicated each generation in Proteus and that the majority of episomes are not replicated at all during a single cell generation. Therefore, various treatments which selectively destroy replicated DNA can be used to enhance the fraction of newly synthesized total cell protein which is coded for by the non-replicated DNA. Furthermore, as there are 12 episomes per cell, this is a favorable system in which to look for the proteins coded by the resistance markers. If we can transfer a normal R and a mutant R to two Proteus cultures, we begin with a 12 fold purification of episomal protein and can further enhance the quantity of episomal protein. The enhancement could be accomplished by P^{32} labelling the host chromosome and allowing decay to take place before testing for protein synthesis in the presence of H^3-leucine and C^{14} leucine. Any other treatment selectively destroying replicated DNA could be used. For example, deoxy 5-BU plus U. V.

If we grow the treated bacteria in the presence of the drugs for

which immunity markers are carried in both mutants, H^3 leucine should be incorporated into protein only in bacteria carrying intact episomes. C^{14} leucine could be used in the case of the mutant resistance marker control. It might be possible to improve the expected yield of episomal protein from 12% to perhaps 60%. As the host proteins should then be a large variety of proteins of individually low concentrations, chromatographic analysis of episomal coded proteins should be uneffected by host-protein contamination.

If 5×10^4 Daltons of DNA codes for one protein, then, after enhancement, each episomal protein should account for about 1% of the total protein. The degree of enhancement could be tested by polyacrilamide gel electrophoresis (Raymond and Wang, 1960) of Proteus, Proteus plus R and treated Proteus plus R proteins.

The identification of the particular protein associated with the mutation could be done as Ptashne (1967) identified the lambda repressor protein. Several mutations of the same marker should be tested to absolutely identify the protein as that associated with the marker.

Preliminary isolation of bacterial cell walls might help to further concentrate the proteins associated with resistance. Once isolated, the effect of these specific proteins on the drugs could be tested. Also, if no effect were found in adding the drugs to the protein, the effect of the protein on cell walls could be tested to see if the proteins merely bind to the cell walls or are enzymatically active in cross-linking the cell-wall structure.

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PROPOSITION 3

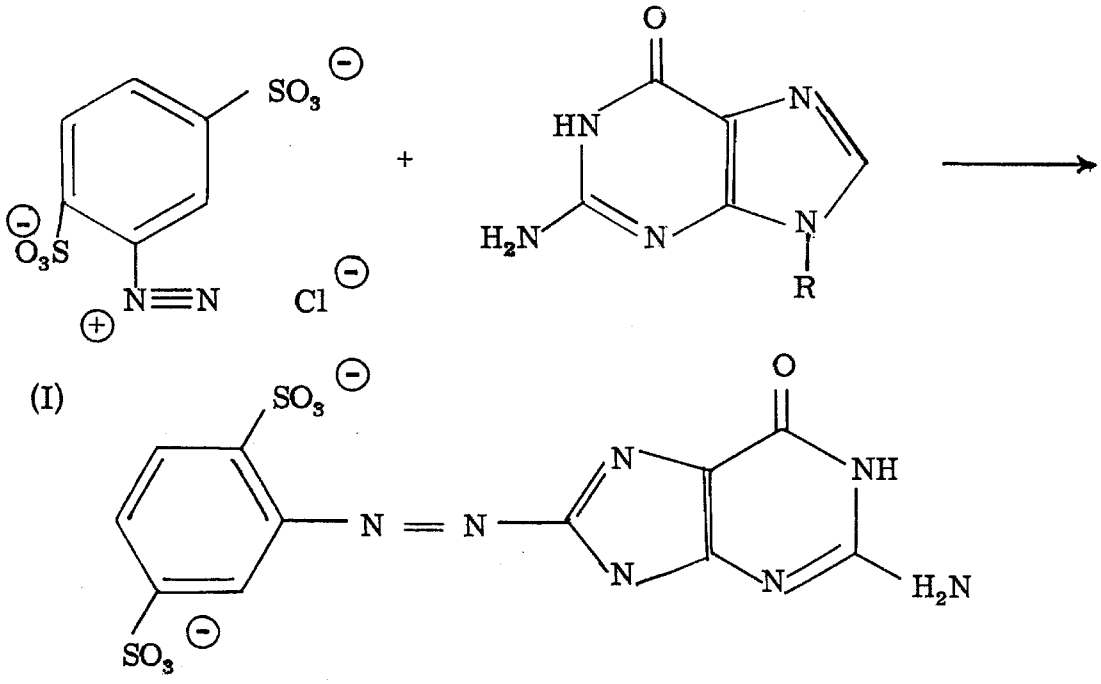
Selective Electron Stain Binding Reagents for Covalent Linkage to Cytosine in DNA

ABSTRACT

The preparation of phenylhydrazine 2, 5 disulfonic acid and 2-chloro-3-phenylpropionic acid hydrazide 2', 5' disulfonic acid is described. One or both of these reagents should specifically add to the amino position of cytosine with displacement of ammonia. The sulfonic acid groups may be used to bind uranyl or other heavy metal stains. The possibility of binding 12-phosphotungstic acid to Girard reagent adducts of cytosine is considered.

Beer and Maudrianakis (1962) first demonstrated a procedure for selective staining of DNA bases for electron microscopic observation. The procedure involves the formation of a covalent linkage between an organic compound and one of the DNA bases. The organic compound is then stained with uranyl ions. The uranyl ions are observed in the electron microscope.

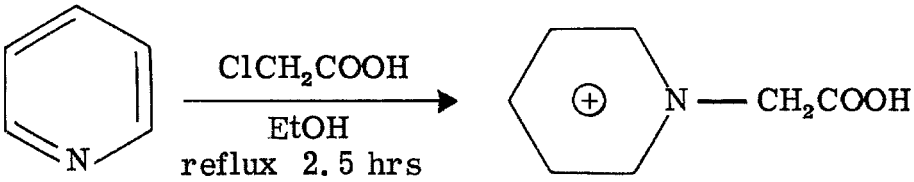
Maudrianakis and Beer (1965) describe the preparation of a diazonium salt which specifically reacts with guanine bases in DNA. 2-amino-p-benzene disulfonic acid can be purchased and diazotized. The diazotized compound (I) specifically reacts with guanine by the following scheme:

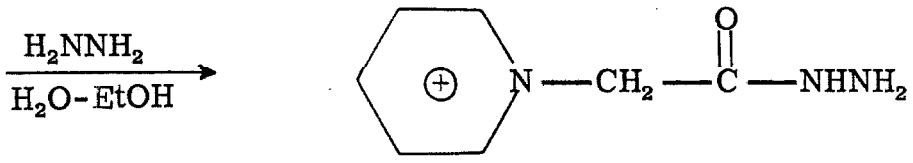


Bartl, Erickson and Beer (1967) have recently used this reaction coupled with uranyl binding to the sulfonic acid groups to determine

the guanine sequence in a transfer RNA.

Verwoerd and Zillig (1963) and Kochetlov, Bodowsky and Shibaeva (1963) have shown that hydroxylamine and hydrazine have a specificity for cytosine. Hayatsu, Takeisha and Ukita (1966) and Hayatsu and Ukita (1966) have shown that semicarbazides specifically react with cytosine without degradation of cytosine or DNA. Kikugawa, Hayatsu and Ukita (1967) have shown that Girard P (II) specifically adds to cytosine without significant chain scission. Girard P (Girard and Sandulesco, 1936) is prepared according to the following scheme:

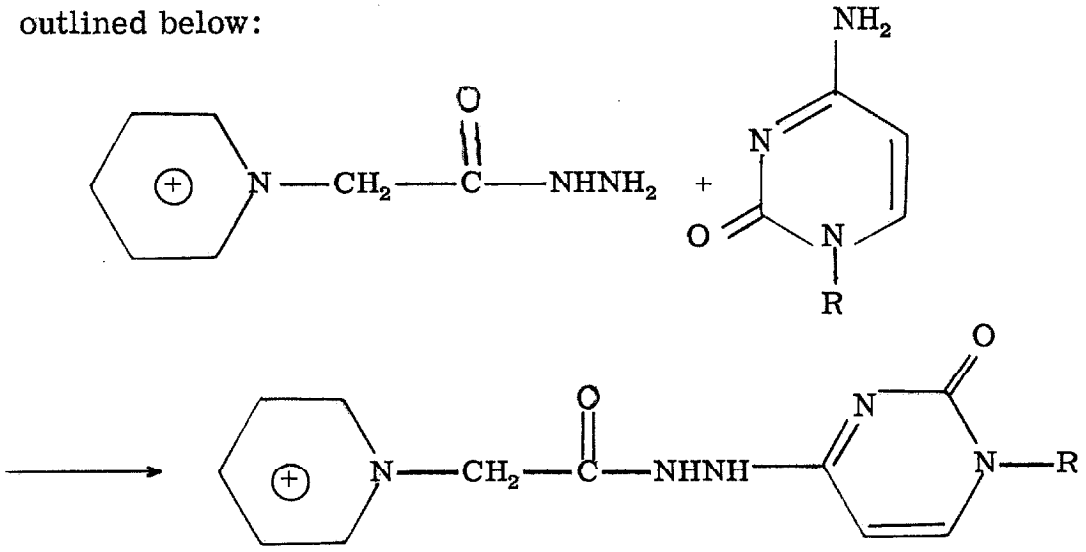




(II)

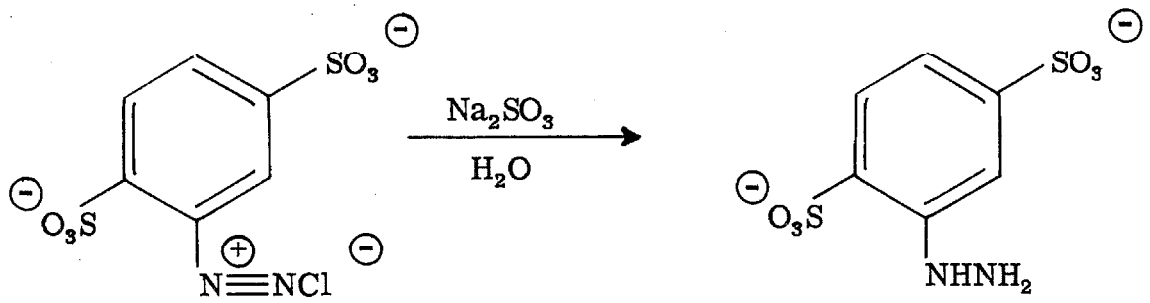
This product (II), after purification, adds to cytosine at 37°C. under mildly acidic conditions. The reaction requires the protonated form of the DNA base. Because of the high pK_a of cytosine, reaction takes place preferentially with cytosine. The reaction follows the scheme

outlined below:

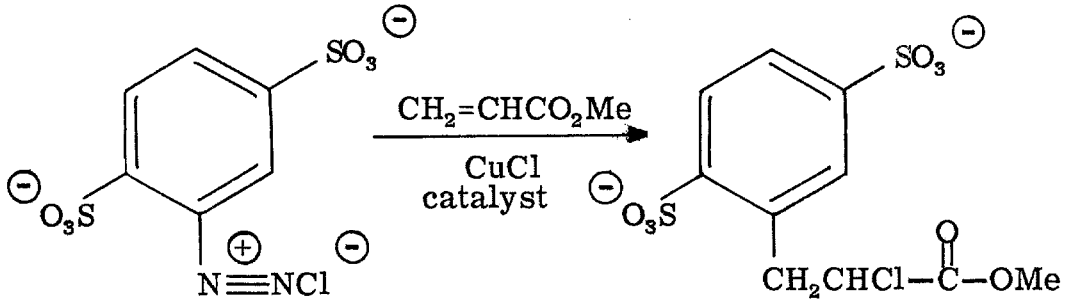


We propose that the binding of phenylhydrazine 2, 5 disulfonic acid (III) with DNA be studied to see if this reagent is specific for cytosine without degradation of cytosine or of DNA. We propose a similar study with 2-chloro-3-phenylpropionic acid hydrazide 2', 5' disulfonic acid, (IV). In the latter case, the specificity of the reaction is certain. However, the stain will be found quite separated from the cytosine moity. If reagent III works, it is preferred. The synthesis of these two reagents is described below. We also will propose a study of the feasibility of binding 12-phosphotungstic acid to Girard reagent adducts to cytosine.

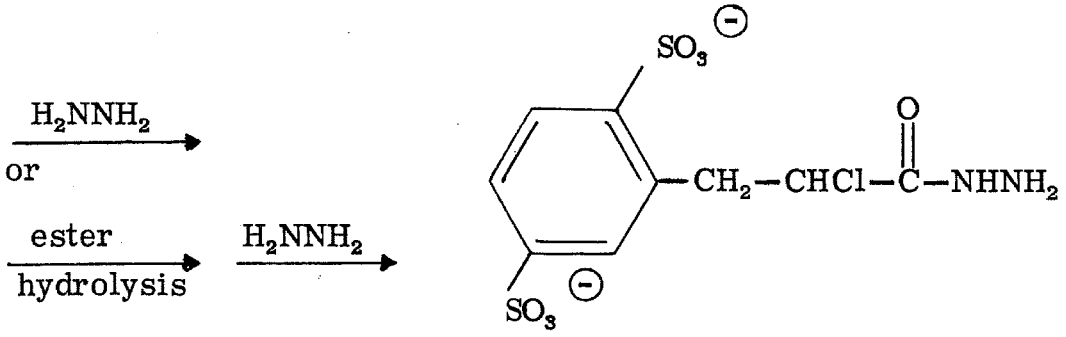
The two disulfonic acid reagents (III, IV) are prepared from the diazonium reagent (I). The reaction leading to the phenylhydrazine reagent (III) was described on a similar compound by Stroh and Westphal (1963). For the production of IV, see the analogous reaction (Meerwein reaction) in Noller (1957) p. 496.


SO3
Na2SO3
H2O
O3S
N≡NCl
SO3
O3S
NHNH2

(I) (III)


SO3
CH2=CHCO2Me
CuCl
catalyst
O3S
N≡NCl
SO3
O3S
CH2CHCl—C—OMe
O

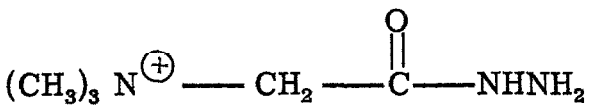
(I)


H2NNH2
or
ester
hydrolysis
H2NNH2
SO3
CH2—CHCl—C—NHNH2
O
SO3

(IV)

Both of these two reagents (III, IV) should be water soluble due to the two sulfonic acid groups. Both reagents should bind uranyl ions as described by Beer and Mandrianakis (1962).

Kahane and Kahane (1930, 1936), Healy (1964) and Asmus (1965) describe the binding of 12-phosphotungstic acid to organic amines. Girard P, which has a pyridinium group, might bind PTA, just as the sulfonic acids bind uranyl. The specific adduct of Girard P with cytosine was mentioned above. Girard T (Girard and Sandulesco, 1936) might also be used. It has the structure:



If such a binding could be made to occur in dilute solutions of PTA in alcohol, alcohol-water or ether, DNA already reacted with Girard P or T and mounted on a support film could be specifically stained with a mass ten times that of uranyl.

The structure of 12-phosphotungstate is described by Linnett (1961). The tungsten atoms are symmetrically disposed about the center phosphate at about 3.6 Å. Almost all of the density of PTA is contained in a sphere of diameter 10 Å. If the resolution of the electron microscope were forced to be limited at 7-10 Å by diffraction at the objective aperture, the scattering cross-section would be increased by a factor of 2 above that required for seeing uranyl with a resolution of 4 Å. The PTA would be seen in an area 4 times that of uranyl, but with 10 times the scattering mass and

2 times the scattering cross-section. Thus PTA would produce about 5 times as much contrast. Using the cross-sections for electron scattering in Hall (1966), we find that PTA would be visible to the naked eye on the fluorescent screen of the electron microscope. The sacrifice of two times in resolution for 5 times in contrast would be justified for preliminary work on cytosine sequences. Considering the size of the organic reagents involved, it might not result in any loss of ability to locate the cytosines. Also, if the DNA chains were sufficiently extended, as the RNA chains were in the work of Bartl, Erickson and Beer, (1967), the resolution might be sufficient for exact sequence work.

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PROPOSITION 4

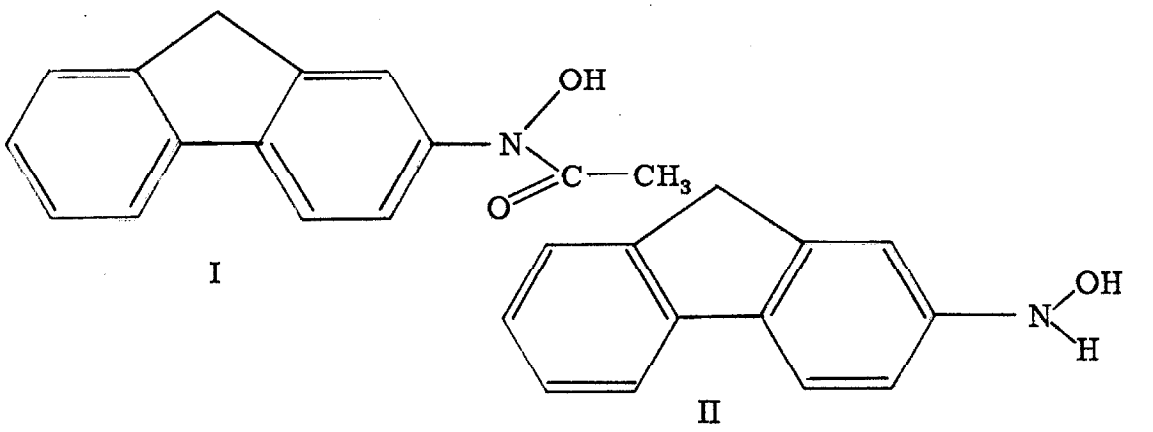
Selective Anionic Electron Stain Binding Reagents for Covalent Linkage to Guanine in DNA

ABSTRACT

To bind anionic electron stains, reagents selective for the DNA bases must contain positively charged groups. Two types of reagents for guanine containing quarternary ammonium groups are proposed. The first is the diazonium salt type previously used by Beer. The second is the N-aryl-N-acetoxy hydroxamic acid type. The use of good leaving groups in this last reagent is suggested to overcome the problem of using such a reagent containing quarternary ammonium groups.

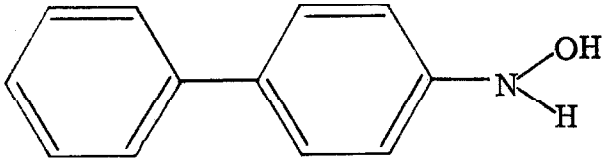
In the previous proposition (Proposition 3), we suggested the use of heavy metal anions instead of cations for staining organic adducts of cytosine. The contrast advantage of highly complex anions over uranyl cations was pointed out. In this proposition, we suggest the synthesis of some possible organic reagents for the base complementary to cytosine, guanine. Beer and Maudrianakis (1962) describe a diazonium reagent specific for guanine at pH 9. This reagent binds heavy metal cations. Bartl, Erickson and Beer (1967) have had some success with this reagent in determining the guanine sequence of a transfer RNA. We propose the synthesis of such reagents containing quarternary ammonium groups. We propose that the guanine specificity be re-examined, as the substitution of quarternary ammonium groups for sulfonic acid groups may alter the specificity. We also propose the synthesis of a different type of guanine specific reagent containing quarternary ammonium groups, an N-aryl-N-acetoxy hydroxamic acid. We shall first consider the pertinent history of this second type of guanine specific reagent.

N-2-fluorenylacetamide (FAA) and the more carcinogenic N-hydroxy metabolite, N-hydroxy-FAA (I), have been shown to become incorporated into DNA and RNA in vivo (Marroquin and Farber, 1962, 1965). Kriek (1965) showed that N-hydroxy-2-fluorenamine reacted better than p-biphenylhydroxylamine (III) which, in turn, reacted better than 2-napthylhydroxylamine (IV). The last two compounds reacted at pH 4. All of these reactions are acid catalyzed.

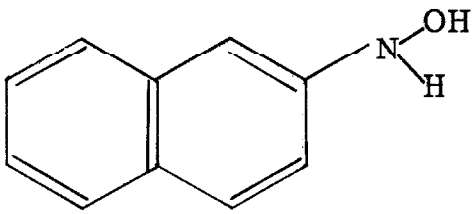


I

II

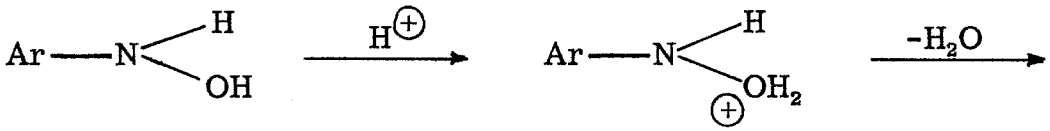


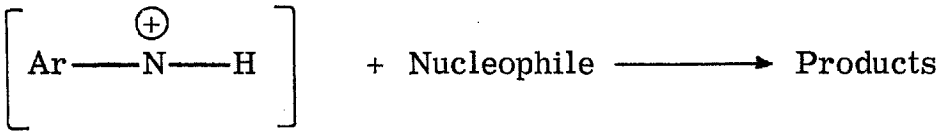
III



IV

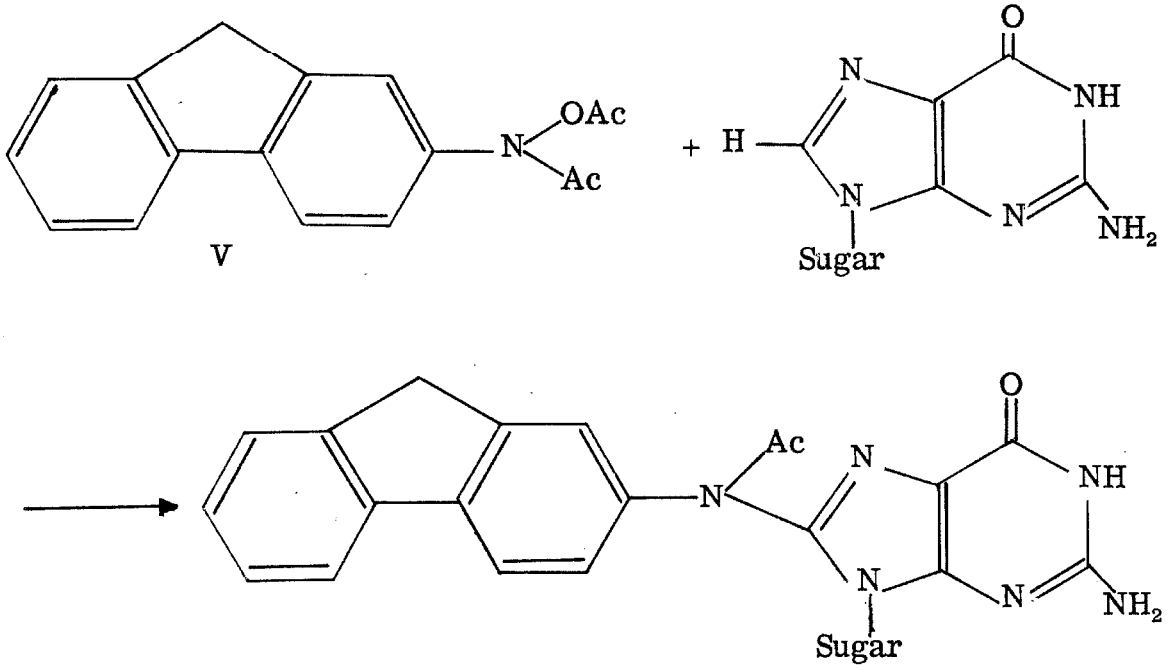
The findings of Kriek (1965) are in agreement with the proposal that hydroxylamines may act as electrophiles (Heller, Hughes and Ingold, 1951) according to the following mechanism:





Irving (1966) showed that N-hydroxy-FAA (I) reacted in vitro with nucleic acids at pH 5-6. The acyl group replacing the N-hydrogen on the hydroxylamine has a small stimulating effect on the reaction. The preparation of the hydroxylamine (II) and the N-acyl derivative (I) is described by Poirier, Miller and Miller (1963) and Miller, Miller and Hartman (1961).

Miller, Juhl and Miller (1966) showed that another possible in vivo metabolite of FAA, N-acetoxy-FAA (V), readily reacted with guanine in DNA at pH 7. The acetoxy compound (V), which they prepared, has a demonstrated specificity for guanine at a pH where diazo compounds do not show this same specificity (Maudrianakis and Beer, 1965). Kriek, Miller, Juhl and Miller (1967) have identified the product of addition and found a C-8 adduct of guanine, just as in the case of diazo addition. The reaction proceeds as follows:

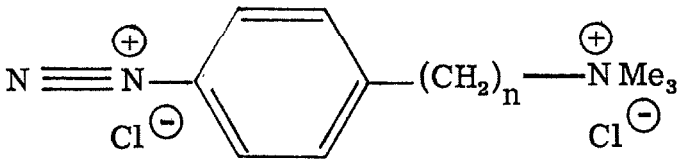


The acetoxy leaving group instead of hydroxyl has a large effect on the reaction rate and the pH below which reaction occurs. This reaction takes place almost as well with native DNA as with denatured DNA.

The selectivity of this type of reagent for guanine suggests that compounds like N-acetoxy-FAA (V), with good leaving groups, could be used for selectively staining guanine in DNA in a manner analogous to the diazonium salt staining reagents.

We shall now describe the synthesis of trial electron stain binding reagents with quarternary ammonium groups, rather than sulfonic acid groups as previously used with diazonium salt reagents. We shall first discuss the diazonium salt type reagents.

Reilly and Drumm (1935) describe the synthesis of the following diazonium salts containing quarternary ammonium groups (VI):



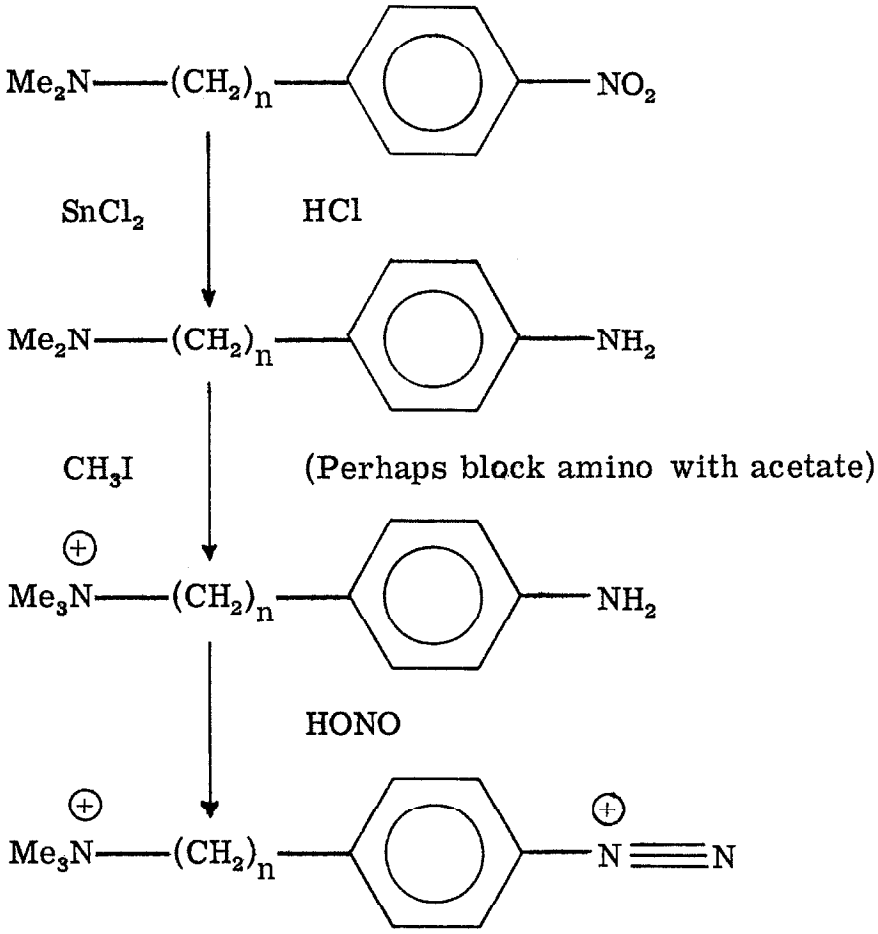
VI $\qquad$ n = 0, 1, 2

The stability of the compounds decreases with the length of the methylene chain. All such compounds are easy to synthesize. The reaction scheme for preparing them is shown in Figure P-1. The quarternary ammonium group has an effect on the stability of diazonium salts and may have an effect on the specificity for guanine. The pH profile of reaction with the bases would have to be reexamined (Maudrianakis and Beer, 1965). It is conceivable that this kind of reagent may not have enough specificity

for guanine. The sulfonic acid reagents only prefer guanine by a factor of 10 and are completely unsuitable at neutral pH or lower.

FIGURE P-1

The Synthesis of Guanine Specific Anion Binding Reagents--Diazonium Salts.

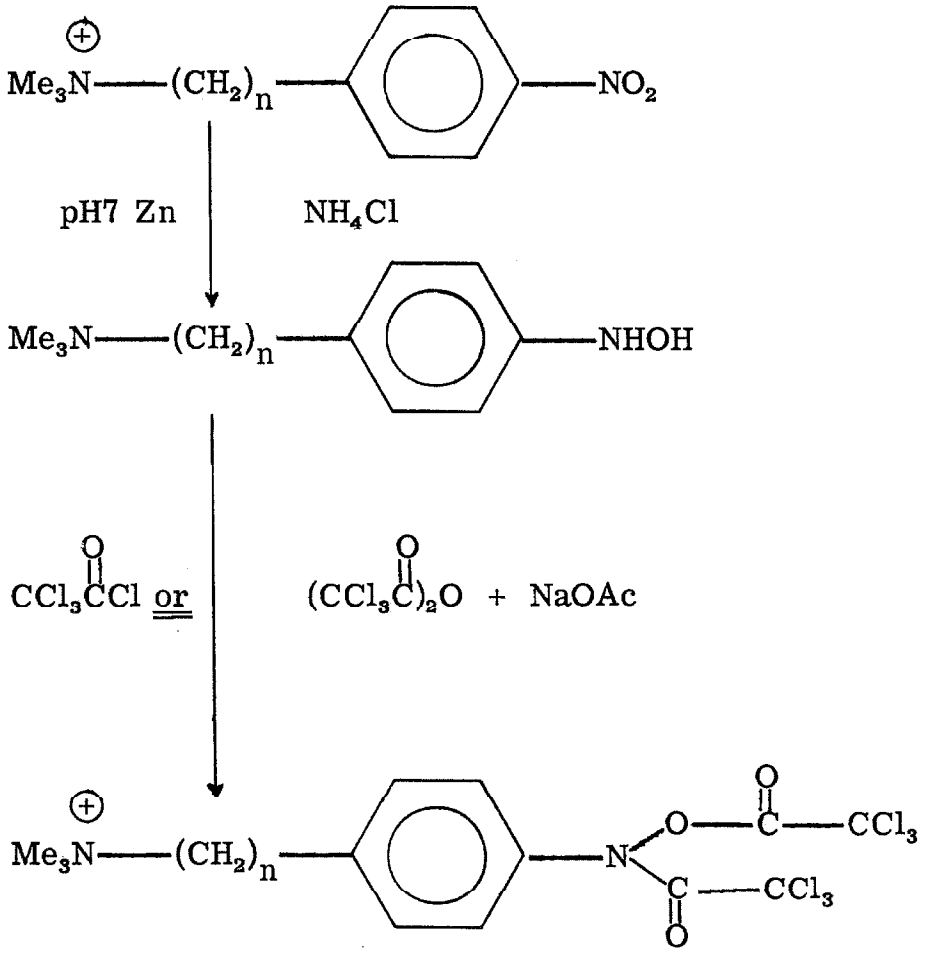


We also propose that the reaction be studied between guanine (and the other bases) and N-aryl-N-acetoxy hydroxamic acid where the aryl group contains the quarternary ammonium substituent(s). This type of reagent has a demonstrated guanine specificity with different substituents (and at lower pH than the diazonium salt reagents), as discussed above. Again a pH profile of reaction would have to be determined. If possible, native DNA should be used as the substrate to minimize possible side reactions with bases other than guanine. The reaction scheme for preparing this type of reagent is outlined in Figure P-2, as described by Smith (1966, Vol. II, pp 15, 94-95). We propose that trichloroacetate be used instead of acetate to provide a better leaving group and help compensate for the adverse effect expected from the quarternary ammonium groups.

If a specificity for C-8 of guanine is maintained with either of these two classes of reagents, then the use of di- and tri-quarternary ammonium substituted reagents could be explored. In these cases, the binding of polyvalent heavy metal anions could be assured.

FIGURE P-2

The Synthesis of Guanine Specific Anion Binding Reagents--
N-Aryl-N-Acetoxy Hydroxamic Acids.



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PROPOSITION 5

Determination of the Existence of an Excluded Volume Effect on the Kinetics of Renaturation of DNA

ABSTRACT

The results reported in this thesis to the effect that the rate of renaturation for a sheared DNA sample increases with the square root of the length of the molecules implies either some sort of a diffusion controlled rate determining step or an excluded volume effect which makes the inside of a large random coil inaccessible for nucleation. Experiments are proposed which will determine the existence or non-existence of an excluded volume effect on the rate of renaturation. The proposed experiment is the measurement of the rate of renaturation when one strand is large and its complement is small. There should be a difference in the dependence of renaturation rate on the size of the small segment in the two types of mechanisms under conditions of extremely different sizes of large and small complementary DNA. Methods of production of and determination of the sizes of different sizes of denatured DNA are considered.

In chapter 3 of this thesis on the kinetics of renaturation of DNA, we demonstrated that the rate of renaturation of DNA was proportional to the square root of the molecular weight of denatured DNA fragments in solution. We discussed excluded volume effects as a possible explanation of this effect. We assumed that the two interpenetrating single stranded DNAs retained their random coil character throughout the interpenetration process. This assumption cannot be completely correct if the DNA chains interfere with each other during interpenetration. We found, with this assumption, that the ratio of the rate of renaturation with an excluded volume effect to the rate of renaturation with no excluded volume effect was given by

$$V_{d \geq d_o} = 1 + \left(\frac{3}{\pi}\right)^{\frac{1}{2}} \frac{d_o}{R_G} e^{-\frac{3d_o^2}{4R_G^2}} - 2\phi\left(\frac{3d_o^2}{4R_G^2}\right)$$

where $\phi(t) = \frac{1}{\sqrt{2\pi}} \int_0^t e^{-t^2/2}\, dt$

R_G is the radius of gyration of one of the random coils. d_o is the closest distance of approach of the centers of mass of the two DNA chains. If we choose the closest distance of approach to be two times the radius of gyration, then the rate of renaturation is only decreased by a factor of three from that expected with no excluded volume effect. We concluded that this was not a large enough effect to account for the observed dependence of the rate of renaturation on the square root of the molecular weight over a range of 400 in molecular weight. Nevertheless, if the assumption about the inter-

penetrating DNAs retaining their random coil character is substantially incorrect, the predicted decrease in the rate of renaturation with an excluded volume effect could be an underestimation of the real effect. Although we believe that excluded volume effects do not account for the dependence of the rate of renaturation on the square root of the molecular weight, we propose a set of experiments which could show whether our belief is, in fact, justified.

We propose that the rate of renaturation of DNA be studied under conditions where the molecular weights of each individual complementary strand may be varied. To perform this experiment, it is necessary to isolate the separate complementary strands of a DNA. We shall discuss this later, but shall first consider the kind of results which may be obtained.

If there is no excluded volume effect and the rate of renaturation depends on the number of bases paired per successful nucleation (proportional to the length of the smaller strand) and the mutual diffusion coefficients of the two strands (or some function behaving in this same way), then the rate of renaturation of large with small complementary strands would be given by

$$\text{rate} \sim \frac{L\,(\text{small})}{2} \left\{ \frac{1}{L\,(\text{small})^{\frac{1}{2}}} + \frac{1}{L\,(\text{large})^{\frac{1}{2}}} \right\}$$

This equation only holds for the cases where the large DNA represents the unbroken complementary strand. Otherwise, we must consider the effects of random scission.

Let $\quad L\ (large) = K\ L\ (small) \equiv Kn$

The effect of random scission on the rate of renaturation is given by the following equation, which gives the ratio of rates (v_{rel}) for the randomly and non-randomly degraded cases:

$$v_{rel} = \{\ 2 \sum_{1}^{n-1} \times^2\ + [(K-1)n + 1]\ n^2 \} \ / \ Kn^3$$

$$v_{rel} = \frac{3K-1}{3K} + \frac{1}{3n^2}$$

For large n,

$$v_{rel} = \frac{3K-1}{3K}$$

For approximately equal sizes of complementary strands, K is about 1 and v_{rel} reduces to 2/3, as previously derived in chapter 3 of this thesis. For the case of random scission without excluded volume,

$$\text{rate} \sim \frac{L\ (small)^{\frac{1}{2}}}{2} \left[1 + \left(\frac{1}{K}\right)^{\frac{1}{2}}\right] \left(\frac{3K-1}{3K}\right)$$

Calculated values of rate/L $(small)^{\frac{1}{2}}$ are given for both the random and non-random degradation cases in Table P-1

The results derived above may be summarized for the case of large K.

a) The rate is approximately proportional to the square root of the size of small molecules.

b) The rate is approximately independent of the molecular weight of the large molecules.

c) The rate is slower than the rate for reaction of small molecules of the same size with more small molecules of the same size.

If there is an excluded volume effect, the predictions listed above would not be expected to hold. It is not obvious how to formulate an expected excluded volume effect. If the large chain possesses an excluded volume, then experimental work should easily demonstrate this fact. However, it should be easier for short segments to penetrate the large segment than for long segments to penetrate the long segment. Consider a case with least dependence on the sizes of both the small and large molecules where the case extrapolates to the observed dependence of the rate of renaturation on the square root of the molecular weight. :

$$\text{rate}' \sim \frac{L(\text{small})}{L(\text{small})^{\frac{1}{4}} \quad L(\text{large})^{\frac{1}{4}}}$$

or

$$\text{rate}' \sim L(\text{small})^{\frac{1}{2}} \, (1/K)^{\frac{1}{4}}$$

or for the random degradation case,

$$\text{rate}' \sim L(\text{small})^{\frac{1}{2}} \, (1/K)^{\frac{1}{4}} \left(\frac{3K-1}{3K}\right)$$

Calculated values of $\text{rate}'/L(\text{small})^{\frac{1}{2}}$ are also included in Table P-1 for both the random and non-random degradation cases. The ratio of expected rates with diffusion control to expected rates with this excluded volume case is included.

TABLE P-1

Effect of Size of Small DNA on the Rate of Renaturation with Large DNA Divided by the Square Root of the Molecular Weight of the Small DNA.

	Non-Randomly Degraded Large DNA:		Randomly Degraded Large DNA:		Diff. Mech./ Excl. Vol.
K	Excl. Vol.	Diff. Mech.	Excl. Vol.	Diff. Mech.	
1	1.00	1.00	0.67	0.67	1.00
2	0.84	0.86	0.69	0.71	1.03
4	0.71	0.75	0.65	0.69	1.06
8	0.60	0.68	0.57	0.65	1.13
16	0.50	0.62	0.49	0.61	1.25
32	0.42	0.59	0.42	0.58	1.40
64	0.35	0.57	0.35	0.56	1.62
128	0.30	0.55	0.30	0.55	1.83
256	0.25	0.53	0.25	0.53	2.16

We see, in this case, that to obtain meaningful data, it may be necessary to produce isolated complementary strands of very different molecular weight. Either the large strand must have a molecular weight of about 13×10^6 Daltons, or the small strands in the case of K equal to 256 will be smaller than previously studied in the work on the dependence of the rate of renaturation on molecular weight (see chapter 3 of this thesis). We have chosen this case to illustrate the difficulty which may be encountered in determining whether there is an excluded volume effect. In other possible cases, the discrepency between the values expected for diffusion control and those observed would appear well before K equals 256. Therefore, smaller K cases should be tried first to see if an excluded volume effect appears. If not, it would be necessary to use these extreme molecular weight differences to see that no excluded volume effect occurs at all.

We shall now consider three available methods for isolating complementary single-stranded DNAs.

A clear plaque mutant of the lysogenic phage α (host β-megatherium)has a DNA with complementary strands of sufficiently different base composition to form two bands in a CsCl density gradient (Tocchini-Valentini, et al., 1963). After self-annealing, pure preparations of separated strands can be produced. The maximum single strand length is then about 13×10^6 Daltons. We desire small pieces with K = 256, that is, with molecular weights of about 5×10^4 Daltons, which is sufficiently long to be

within the range of the DNA sizes previously studied in renaturation kinetics work (chapter 3). Gruenwedel and Davidson (1966) describe the preliminary work on the use of methylmercuric hydroxide-DNA binding for enhancing the separation between single-stranded DNAs of different base composition. The method has not yet been fully developed. In addition, there is the complication that many DNAs may not ever be separable if they contain complementary strands too much alike.

Another special case where separate strands may be isolated is ϕ x 174 DNA. Sinsheimer (1959) demonstrated that ϕx174 DNA contained only one of two complementary strands. The double stranded in vivo form (RF) has a molecular weight of 3.4×10^6 Daltons (Kleinschmidt, Burton and Sinsheimer, 1963). The negative strand could be isolated by renaturation of sheared RF with excess ϕx174 DNA and sedimentation in sucrose first at neutral pH and then at alkaline pH. Also, N^{15} labelling and equilibrium centrifugation (Meselson and Stahl, 1958) could be used for separations, again involving reannealing.

In the case of ϕx174, with K = 256, the single-stranded small pieces would contain less than 20 bases. This size is outside the molecular weight range for which renaturation kinetics have previously been investigated. Also, the melting properties of such small segments would be different from those of the normal more highly polymerized segments, and the reaction could not be studied under normal renaturation conditions. Crothers, Kallenbach and

Zimm (1965) describe melting temperature and melting profile changes on DNA segments much larger than those needed for study of ϕx174.

Opara-Kubinski, Kubinski and Szybalski (1964) and Kubinski, Opara-Kubinski, and Szybalski (1966) have demonstrated strand separation by banding denatured DNAs interacted with synthetic polyribonucleotides and RNA. This procedure,followed by self-annealing offers the best possibilities of the three methods. T1 DNA, T7 DNA, B. subtilus DNA, and to a less extent other DNAs can be used with poly G or poly IG to lead to isolation of separate strands.

We propose that the technique of Szybalski and coworkers be used for the isolation of the separated strands of T7 DNA. After self-annealing and re-banding in CsCl, the pure denatured DNA strands would be isolated. With T7 DNA, the range of molecular weights from K = 1 to K = 256 would be the same as with phage α DNA. If the whole T7 strand is used as the large DNA, then we have the case of non-randomly degraded DNA. After breaks are put into the large T7 strand, the results would approach those of randomly degraded DNA. This effect would have to be taken into account.

The breakage of isolated single-stranded DNA can be accomplished by alkali shear (Davison and Felsenfeld, 1966) and shear in alkali in a French Pressure Devise (McCarthy and Bolton, 1964) or sonication in alkali, or by heat degradation followed by alkali treatment.

The size of the small DNAs could be determined by alkaline band velocity sedimentation (Studier, 1965). The kinetics of re-

naturation could be determined as described in chapter 1 of this thesis.

The experiment consists of determining the rate of renaturation of one whole T7 strand with varying sizes of the complementary strand and, as well, the rate of renaturation of one small size T7 DNA strand with the large complementary DNA strand of varying size. The results should be compared with the rate of renaturation determined where both the T7 strands are of the small size. The results are compared with those previously predicted.

This experiment could answer the question of the existence of an excluded volume effect on the kinetics of renaturation. Also, this study could be used as a model for the case of the kinetics of DNA-RNA hybridization, where the RNA molecule is much smaller than the complementary DNA molecule.

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