THE HELIX-COIL TRANSITION IN DNA: EFFECTS OF THE INTERACTIONS WITH SMALL IONS AND OF THE COMPOSITION OF DNA.

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The individual influences of some friends and some teachers are akin to the intangible inspiration for which I should like to thank my parents.

Author's Preface

The advent of interdisciplinary sciences presents a problem to the writer. This problem stems primarily from the development of specialized terminologies in the established disciplines; there is no reason in principle why concepts in one area should be incomprehensible to workers in another. I have tried to attack this communication problem by presenting the material in this thesis with some sort of continuity so that one may progress from structural to chemical to biological considerations. Since I have not been able to do this completely, I have included a glossary in the Appendix.

The exponential growth of the volume of scientific literature requires both omission and selection in reviewing previous work. My attitude in this thesis has been to cover only cursorily the areas in which good reviews exist, and to look closely at critical areas where there is controversy or where there exists no thorough discussion in the literature.

Abstract

We have studied the effects of ion binding and DNA composition on the helix-coil denaturation of the DNA macromolecule. Among the systems studied, none gave a marked increase in the composition dependence of the denaturation conditions, an increase which would allow extensive fractionation of a compositionally disperse DNA sample.

The binding of protons to DNA is extensive even before hyperchromicity and irreversible changes in intrinsic viscosity are observed, and includes protonation of the cytosine heterocycle. It is intriguing that this cytosine protonation seems to require the breaking of hydrogen bonds in the Watson-Crick structure.

The binding of Mg^{++}, Co^{++}, or Ag^{+} at low ionic strengths markedly stabilizes the helical conformation. We have evaluated the extent to which high concentrations of Na^{+} ion reduce the binding of Mg^{++} and H^{+} ions.

Broad transitions are observed at low ionic strengths of Na^{+} ion, and in the presence of the equivalent ratios $r = 0.2$ Ag^{+}, $r = 0.5$ Co^{++}, or $r = 0.5$ Mg^{++}. In the latter two cases, this broadening may be due to the selective binding of cations to native DNA, but the broadening observed at low Na^{+} concentrations may be due to a change in Zimm's parameters σ_o and/or σ_j.

The inactivation of the transforming ability of pneumococcal DNA for three characteristics was studied at 0.1 and 3×10^{-4} ionic strengths. Differences in denaturation temperature were observed which could not be correlated with the compositions deduced by Rolfe and Ephrussi-Taylor from density differences. At low ionic strengths, extremely broad inactivation curves were observed, and can be explained by the renaturation made possible by the existence of short helical regions in low ionic strength denaturations.

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I. Introduction.

The general goal of the research reported in this thesis is to study the structure of DNA in aqueous solution in the hope of finding techniques which are sensitive to the differences in structure between the various molecules obtained from an organism. We have focussed our attention on two properties of the DNA molecule: its denaturation and its interactions with small molecules.

The progress made to date on the structure and function of DNA has concerned general features of its structure and biological replication. One of the roadblocks to further progress lies in the fact that all current means of preparation of DNA give a collection of molecules representing the entire DNA complement of a cell. This heterogeneity stands in the way of an understanding of the structure of individual genes in non-viral organisms. Only in viral organisms has it been possible to isolate a single DNA molecule from one organism.

The process of denaturation has been shown to depend to some extent on the composition of DNA, and on the presence of small ions and molecules. These small species often show compositional specificity in their interaction with DNA. Therefore, we have been interested in finding situations in which specific denaturation can be obtained. Since the physical and chemical properties of native and denatured DNA differ noticeably, it is possible to separate native DNA from denatured (Meselson, 1957, and Stevens and Duggan, 1957). Therefore, if one could specifically denature molecules in a preparation, one could separate molecules effectively.

In the course of this work, it has been possible to learn much about the process of denaturation and the interaction between DNA and small molecular species. We shall present a fairly thorough study of acid and alkaline denaturation, and of thermal denaturation in the presence of varying concentrations of sodium, magnesium, cobalt (II), and silver ions.

Since our primary concern has been whether any of these denaturations offer great compositional specificity, so that one could resolve

heterogeneity, we have not made a complete study of these processes. Wherever it is appropriate, we have tried to point out alternative hypotheses to account for the unsolved problems we have unearthed.

II. Introductory discussion.

A. The structure of DNA.

The current notions about the structure of DNA rest on crystallographic, hydrodynamic, optical, titration, and analytical data. (See, for example, Langridge, Wilson, Hooper, Wilkins and Hamilton 1960.)

The pertinent crystallographic facts are that the two-dimensional x-ray diffraction patterns from DNA fibers at high humidity suggest that DNA has a helical configuration with short and long repeat distances of 3.4 Å and 34 Å along the fiber axis (Wilkins, Gosling, and Seeds, 1951). This pattern is only one of the many produced by DNA under different conditions. It is, however, of primary interest because it occurs at high humidity, and therefore may correspond to the conformation of DNA in aqueous solution. In addition, Wilkins and Randall (1953) found this structure in cell nuclei.

A second crystallographic requirement is a dimension of 18 Å normal to the helical axis, corresponding to the diameter of the helix. This constant diameter restricts the mode of linking across the helix to links of constant length.

Hydrodynamic experiments indicate that DNA is an anisotropic macromolecule. Values of the intrinsic viscosity and sedimentation coefficient indicate that bacterial and mammalian samples have molecular weights of the order of 10^7. The precise measurement of these molecular weights by hydrodynamic means is difficult because of the anisotropy of the molecules and because their size makes sedimentation equilibrium measurements difficult.

The anisotropy of these molecules is indicated by their flow birefringence and dichroism. Their ease of orientation in a shear gradient is exemplified by the pronounced non-newtonian viscosity behavior of DNA solutions. Doty, McGill, and Rice (1958) found that DNA molecules of various sizes, prepared by sonication, obey the relationship $S \propto M^{0.37}$. This indicates that DNA is gently kinked and not strictly a rigid rod which would show no molecular weight depend-

ence of its sedimentation coefficient.

Optical data includes light-scattering measurements, which indicate a radius of gyration for native calf thymus DNA of 3000 Å for a sample with an estimated molecular weight of 6×10^6. Optical rotation measurements indicate that the optical activity of the native DNA molecule differs from that of the constituent nucleotides. Ultraviolet absorption measurements indicate that the nucleotides absorb less strongly when they are arranged in the DNA molecule. This is the so-called hypochromic effect.

Analytical data indicates that DNA is composed of four nucleotides polymerized as 3', 5'-phosphodiesters of deoxyribose, and that the mol fractions of adenylic acid (A) and thymidylic acid (T) are equal, as are those of guanylic acid (G) and cytidylic acid (C), for a sample of DNA. It should be pointed out that these data apply to the mean properties of a group of molecules which are probably dissimilar (*vide infra*: DNA heterogeneity).

These structural data can all be explained by an hypothesis of Watson and Crick (1953) stating that all DNA molecules in such samples are composed of two helical strands wound around a common axis and connected by hydrogen bonding between the bases which occur only in the pairs AT and GC (see Appendix I). There are ten base pairs per helical turn, and the pitch of the helix is 34 Å. The diameter of the helix is 18 Å, and the GC and AT pairs can be placed on the helical staircase interchangeably, without bulging or crimping.

Donohue and Trueblood (1960) have found other base-pair structures which will fit these dimensions. The chief objections to introducing other base pairs are the complementary mean base ratios and the simplicity of the genetic replication scheme based on the Watson-Crick structure. Neither of these objections is rigorous in excluding alternative pairing schemes.

B. Denaturation.

1. The phenomenon of denaturation.

The phenomenon of denaturation of DNA involves a change in macromolecular properties without extensive change in molecular weight. Whereas native DNA can be described as an extended, ordered, 40% hypochromic structure, denatured DNA is much less extended, less hypochromic, less helical by optical rotation, and with vanishing biological activity. The extent of change in molecular weight upon denaturation is unclear because of experimental difficulties. There is evidence (Doty, Marmur, Eigner, and Schildkraut, 1960) that the molecular weight falls immediately to half its original value upon denaturation, and that there is subsequently a further decrease in molecular weight. Under some conditions, there appears to be aggregation upon denaturation. At any rate, we can say that there is no extensive change in molecular weight upon denaturation.

The transition from native to the denatured conformation occurs as a function of the variables T, pH, ionic strength, and concentration of small molecules such as alcohols, urea, and formamide. In that this transition generally has a temperature-breadth of only a few degrees (or a small change in composition of the solution), it approximates an ordinary phase transition, and one can draw phase diagrams for native and denatured DNA as functions of these intensive variables. The variables T, pH, μ, and urea concentration are discussed by Sturtevant, Rice, and Geiduschek (1958), and formamide concentration by Helmkamp and Ts'o (1961).

The denaturation reaction is relatively fast. Geiduschek and Sturtevant (1959) find reaction times of the order of one second for acid denaturation at 0° and 25° C, and thermal denaturation seems to be complete in less than ten minutes at $0.1\ \mu$ by biological criteria (Ginoza and Zimm, 1961). There do exist slow reactions, such as depurination and depolymerization by hydrolysis (Thomas and Doty, 1956), both of which are more rapid in acidic solution than in neutral or alkaline solution, but which have half-lives of the order of hours

under conditions of denaturation.

Thus, the states of denaturation generally observed represent steady states except that they change with time by these slow degradations. The molecular nature of states of partial denaturation will be discussed in detail in this thesis, but we should now bear in mind that these states may represent one of several possible molecular conditions:

1. Dynamic equilibrium, in which at any instant each molecule has some helical regions and some denatured regions. These regions reversibly change state by random fluctuations.
2. Strict heterogeneity, in which denatured regions, which may be as large as entire molecules, have denatured irreversibly. The state of partial denaturation then represents the state in which some regions have passed critical conditions and others have not. This heterogeneity may be of two forms:
   (a) Macromolecular heterogeneity: differences in ease of denaturation between entire molecules. This might involve differences in composition, conformation, or size.
   (b) Internal (micro-) heterogeneity: differences in ease of denaturation between small regions of molecules. This might be due to differences in composition, sequence, or position in the molecules. The present knowledge of heterogeneity in DNA will be discussed later.

To state that the observed states of denaturation are equilibrium states is misleading because these states ordinarily cannot be approached from both sides. If one heats a sample of DNA through its denaturation transition and then recools, apparently irreversible changes are observed. The recooled material is hyperchromic with respect to the native material at the same temperature, and much less extended. The same behavior is observed on acidic or alkaline titration, or in formamide denaturation. The transitions which are readily reversed are those in alcohols (Geiduschek and Gray, 1956) and with Hg(II) ion (Yamane and Davidson, 1961, and Dove and Yamane, 1960). These may

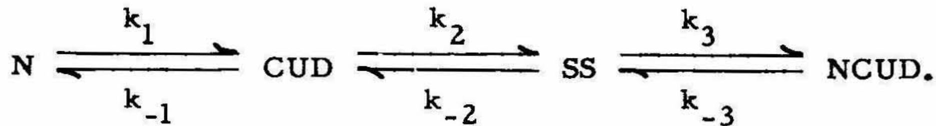
involve no breakage of hydrogen bonds.

Marmur and Lane (1960) and Doty et al (1960) have found this irreversibility to be much reduced by cooling slowly, and attribute the residual denaturation to the slow processes mentioned above. We could account for the behavior observed in the fast cooling in the following way:

There exist many conformations of DNA involving extensive hydrogen bonding, which are not completely complementary and therefore highly kinked. There exists one perfectly paired, native structure which, at room temperature, has the lowest free energy of any conformation. On cooling at a finite rate, the molecule has a high probability of achieving a conformation of suboptimal stability, at least temporarily. At room temperature, the rate of transition from this denatured conformation to the native structure of optimal stability is too slow to be observed. At the elevated temperatures involved in renaturation, the rate of dissociation and rearrangement of the suboptimal state is high enough that the molecules can find the optimal, native state in an observable time.

In this discussion, we have not mentioned whether the strands of the double helix have separated in passing through denaturation. The work of Schildkraut, Marmur, and Doty (1961) indicates that this is true, and that renatured molecules result from strand reunion. Some objections can be raised to their interpretations (Delbrück, 1961), and we prefer to reserve judgement until the required critical experiments are done. At any rate, we can extend our discussion to include strand separation. There exist suboptimal strand-separated states whose free energy is comparable to that of suboptimal, strand-united states, but which require longer to attain the complementary native state because they must find their partner strand. Renaturation depends again on the rate of leaving the suboptimal state, and again we can understand the requirement for elevated temperatures for renaturation.

Let us summarize this one possible interpretation of the situation diagrammatically. Consider the reaction scheme



$$N \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} CUD \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} SS \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} NCUD.$$

Here N represents native, complementary DNA; CUD is denatured DNA in which the complementary strands are still united; SS is denatured DNA in which the strands are all separated; and NCUD is denatured DNA in which non-complementary strands are united (i.e., non-specific aggregation).

The relative free energies of these states would be somewhat as indicated in figure 1. At room temperature, the native structure is much more stable than any other structure; at the renaturation temperature, the native structure is only slightly more stable. At the denaturation temperature, above this renaturation temperature, the free energies of all states are about equal.

The rates of reaction may obey the relationships shown in figure 2. The temperature dependence of k_1, the collapse step, is quite pronounced, whereas all other rate constants have temperature dependences similar to that in diffusion. By both the theories of Kuhn (1957) and of Longuet-Higgins and Zimm (1960), the untangling (and retangling) of strands is a diffusional process. On the other hand, the severe temperature dependence of the rate of collapse, k_1, is ordinarily found in such cooperative situations, since the breaking of one secondary bond enhances the breaking of another. This situation is observed in the rate of denaturation of proteins (Moore, 1955).

To understand the phenomena observed in this system of denaturation, we must also discuss the relative importance of each step in the reaction sequence; in one situation, k_{-1} may be the rate limiting step in renaturation, while in another situation, it may be k_{-2} or k_{-3}.

Figure 1. Relative free energies of various conceivable states of DNA.

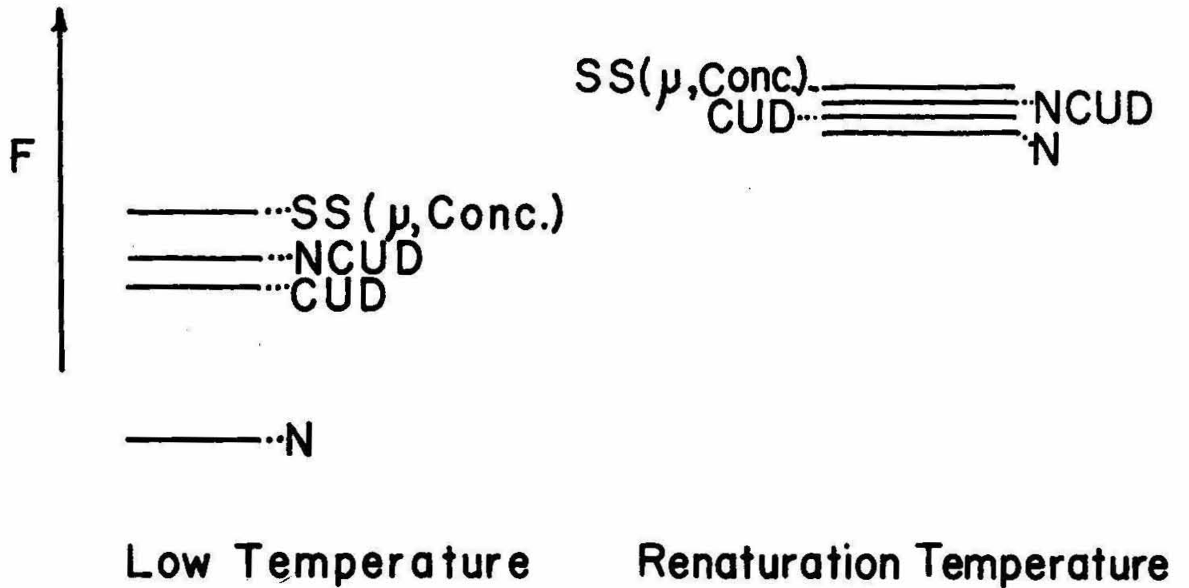
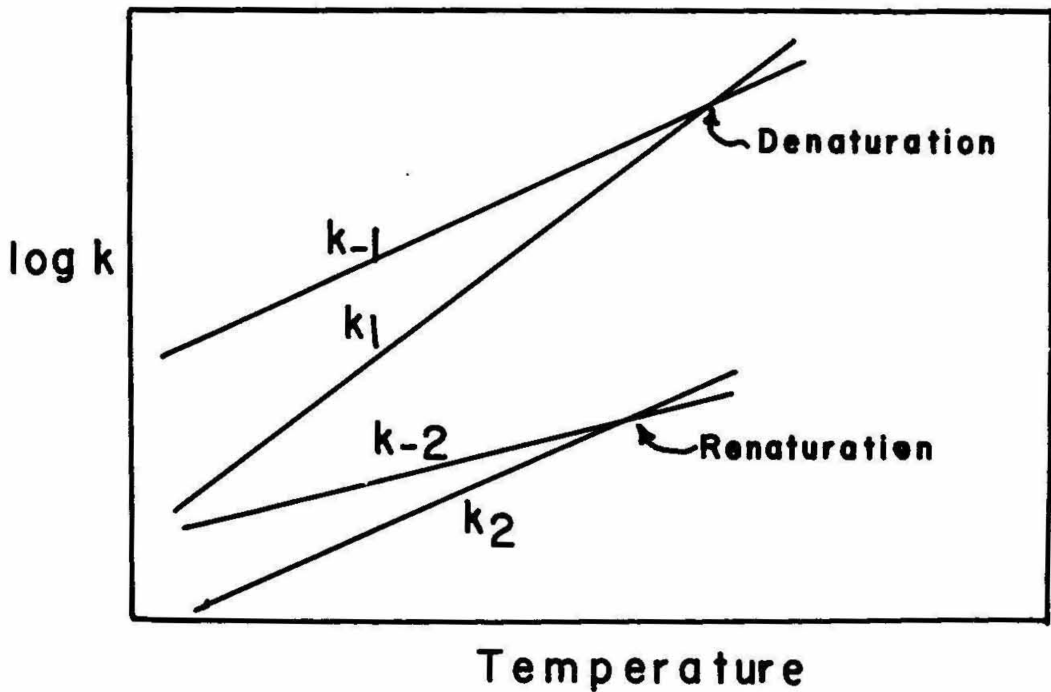


Figure 2. Proposed temperature dependence of reaction rates for conversion of DNA conformations.



The internal heterogeneity would be expected to influence the importance of the k_1's vis-a-vis the k_2's. In a sample with no internal heterogeneity, the reaction CUD $\longrightarrow$ N would not be difficult, and the rate-limiting step in renaturation would be that measured by k_{-2}. This will be discussed in relation to the union of polyadenylic acid and polyuridylic acid.

The molecular heterogeneity would be expected to influence the importance of the k_3's vis-a-vis the k_2's. For a heterogeneous sample, non-complementary unions are much more probable than complementary ones, and renaturation would be difficult. This, in fact, is the reason given by Doty, et al. (1960) for the failure of calf thymus DNA to renature.

The length of the molecular strands would be expected to influence the importance of k_2 in denaturation. Long strands would have a more difficult time becoming disentangled than would short strands. This is the reason proposed by Delbrück (1961) for the observation that viral DNA's renature more readily than bacterial DNA's: they merely could not complete the reaction CUD $\longrightarrow$ SS before being recooled. The theory of Longuet-Higgins and Zimm predicts that the time required for the strands to separate is proportional to the 5/2 power of the molecular weight.

We now see that this model of the denaturation reaction scheme can be used to discuss the results very flexibly. The difficulty is that experiments to measure these individual steps in denaturation, or even to identify them, are difficult and have not been done satisfactorily. Ross and Sturtevant (1960) have measured the rate of helix formation between poly A and poly U as a function of temperature, and found this rate to decrease with increasing temperature. If we take this result over to the DNA case directly, we would expect renaturation to take place more rapidly at room temperature than at the elevated renaturation temperatures. That this result cannot be taken over blindly to the DNA case should be clear from our discussion.

The result of Ross and Sturtevant, fitted to a nucleation kinetic model by Saunders and Ross (1960), can be understood in the following

way: there is no internal heterogeneity in the complex, and therefore the rate-limiting step in the formation of N from SS is the union of strands. This union is stable when n(T) base pairs are formed at temperature T. Complexes with less than n base pairs dissociate at this temperature. Thus, at any temperature T, Ross and Sturtevant's experiment measures the difference in rate between the formation of an n-pair complex and the dissociation of an (n-1)-pair complex. The former process has a rate behaving somewhat as does k_{-2}, while the latter process has a rate behaving like k_2. This net rate, then, increases as one lowers the temperature. We could say that, at fairly high temperatures (not necessarily above the helix melting temperature) the rate of formation of helix is low because it is improbable that a poly A strand and a poly U strand could form hydrogen bonds in enough places to stay together long enough to wrap up into a helical complex. At low temperature, the complementary strands have a high probability of forming a transient complex long enough to wind up into a helix.

The rate of renaturation of DNA need not increase with decreasing temperature, however, because in this case there may exist CUD states which become quite stable and, unlike any imperfect poly A-poly U states, cannot easily slide into the native state which is the most stable. Also, there may exist the NCUD states in the case of DNA.

Thus, the use of poly A-poly U complex formation as a model for the DNA system can be interpreted on our reaction scheme, and certainly does not involve all of the processes which we must consider in the DNA case.

We should now be aware of the possible effects of these renaturation phenomena on our observations of DNA. There may be both reversible components and irreversible components in a denaturation experiment. Perfect renaturation reduces the irreversible component to zero. To what extent renaturation actually takes place is at present unclear, and its understanding hinges on an understanding

of the kinetic relationships outlined here. Despite lack of understanding, it is important to bear in mind which component of the denaturation process a particular observation measures.

2. Denaturation midpoint and breadth.

We have now described the phenomenon of denaturation and formulated a reaction scheme with which to discuss it. We shall now investigate the information given us by a measurement of the midpoint of the transition and of the breadth of the transition, before outlining the statistical mechanical treatment which quantitatively predicts the midpoint and breadth.

The midpoint of the helix-coil transition is useful in discussing this as a phase transition and gives information on the relative free energies of native and denatured conformations in a particular environment. A finite transition breadth is also observed, and can be measured by a standard deviation σ_T if we approximate the transition profile as a normal probability integral. This transition breadth seems to be a function of the compositional heterogeneity of the sample, being greater for calf thymus DNA than for microbial DNA, which in turn is greater than for bacteriophage DNA. It also seems to be non-zero for the most homogeneous DNA samples available--phage DNA and synthetic adenine-thymine (AT) polymer. We shall call the breadth of a homogeneous DNA the intrinsic breadth, and the additional breadth heterogeneity breadth. If the heterogeneity and intrinsic transition profiles are both gaussian, and they seem to approximate this, then, as Sueoka (1959) has shown, the variance of the total transition is the sum of the variances of these components;

$$\sigma^2_{tot} = \sigma^2_{het} + \sigma^2_{intr} .$$

It should be recalled that there may well exist sources of internal heterogeneity, which contribute to the intrinsic breadth as we have defined it, and which are certainly different in the synthetic AT polymer than in a molecularly homogeneous viral DNA.

3. DNA heterogeneity.

Current information on the heterogeneity of DNA preparations indicates both compositional and size heterogeneity in samples prepared by current techniques. Analysis of the DNA content of different cells indicates that cells from sources of different biological complexity contain amounts of DNA increasing with the complexity of the source, as shown in table 1.

Table 1. DNA content of cells from organisms of different complexity.

Source	DNA content (g)	Molecules (MW 6×10^6)	Reference
ØX174 phage	2.6×10^{-18}	0.26	Sinsheimer (1960)
T7 phage	$\frac{0.6}{0.9}\times10^{-16}$	7.5	Sinsheimer (1960)
T4 phage	2.5×10^{-16}	25	Sinsheimer (1960)
D. pneumoniae (bacterium)	1.5×10^{-15}	150	Fox (1957)
Calf thymus (mammalian)	-	>1000	Doty et al (1960)

If we start with the assumption that all cells or viruses of a given type have identical DNA complements, then a sample of bacterial DNA may have as many as 150 molecules of molecular weight 6×10^6, whereas a sample of T4 DNA will have only 25 molecules of molecular weight 6×10^6. There is, indeed, evidence that one can prepare a single molecule of weight 150×10^6 from a T4 particle.

Compositional differences between fractions of calf thymus DNA have been demonstrated in salt extraction of denatured DNA and its nucleoprotein, and between fractions isolated from the slightly basic histone and ecteola cellulose columns (Butler, 1958). These chromatographic separations appear to be unreliable in that some-

times denaturation is produced, and fractions do not rechromatograph reproducibly.

Physical differences between fractions of calf thymus DNA have also been demonstrated (Butler, 1958), with preparative sedimentation fractions exhibiting a range of sedimentation coefficients and intrinsic viscosities.

Recently, compositional and density heterogeneity have been established not only for calf thymus DNA, but also for bacterial DNAs (see Sueoka, 1961). Thymus DNA exhibits a bimodal band in density gradient centrifugation (Meselson, 1957). By comparing the variance of the density gradient band with the variance expected from the MW determined by hydrodynamic methods, Sueoka (1961) calculates a σ_{GC} for density heterogeneity of D. pneumoniae DNA corresponding to 2.0 % in GC mol fraction. Sueoka, Marmur, and Doty (1959), and Rolfe and Meselson (1959) had previously shown that there exists a linear relationship between buoyant density and mol fraction GC in native DNA.

Marmur and Doty (1959) have also established a linear relationship between T_m and mol fraction GC for DNAs of mammalian, bacterial, and viral origin. Sueoka et al (1959) have calculated the heterogeneity in GC content in a single preparation using this relationship and the observed transition breadth. This gives a higher estimate of GC heterogeneity, $\sigma = 4\%$ GC, than that given by density heterogeneity. Rolfe and Ephrussi-Taylor (1961) have measured density differences between four markers in D. pneumoniae DNA, finding that each marker bands as a homogeneous species of MW 9×10^6 (sodium salt), and that the four markers cover a region 1.0% GC in width on the heavy side of the band. This magnitude of density heterogeneity is compatible with Sueoka's (1961) estimate, although it is somewhat surprising that all markers should be significantly more dense than the mean. These results will be discussed in greater detail later in light of our studies on some of the same markers.

The chromatographic fractions of Beiser, et al (1959) give

values of the GC heterogeneity of D. pneumoniae DNA (Sueoka, 1959) higher than these lower estimates. This may be attributable to degradation.

Thermal denaturation results at 0.15 ionic strength on pneumococcal DNA also have indicated some fractionation. Doty, Marmur, and Sueoka (1959) have published preliminary reports of differences in sensitivity between the streptomycin and bryamycin markers. The experiments of Ginoza and Zimm (1961) indicate differences in the critical temperatures of different markers, and Roger and Hotchkiss (1961) have reported marked differences between pneumococcal markers which gave extremely sharp denaturation transitions.

In general it seems that the effect of density differences between molecules on their position in the density gradient is too small to allow extensive fractionation in this way. The effect of differences in composition on the denaturation transition seems also too small with respect to the intrinsic breadth to achieve selective denaturation and subsequent fractionation by separating native from denatured DNA. It might be possible to magnify the effect of differences between molecules on either their buoyant density or on their stability, and thereby achieve significant separation of species. This latter possibility will be discussed for several cases in this thesis.

4. Statistical mechanical treatment of the denaturation of a homogeneous sample.

The statistical mechanical theory of the denaturation of a double-helical, DNA-like molecule has been developed by Rice and Wada (1958), Gibbs and DiMarzio (1959), Hill (1959), and most recently by Zimm (1960). We shall give a qualitative discussion of Zimm's treatment to enable us to view our denaturation experiments critically.

The relative stabilities of helix and coil determine the midpoint of the transition, while the number of ways one can construct states of intermediate denaturation, weighted by the Boltzman factors

for their energies, determines the breadth of the transition. The midpoint of the transition occurs at that state in which the free energies of helix and coil are equal. The transition around this midpoint is very broad if one can intersperse helical and denatured regions along a molecule at will. The transition is sharp, a cooperative phenomenon, if one must keep all helical segments continuous.

In thermodynamic notation, we can discuss the midpoint by writing out the contributions to the free energies of helix and coil.

$F_h(T, pH, X_{GC}, \mu) = H_h(pH, X_{GC}, \mu) - TS_h$, where we have neglected the heat capacity of the hydrogen bonds in limiting temperature dependence only to entropic factors. The pH dependence stems from breakage of hydrogen bonds by protonation, and from electrostatic interactions between protons in native DNA and between these protons and the negatively charged phosphates. The X_{GC} dependence, reflected in the results of Marmur and Doty (1959), rests on the greater energy of interaction in GC pairs than in AT pairs, perhaps simply because there are three hydrogen bonds in the former and only two in the latter. Alternatively, this is due to the interaction between the permanent dipoles in these nucleotides (Tinoco,1961). The μ dependence includes the free energy of the cloud of counterions about the helical molecule, and the electrostatic self-energy of a charged helical structure, which is a function of the screening provided by the medium in which the molecule is immersed.

$F_c(T, pH, \mu) = H_c(pH, T, \mu) - TS_c$. We have allowed no hydrogen bonds in the denatured molecule, since we are interested in the temperature region around the transition point. The pH, ionic strength, and temperature dependence of the coil are displayed by the typical swelling properties of charged flexible macromolecules due to charge repulsions and configurational enthalpy. The ionic strength dependence includes also the electrostatic free energy of the counterion cloud.

At denaturation, neglecting kinetic barriers, we have $F_h = F_c$. We would expect $S_c > S_h$ on statistical grounds, since the coiled con-

formation can be achieved in many ways. Thus, the temperature effect in denaturation is chiefly entropic. The theory of Zimm includes the entropy term explicitly by introducing a statistical weight σ_j for a loop of j segments, evaluating the entropy of this segment by the theory of Jacobsen and Stockmayer (1950). This entropy is compared to the entropy of a molecule with the same number of unbonded pairs, but at the end of the molecule. The energetic terms are represented in Zimm's treatment by a parameter s, defined by $s = \exp(-\frac{E}{kT})$, where E is the free energy difference between a helical pair and an unbonded pair.

For treating the cooperative nature of the transition, Zimm introduces a parameter σ_o, which is defined by $\sigma_o = \exp(\frac{e}{kT})$; e is the difference in energy between states I and II in figure 3. The stacking energy e is a negative quantity because of the energy of "crystallization" of base pairs on top of one another. In crystals of aromatic hydrocarbons, this energy is of the order of 1 kcal per ring per mole, and so Zimm takes a value of 2-3 kcal per base pair for DNA.

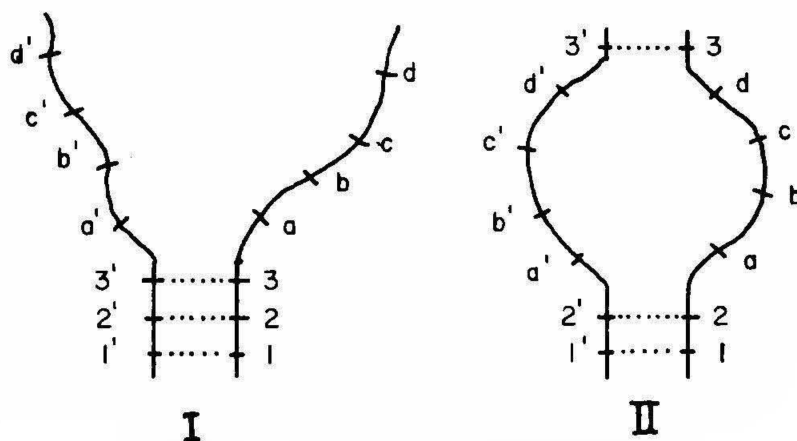


Figure 3. The definition of Zimm's cooperative parameter σ_o.

For $\sigma_o = 1$, there is no difference in energy between the situation with all helical regions continuous and the situation with helical regions dispersed. The transition is rather broad, as many intermediate

states can exist. The only cooperative influence remaining is the fact that loops have lower entropy than free ends of the same number of unbonded segments. For $\sigma_0 \ll 1$, the transition is more cooperative and hence sharper, since helical regions tend to remain continuous.

5. Relationship between hyperchromicity and fraction of unbonded segments.

Zimm's theory predicts the change in the fraction of bonded pairs with temperature around the transition point. Whether the hyperchromicity of the macromolecule changes in the same way depends upon whether this effect is a linear function of the fraction of bonded base pairs. Tinoco (1960) has explained the hypochromic effect on the basis of the interaction between excited dipoles in neighboring chromophores along the linear "crystal". These interactions are significant over distances not longer than seven base pairs.

Applequist (1961) has recently calculated the non-linear relationship between hyperchromicity and fraction of broken base pairs. However, in the transitions we shall be concerned with, helical regions are, on the average, never shorter than twenty base pairs, according to the Zimm theory. Therefore, this non-linear effect should not be important in our experiments. This would become significant only when the average helical region becomes as short as seven base pairs in length.

In this introduction we have discussed the current notions of the structure of DNA briefly, and the sort of experiments on which they rest. We have looked carefully at the phenomenon of denaturation in DNA, formulating a reaction scheme and describing qualitatively a system of rate constants and temperature dependences which will account for the phenomena observed to date. We have discussed the evidence for heterogeneity of DNA, and mentioned its effect upon denaturation. Finally, we have presented Zimm's theory of denaturation of a homogeneous double stranded molecule such as an individual DNA molecule. We have explicitly mentioned the sources

of temperature, ionic strength, composition, and pH dependence in denaturation, and the causes of the cooperative nature of the transition.

III. Experimental methods.

A. DNA.

1. Sources and preparation.

Highly polymerized calf thymus DNA was obtained from Nutritional Biochemicals Corporation. AT polymer, prepared by C. D. Radding, was kindly donated by Dr. A. Kornberg. Bacterial DNAs were prepared by the method of Marmur (1961) from the following strains: *D. pneumoniae*, antibiotic resistant mutants and transformants of strain R36A from Dr. H. Ephrussi-Taylor; *B. megatherium*, strain KM (lysable) from Dr. S. Spiegelman via Dr. M. Meselson; *E. coli*, strains K12 and B, from Drs. R. Rolfe and A. Roller, respectively; *M. lysodeikticus*, from Dr. R. Sinsheimer.

The Marmur method was modified in the use of lysozyme followed by duponol in lysing *M. lysodeikticus*. *B. megatherium* was lysed only by formation of protoplasts in 0.5 M sucrose by lysozyme, followed by dilution into buffer and addition of duponol.

The quality of the DNA preparation was found to be highly dependent upon the state of the culture when lysed, as saturated cultures gave DNA in very low yield. The aerobes *E. coli*, *B. megatherium*, and *M. lysodeikticus* were grown in aerated Hershey broth, while *D. pneumoniae*, which is micro-aerophilic, was grown in capped bottles in medium 1 (*vide infra*). The cells were harvested just before their final logarithmic division, which was at titers of 3×10^9 for *E. coli* and *M. lysodeikticus*, 3×10^8 for *B. megatherium*, and 4×10^9 for *D. pneumoniae*. In the last case, high titers were obtained by making 10^8 cultures in medium 1 and then adding glucose up to 0.75%. These forced cultures were kept neutral as indicated by phenol red by the addition of sodium hydroxide. The pneumococcus cultures are very sensitive to autolysis, a problem which seems to stem from trace amounts of detergent in the glassware.

The Marmur method was modified also in that a cloudy residue, probably of protein, was removed before the final precipitation by cen-

trifugation at 12,000 RPM in an SW 39 rotor for one hour.

Worthington RNase was used, and freshly dissolved before use.

Duponol (DuPont) was purified by recrystallization from ethanol.

2. Solutions.

DNA was dissolved by addition of small increments of solvent until a clear gel of concentration 0.5 - 1 mg/ml was obtained. These stock solutions, stable only when so concentrated, seem to suffer no degradation in one year if kept at 4° C, with a few drops of chloroform to prevent growth of organisms.

Experimental solutions were prepared by dialysis of aliquots of these stock solutions (vide infra) against the required solvent, and then dilution into this solvent.

3. Dialysis.

Visking tubing was used, after removal of ultraviolet absorbing impurities by boiling for a total of 25 minutes in two changes of 0.1 F versene, pH 8, and three changes of 10^{-3} F cacodylate, pH 7. Tubing was stored in conductivity water (vide infra). Dialysis was performed at 4° for a total of seven days against two changes of 0.1 F versene, pH 8, and three changes of 10^{-3} F cacodylate, pH 7, the last change being run for three days.

B. Spectral titrations.

1. Solutions.

$HClO_4$ and NaOH, analytical grade, were used for all acid-base titrations of DNA reported, except that HCl was used in the early work reported in Dove, Wallace, and Davidson (1959) (q.v.). The primary standard used was potassium acid phthalate. The indicator was phenolphthalein.

For DNA titrations in the alkaline region, solutions in 10^{-3} F phosphate buffer at pH 6.7, 0.1 F $NaClO_4$, were titrated with NaOH.

$NaClO_4$ was G. F. Smith, sodium phosphates were Mallinckrodt Analytical, NaOH was Baker Analytical, and $HClO_4$ was Mallinckrodt Analytical.

2. pH measurement.

In the acid region, titrations of DNA by F. A. Wallace (1961) afford knowledge of the extent of proton binding and the apparent activity coefficients of hydrogen ion down to 10°. Using these data, one can correct the pH_F calculated from the formal concentration of acid to the pH read by a Beckman General Purpose glass electrode (1190-80) with a Beckman Model G pH meter. At the pH of denaturation, the number of protons bound in a 10^{-4} M DNA solution is about 5×10^{-5} F, and this is negligible at pH less than 3. Apparent activity coefficients determined by Wallace were 1.0, and so we can use pH_F as a good approximation to pH (pH_F is certainly a more useful quantity experimentally).

In the alkaline region, a Beckman 40495 blue-glass electrode was used to determine the pH in blank titrations of phosphate buffer with NaOH, as a function of the ratio of these substances. Since this titration shows a buffer region in the area of interest in these experiments, and since DNA is dilute in these experiments, we can use the pH values from the blank titrations to calculate the pH during a DNA titration.

3. Hyperchromicity and calculation of protonation.

Spectra for neutral and for titrated samples of DNA were calculated from the nucleotide spectral data of Bock (1956), and the composition, without considering hyperchromicity. These predict an isosbestic point for protonation of 265 mμ which shifts slightly on guanine protonation. On alkaline titration, the isosbestic point is sensitive to composition, but lies around 270 mμ. One can therefore take absorbance at these wavelengths to measure hyperchromicity independent of titration, and to give an estimate of the degree of

denaturation.

Since cytosine is the only base which changes its spectrum drastically on protonation, the extent of protonation of cytosine can be estimated by measuring changes at a wavelength such as 280 mμ and correcting for any hyperchromicity.

Therefore, we use the following indices:

For acid denaturation, $$h_{265} = \frac{a_{265}(pH)}{a_{265}(pH\ 7)} .$$

For alkaline denaturation, $$h_{270} = \frac{a_{270}(pH)}{a_{270}(pH\ 7)} .$$

For protonation of cytosine, $$p = \frac{a_{280}(pH)\,(h_{265})^{-1} - a_{280}(pH\ 7)}{a_{280}\,(pH\ 7)} .$$

p is the fractional change in absorbance at 280 mμ, corrected for the hyperchromicity of the sample. To calculate the fraction of protonation of cytosine from p, we must refer it to the p for full protonation. This p_{max} can either be taken from the limit of the observed p as DNA is titrated to low pH, or it can be calculated from the nucleotide spectra and the composition of the DNA. These two values, p_{max}(obs) and p_{max}(calc) do not, in general, agree for the DNA samples we have studied. This is indicated in table 2.

Table 2. Values of protonation index for various DNAs.

DNA source	p_{max}(obs)	p_{max}(calc)	% GC
D. pneumoniae	0.2	0.266	39
Calf thymus	0.3	0,275	42
E. coli	0.3	0.378	50
M. lysodeikticus	0.55	0.343	72

For low GC DNAs, the spectral changes observed at 280 mμ are less than would be expected on the basis of the nucleotide spectra, while for the GC rich DNA of M. lysodeikticus, the spectrum changes at 280 mμ on protonation much more than expected. The significance of this is unknown.

Hyperchromicity seems also to be a function of composition, as one can see in table 3 (page 43), and from the data of Marmur and Doty (1959). These effects make it inadvisable to make quantitative calculations from the spectral changes observed in DNA.

4. Method for spectral titrations.

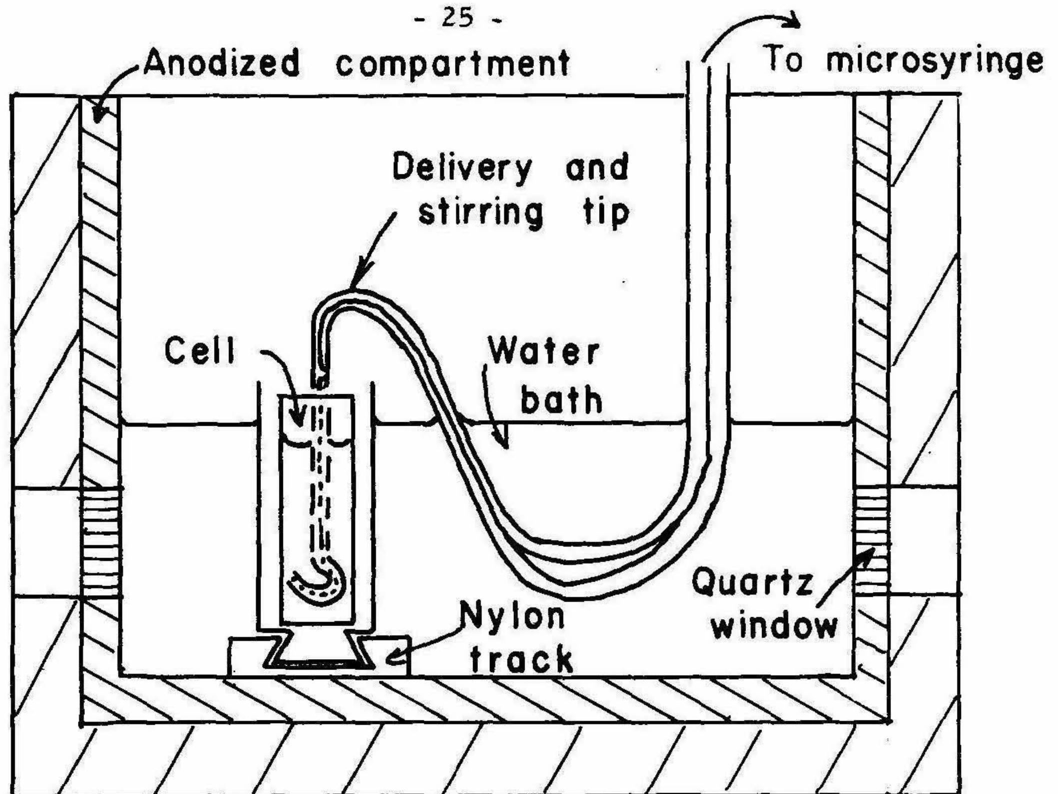
DNA samples were made up at concentrations about 30 μg/ml in 0.1 F $NaClO_4$ and, in the case of alkaline titrations, 10^{-3} F phosphate buffer, pH 6.7, and placed in a Pyrocell microcell holding 1 ml. The cell was placed in a special thermostable cell holder for the Beckman DU spectrophotometer, and titrated by delivering titrant from a microsyringe (Agla) through a connection of polyethylene tubing to a heavy walled glass capillary dipping into the cell. Stirring was accomplished by moving this capillary up and down during delivery of titrant, since it had a curved end. Temperature control was provided by surrounding the cell with water containing 10% alcohol, and pumping water from a Precision Scientific Co. water bath through a cooling coil in this compartment. The water bath maintains a constant temperature within $\pm$ 0.2°, and a thermometer in the cell compartment indicated that this was the accuracy of the temperature control. Stirring of the water in the cell compartment (except during absorbance measurements) provided uniform temperature.

This compartment and titration apparatus are drawn schematically in figure 4.

C. Heating curves.

1. Solutions.

Stock DNA solutions, dialyzed as described above in



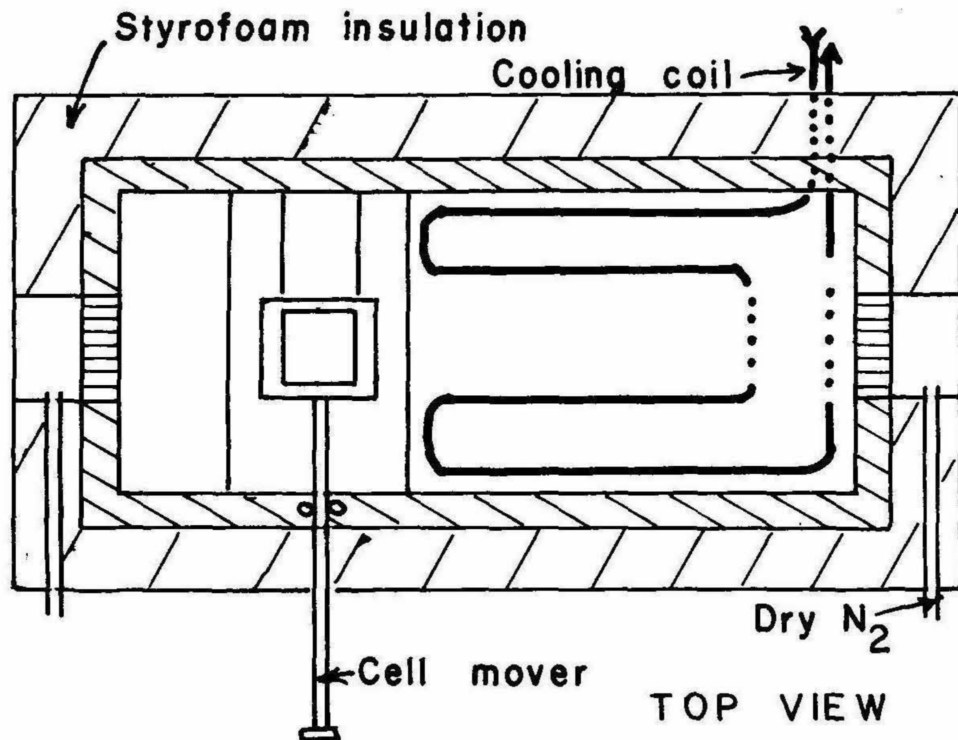


Figure 4: Spectral titration cell.

solutions made exclusively from conductivity water (vide infra), were stored in 10^{-3} F cacodylate buffer at pH 7, at DNA concentrations $1\text{-}2 \times 10^{-3}$ M (P). These solutions require no chloroform to prevent growth of organisms, presumably because of the toxicity of cacodylate and the low ionic strength.

Solutions of low ionic strength were prepared by Mr. T. T. Bopp, using conductivity water, technical grade cacodylic acid, Mallinckrodt Analytical $MgCl_2$, G F Smith $Co(ClO_4)_2$, and Baker Analytical NaOH for neutralization to pH 7. Their compositions are:

Sodium stock:	1.56×10^{-4} F $NaClO_4$, 2.64×10^{-5}F Cacodylate pH 7 $\mu = 1.7 \times 10^{-4}$
Magnesium stock:	5.20×10^{-5}F $MgCl_2$, 2.64×10^{-5}F Cacodylate pH 7 $\mu = 1.7 \times 10^{-4}$
Cobalt (II) stock:	5.20×10^{-5}F $Co(ClO_4)_2$, 2.64×10^{-5}F Cacodylate pH 7 $\mu = 1.7 \times 10^{-4}$
Silver (I) stock:	1.05×10^{-4}F $AgNO_3$, 9.0×10^{-5}F Cacodylate pH 7 $\mu = 1.7 \times 10^{-4}$

2. pH measurement, low ionic strength samples.

Aliquots of samples are tested with the Beckman 40316 one drop electrode after experiments. Stable readings of pH are obtained with this electrode at low ionic strength, and with other electrodes if shielding is provided.

All samples gave pH values of 7.0 ± 0.3 when prepared from these solutions.

3. Conductivity water technique.

Puritas water was redistilled in pyrex equipment, and found to have a conductance of 1.15 μmhos/cm, an improvement of 50% over the tap distilled water. All equipment for low ionic strength experiments was exhaustively cleaned with boiling water of this sort

(conductivity water). It was found that this was required to obtain reliable results, presumably because trace contaminants are strongly bound by DNA at low ionic strengths.

4. Method for heating curves.

Samples prepared at 1×10^{-4} M (P) as described above were degassed by placing them in a chamber evacuated to 30 mm pressure, at which pressure dissolved air came out of solution but no extensive evaporation was observed. This step is necessary to prevent formation of air bubbles in the light path during heating experiments.

A Pyrocell microcell carefully cleaned with conductivity water was filled with such a solution, and capped with a glass stopper. Such cells were made by cementing tops on ordinary 0.6 ml cells with Resiweld resin and curing above 100°.

The cell was placed in a heating compartment constructed for the Cary Model 14 spectrophotometer. This compartment was insulated by polyurethane foam, heated by electrical heating tape controlled by a variac, and kept dry by a stream of argon through the entire Cary compartment. Temperatures near the sample cell were read by copper-constantan thermocouple whose junction was placed next to the cell. Water-ice was used for the reference junction, and the potential read on a Leeds and Northrop Model K-2 potentiometer. Calibration of the thermocouple was made against a thermometer standardized by the National Bureau of Standards, and it was found that recalibration was necessary for each thermocouple used.

Samples were heated at a constant rate of 0.5 deg/min. An experiment at 0.1 deg/min showed no significant difference in result from one done at this rate. Temperatures and spectra were taken on the fly. Samples could be heated to 95° in such an experiment with no detectable evaporation.

Hyperchromicity was calculated at 260 mμ for these samples. Blanks were run in the heating compartment and all spectra calculated

by difference.

The heating compartment is described in figure 5.

5. Calculation of σ_T.

The area of the normal curve outside $\pm$ 0.5 σ is 0.618. Therefore, the range of temperature about the midpoint of the transition which includes $\pm$ 0.5 σ is $\pm$ 19.2% of the maximum hyperchromicity. We have therefore calculated σ_T by

$$\sigma_T = T(\bar{h} + 0.192\, h_{max}) - T(\bar{h} - 0.192\, h_{max})$$

where $\bar{h}$ is the hyperchromicity at T_m.

D. Biological transformation experiments.

Transformation experiments were performed by the technique of H. Ephrussi-Taylor, which will be summarized because it has not been published. The compositions of the media used are described in Appendix II.

1. Transformation system.

Recipient strains of D. pneumoniae must, of course, lack streptomycin (S), optochin (Q), and bryamycin (B) resistance. Either Cl#3 of R36A, a strain descended from that used by H. Ephrussi-Taylor and by R. Hotchkiss, or Am, was used as the recipient.

Donor DNA was prepared from a strain $S^rQ^bB^m$, resistant to all three antibiotics at the levels 200 µg/ml, 5 µg/ml, and 1.5 µg/ml respectively. This strain was obtained by transformation of the strain S^rQ^b, from H. Ephrussi-Taylor, by DNA from a strain S^rB^m. This bryamycin marker originated from DNA sent by Dr. J. Marmur, and is not necessarily identical to the marker used in the experiments of Rolfe and Ephrussi-Taylor, which had been prepared from a mutant isolated by W. F. Dove.

The streptomycin marker thus has a pedigree including the

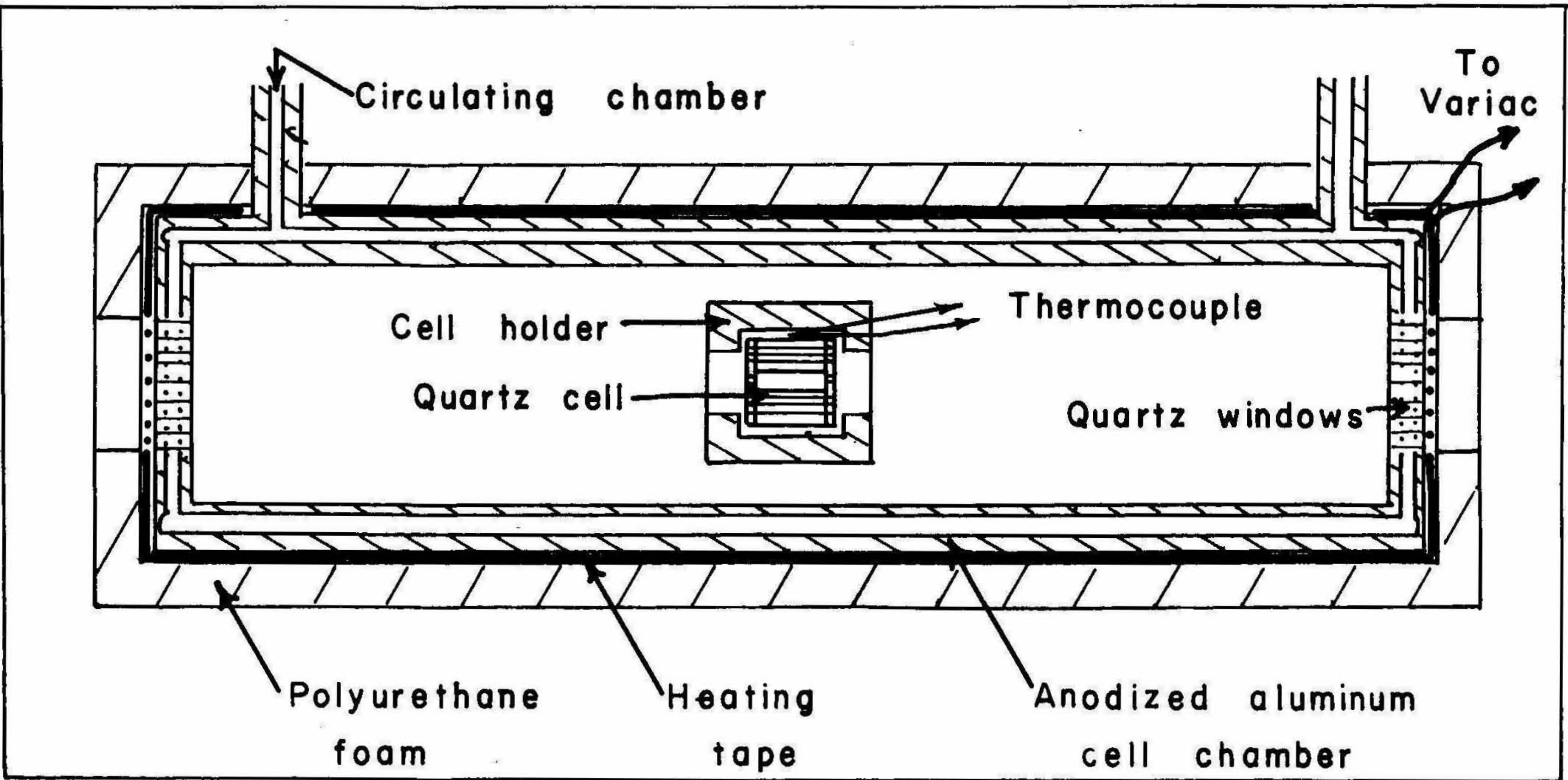


Figure 5. Compartment for heating experiments.

marker used in the laboratories of Hotchkiss, Marmur, Ginoza, and Ephrussi-Taylor. The optochin marker is used in the laboratory of Ephrussi-Taylor. The bryamycin marker is used by Marmur, Ginoza, and Hotchkiss.

Transformation is effected by transfer of a fresh liquid culture of recipient bacteria into a preculture of medium P, inoculated at 1:20. After 90 minutes growth (titer 10^8), the competence medium NS is inoculated at 1:25. Cells become competent (transformable) at a time determined by calibration on the previous day. At this time, aliquots are withdrawn and pipetted onto the DNA solutions to be assayed. Reaction for 20 minutes is followed by chilling, and then dilution for plating. All cultures are incubated at 37°, and good temperature control ($\pm$ 0.1°) is necessary to accurately judge the time of competence.

This procedure can be modified by freezing the precultures with 10% glycerol and storing at -20°. These frozen precultures can be used for several weeks to inoculate competence cultures.

Plating is done in blood agar (0.2 ml defibrinated horse blood to 10 ml plate) with 0.2 ml fresh yeast extract for the Am strain. Plates are incubated for two hours to allow genetic incorporation and phenotypic expression before challenging the cells by overlayering with a measured volume of agar containing the amount of antibiotic required to give the correct final concentration.

Transformants can be counted after 24-30 hours by the appearance of haloed clones. Plating is done so that between 100 and 600 clones will appear per plate. The error arising just from sampling is, of course, $(n)^{1/2}$, and is therefore 10% at a count of 100.

Pipetting, plating, and unknown errors make such experiments reliable to 20%.

2. Antibiotics.

Dihydrostreptomycin sulfate is obtained from Squibb.

Optochin (ethyl hydrocupreine•HCl) is obtained from L. Light and Co., Colnbrook, England. Bryamycin is obtained from Bristol Laboratories.

IV. Results and Discussion: Chemical experiments.

We shall discuss now a number of topics which we have investigated experimentally. These topics are unified in that they are all concerned with the denaturation of DNA, the interaction of DNA with small ions, and the heterogeneity of DNA. Each set of experiments will be discussed as it is presented. A general summary will be given at the end of this thesis.

A. The acidic denaturation of DNA.

1. General studies.

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SPECTROPHOTOMETRIC STUDY OF THE PROTONATION OF UNDENATURED DNA

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The denaturation of DNA by controlled treatment with mild acid generally resembles the heat denaturation and results in the destruction of the characteristic two-stranded helical structure. This is shown by the marked decrease in viscosity, by the hyperchromic effect, and by the increased availability of the bases for acidic and basic titration (for reviews see Peacocke, 1957; Sturtevant, et al., 1958). Light scattering indicates no significant decrease in molecular weight for thymus DNA (see, however, Cavalieri, et al., 1959). However, at 0° C, calf thymus and herring sperm DNA can be brought to a pH of 2.6 with the extent of protonation being ca. two protons per four P, without denaturation, as evidenced by the reversibility of the titration curves and the unchanged and high intrinsic viscosity after reneutralization (Peacocke and Preston, 1958; Geiduschek, 1958; Cavalieri and Rosenberg, 1957).

We have made a spectrophotometric study of the acidification of calf thymus DNA over the range of temperatures, 0°-30°, and ionic strengths, 0.1-0.5 M (NaCl), in order to obtain further information about the sites of protonation and the effects of protonation on the ordered structure of native DNA.

*Contribution No. 2524

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Fig. I displays the essential results. As the pH is lowered, resulting as we shall see in the protonation of DNA, the absorbance at 257 mμ falls whereas that at 280 mμ increases. As more acid is added, a point is reached where the light absorption at all wave lengths rises rapidly with decreasing pH and the ratio $\epsilon_{280}/\epsilon_{265}$ continues to increase.

It should be recalled that of the three basic nucleosides, cytidine (pK_a=4.2) shows a decrease in ϵ_{257} and an increase in ϵ_{280} with protonation, with an isosbestic point at 265 mμ. The spectrum of adenosine (pK_a=3.5) is little affected by protonation, whereas the weakest base, guanosine (pK_a=1.6), shows significant changes only below 260 mμ and above 280 mμ.

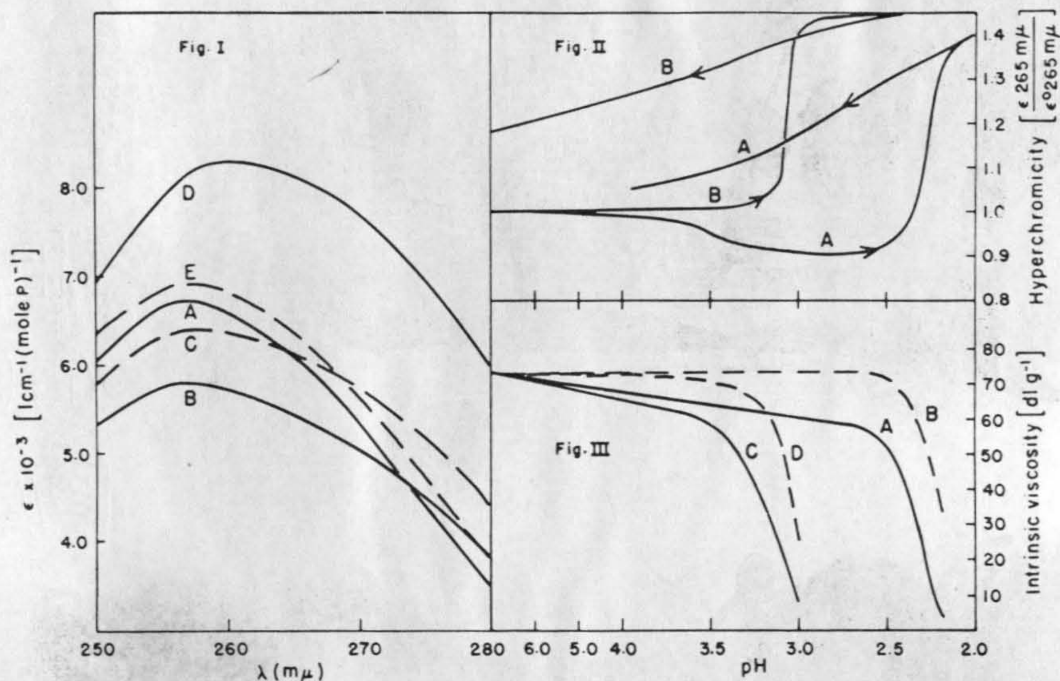

Fig. I. Absorption spectra of DNA at 0.0(± 0.2)°C in 0.1 M NaCl. A, original DNA, pH 6; B, pH 2.80; C, pH 2.42; D, pH 2.25; E, reneutralized, pH 8.

Fig. II. Hyperchromicity vs. pH on forward and back titrations at 0° (A) and 30° (B).

Fig. III. Intrinsic viscosity in acidic and reneutralized form vs. pH. A, 0° at pH indicated; B, 0°, reneutralized from indicated pH; C, 30°, at pH indicated; D, 30°, reneutralized from indicated pH.

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The general increase in absorption in the more acid solutions is a hyperchromic effect, indicating denaturation, and is well known from previous studies of the acid and heat denaturation. The changes in spectra before denaturation, i.e., below pH 2.59 at 0° and pH 3.32 at 30°, are like the spectral changes on protonation of cytidine. Since cytidine and adenosine are stronger bases than guanosine, we attribute these changes to the protonation of cytosine in the DNA. We believe that the site of this protonation is the carbonyl oxygen of cytosine and that protonation here need not disrupt the Watson-Crick pattern of hydrogen bonds. Briefly, the principal evidence for this hypothesis is the shift in spectrum of cytidine towards the red on protonation. Protonation of a sigma electron pair on the N-1 ring nitrogen would have relatively little effect on the spectrum; protonation of the amino group would suppress the resonance of this group with the ring and shift the spectrum toward shorter wave lengths; whereas protonation of the carbonyl oxygen would increase the amount of resonance in the ring and cause the observed spectral effects. The spectra of most model compounds confirm the above statements. There are however some anomalies and exceptions, and we regard our hypothesis as being probable, but not certain.

By assuming that the shapes of the absorption curves of the bases are the same in native DNA as for the free bases, we calculate from the $\epsilon_{280}/\epsilon_{265}$ ratios, plus the fact that thymus DNA contains 25 mol % cytosine, that at pH 2.7 at 0° C (μ=0.1 M) about 90% of the cytosine is protonated. At this point, according to pH-titrations, there are about 1.7 H^+ per 4P atoms (in general agreement with the Peacocke and Preston titrations). The intrinsic viscosity of the reneutralized material is the same as that of the starting material in agreement with Geiduschek, and Cavalieri and Rosenberg. It is noteworthy that the isosbestic point for protonation of the undenatured material shifts from 265 mμ at 30° to 274 mμ at 0°.

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The general hypochromic effect in native DNA is not understood, but it appears to be due to the compact packing of the bases. The spectra then indicate that this compact packing is not destroyed by protonation up to the denaturation point.

We are tempted to conclude that at 0° the molecule is stable with one proton on each adenine and one on each cytosine, but that denaturation occurs when protons are added to the guanine partners of some of the cytosines.

The pH of denaturation behaves in the expected way as a function of temperature and ionic strength (Table I). The optical changes on denaturation (Fig. II) agree with those reported previously (Lawley, 1956) and indicate a partial recovery of order on reneutralizing the denatured material.

Table I

Denaturation pH

T μ	0° C 0.1	0° C 0.5	10° 0.1	20° 0.1	30° 0.1	30° 0.5
pH_{id}	2.59	2.44	2.73	3.13	3.32	2.92
% CyH^+	90	83	90	80	75	-
$H^+/4P$	-	-	1.6	1.5	1.3	-
pH_{50}	2.25	2.11	2.57	2.79	3.07	2.69
% CyH^+	95	91	89	87	87	-
$H^+/4P$	-	-	2.0	2.0	1.9	-

pH_{id} is pH of incipient denaturation; pH_{50} is pH for 50% denaturation; % CyH^+ is % cytosine protonated at these points.

By controlling the changes in either pH or temperature, it is possible to produce partial denaturation. This is clear evidence for heterogeneity of calf thymus DNA in agreement with the observations on

its heat denaturation and density-gradient centrifugation (Doty, et al., 1959).

The table shows that the effect of ionic strength is principally an effect on the titration curves. The higher the ionic strength, the lower the proton affinity of the bases because of the neutralization of the negative charge on the peripheral phosphate groups by the ionic medium. At an equal degree of protonation the sensitivity to denaturation is about the same at high salt and low salt, indicating that the external salt does not greatly affect the repulsions between positively charged base groups.

Calf thymus DNA, prepared by a modified Mirsky-Pollister procedure, was supplied by the Nutritional Biochemicals Corporation: phosphorus 7.3% of bottle weight, protein, 1-3%; $\epsilon_{260}=6.58 \times 10^3 \ell$ cm^{-1} (mole P)$^{-1}$; $[\eta] = 73 d\ell g^{-1}$. Sedimentation patterns of the acid-denatured, reneutralized material indicated a small amount of hydrolysis to fragments of lower molecular weight, but no significant formation of nucleotides.

We are glad to acknowledge support from the U.S. Public Health Service (Grant No. A-2145C), the technical assistance of Mr. Herbert Rice, and an NSF predoctoral fellowship for one of us (WFD). We are grateful to Professor George S. Hammond for advice and discussion about the spectra and mode of protonation of the nucleotides.

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2. The sites of protonation in DNA.

The arguments referred to in this paper concerning resonance forms resulting from protonation of cytosine in various positions are presented in detail by Wallace (1960). We shall discuss this in the light of ideas subsequently presented by Dekker (1960) and we shall mention some experimental results also recently published which bear on the question of the site of protonation in DNA.

In figure 6 we have written out the resonance forms corresponding to N-3, N-7, and 0-2 protonation. It can be seen that protonation of N-7 localizes the π electrons on this atom and would be expected to shift the absorption spectrum to shorter wavelengths. Indeed, the absorption spectrum of aniline shifts in this way on protonation.

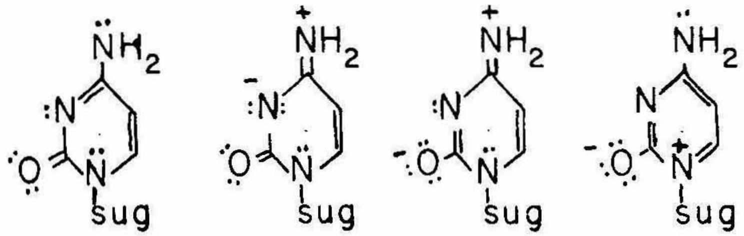
Protonation of N-3 localizes a pair of σ electrons on this atom. To the slight extent that these electrons are delocalized before protonation, a shift to shorter wavelengths would be expected, but certainly not a shift to longer wavelengths. If pyridine is a model for this case, we are encouraged by the fact that no great spectral shift takes place on the protonation of pyridine.

However, Bayzer (1957) reports that the spectrum of 2-amino-pyridine undergoes a shift on protonation very similar to that observed for cytosine. Dekker (1960) has pointed out that protonation of N-3 in cytosine allows one to write resonance forms with the positive charge on N-1, N-3, or N-7. The important fact is that this allows the amino group in cytosine (and in 2-amino-pyridine) to contribute its π electrons without charge separation. This might result in a shift of the absorption spectrum to longer wavelengths, and it does in the model compound.

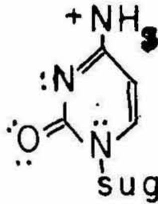
Protonation of O-2 allows one to write an aromatic pyrimidine structure with the positive charge on N-1. This increases the aromaticity of the system, and one would expect a shift in absorption to longer wavelengths. However, Dekker (1960) has reported that the protonation of cyclooctocytidine (I) (see figure 6) produces a spectrum

Resonance forms in cytidine.

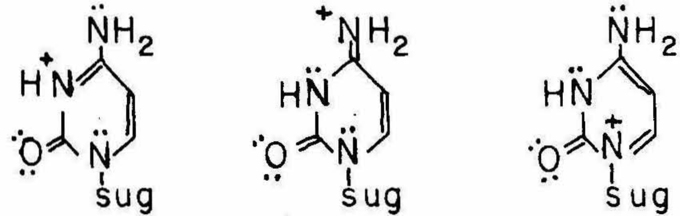
Unprotonated:



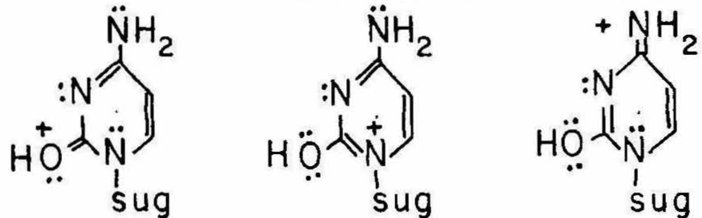
N-7 protonated:



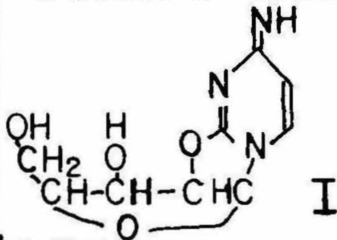
N-3 protonated:

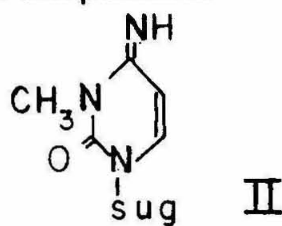


O-2 protonated:

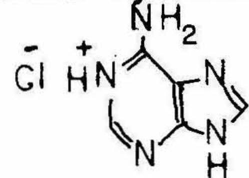


Decker's model compounds:

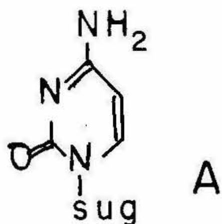


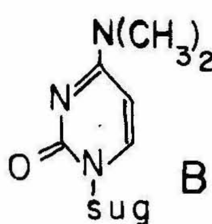


Adenine hydrochloride



Miles' model molecules:





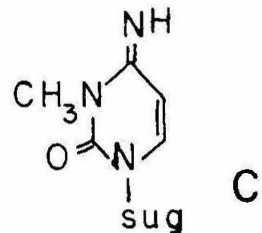


Figure 6. The protonation of cytidine and adenine.

shifted to the blue with respect to cytidine, whereas protonated $3-CH_3$-6-iminocytidine (II) has a spectrum very much like that of protonated cytidine. This reported result seems to implicate N-3 protonation of cytosine both in the free base and in DNA, and also seems to rule out O-2 protonation in both cases.

Some additional information on the site of protonation of cytosine may be contained in the work of Jardetzky and Jardetzky (1960). They have studied NMR spectra of cytidine and 1-methylcytosine in acid media. They find that the C-6 proton undergoes a somewhat greater chemical shift during protonation than does the C-5 proton, and therefore conclude that the positive charge must lie partly on N-1. It seems that this result does argue against N-7 protonation in cytosine, but does not distinguish between O-2 and N-3 protonation.

Miles (1961) has taken infrared absorption spectra in D_2O for a series of compounds A, B, and C (see figure 6). In acid solution they have very similar spectra between 1.6 and 2.2 μ. This also indicates that cytosine protonates on N-3 in solution.

We must conclude then that the spectral changes observed in DNA protonation reflect N-3 protonation of cytosine. Subsequent to our work reported above, we have found that AT polymer shows no spectral changes on acidification until a general hyperchromic effect occurs. Experiments with bacterial DNAs of different composition indicate that one cannot quantitatively analyze the spectral changes to find the extent of cytosine protonation, since samples of high GC content demonstrate much larger changes at 280 mμ than would correspond to total protonation (see table 1, page 13).

If we take N-1 as the site of protonation of adenine, as it is in solid adenine hydrochloride (Cochran, 1951, and Broomhead, 1951), then it is necessary to break Watson-Crick hydrogen bonds each time we protonate one of these bases. It is quite intriguing that there is no hyperchromicity during the protonation prior to irreversible denaturation.

The situation is different for thermal denaturation. In unpublished experiments by Dekker and Barrett (1961) (which have been confirmed in this laboratory by R. Jensen) a calf thymus DNA sample at 0.1 μ is heated to a temperature where it displays 50% of its full hyperchromicity. This sample will largely renature upon slow cooling. Thus, in this case too we can break half the hydrogen bonds without irreversibly denaturing the molecule. But, in contrast to denaturation by acid, there is hyperchromicity in the state where half the hydrogen bonds are broken.

3. Compositional selectivity.

a. Low pH, 25°.

Our studies of the protonation of DNA in acid denaturation were performed primarily on calf thymus DNA. To determine whether these denaturations exhibit any compositional selectivity, we have performed spectral titrations on DNA samples of various compositions. It is found in this case that GC rich DNAs are more stable to acid denaturation at 25° than AT rich DNAs. The magnitude of the differences observed is not large enough to encourage fractionation by this method. Simultaneous experiments by Bunville and Geiduschek (1960a) quantitatively agree with the results presented in table 3.

b. Low pH, 0°.

The fact that the cations of adenine and cytosine have pK_a's of 4.2 and 4.5 (Peacocke, 1958), while guanine cation has a pK_a of 3.2, indicates that adenine and cytosine are the first bases to protonate, as discussed above. Thymine protonates only in strong acid. At 0°, one must protonate about two out of four bases completely to cause denaturation, and it may be that guanine protonation becomes a significant factor in denaturation. If such is the case, a

Table 3. pH denaturation of DNAs.

Sample	X_{GC}	Acid		Alkaline	
		pH_F, 25°	pH_F, 0°	pH, 20°	pH, 2°
D. pneumoniae	0.39	2.92	2.31	11.78	-
Calf thymus	0.42	2.95	2.31	11.77	11.96
E. coli	0.50	2.86	2.23	11.92	12.06
M. lysodeikticus	0.72	2.74	2.20	11.99	12.25

Sample	h_{max} acid, 25°	h_{max} 0°
D. pneumoniae	1.51 1.45	1.45
Calf thymus	1.45 1.43	1.40
E. coli	1.51 1.40	1.27
M. lysodeikticus	1.35 1.26	1.23

high GC content would begin to be a liability to a native molecule. One would then expect the dependence of pH of denaturation on GC content to be reduced and perhaps eventually reversed at low temperatures.

Experiments at 0° indicate that the pH dependence of denaturation on GC content is indeed reduced, but not reversed. This is consistent with these notions about the order of protonation in DNA. The results, also summarized in table 3, are in agreement with subsequent unpublished results of Bunville and Geiduschek (1960b).

B. The alkaline denaturation of DNA.

1. General studies.

The alkaline denaturation of calf thymus DNA was studied by spectral titrations and by observing changes in the intrinsic viscosity of solutions brought to a given pH and then reneutralized. As in the case of acid denaturation, irreversible changes in viscosity can be correlated with the onset of hyperchromicity, shown in figure 7.

In the case of alkaline titrations, there seems to be a marked component of denaturation at early stages in the titration. This may be simply due to the mixing problem, which would be made more serious if the rate of denaturation is greater for alkaline denaturation than for acid. If this is due to mixing, then our results indicate that the sensitive way to bring a sample to a given pH is to dilute an aliquot of a DNA stock in a buffer of the required pH, at 0° C, and then to warm up to 20°. By this technique, a sample will never experience more severe conditions than those under which it is to be tested.

2. Compositional selectivity at high pH, 2° and 20°.

Guanine and thymine both have acid dissociation constants with $pK_a = 10$. In DNA, however, guanine interacts more strongly with its partner than does thymine. This might selectively raise the pK_a of guanine. We would then expect to find increased relative sensitivity to denaturation in AT rich molecules in the alkaline region.

D. pneumoniae
Calf thymus
E. coli
M. lysodeikticus

h_{270}

1.5
1.4
1.3
1.2
1.1
1.0

7 8 9 10 11 12

pH

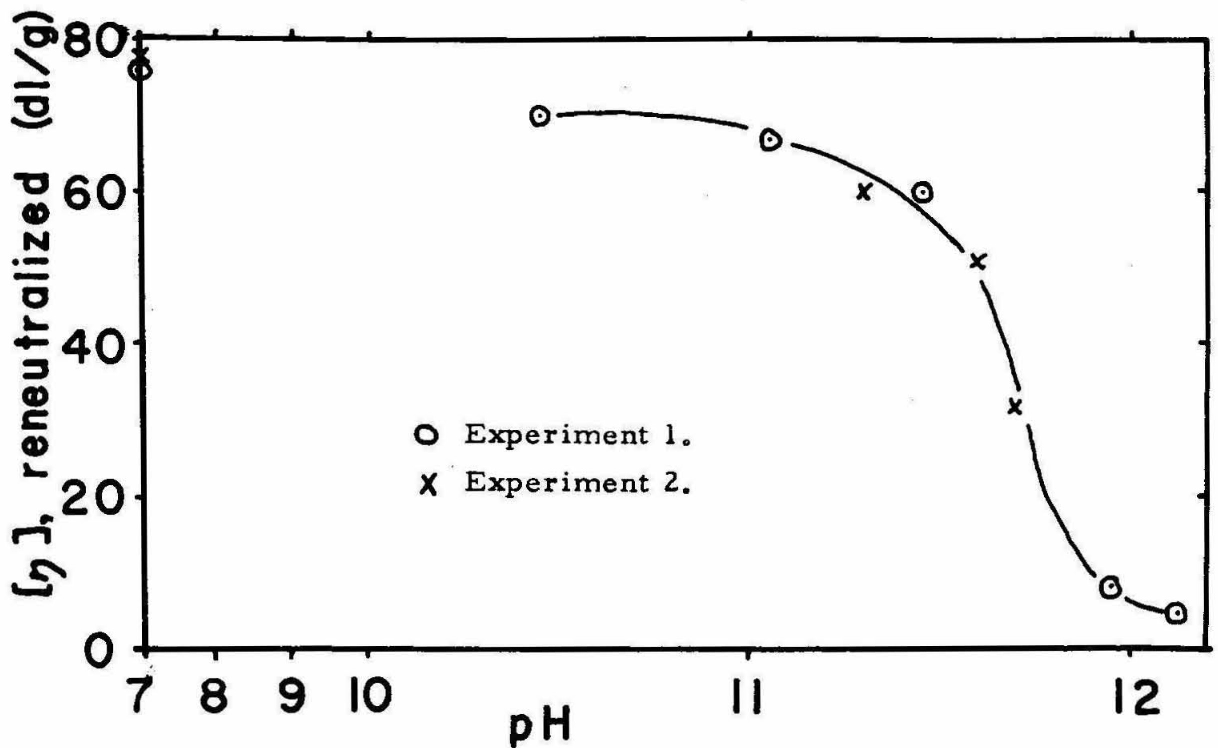


Figure 7. Spectral titrations in alkaline region for various DNAs, 20° C, and irreversible viscosity changes in calf thymus DNA.

Spectral titrations in phosphate buffer, $\mu = 0.1$, gave rise to sharp transitions, as seen in figure 7. Spectral changes due to titration result in there being no stable isosbestic wavelength, and the calculation of hyperchromicity is therefore approximate.

The denaturation pH's for these samples at 2° and at 20° are listed in table 3. At 20°, no large differences in denaturation pH was observed, and our hope of finding a significantly enhanced pH dependence on GC content is not substantiated. It is interesting to note, further, that at 2°, no change in the pH dependence on composition is observed, although one has to titrate the molecules to higher pH to cause denaturation. This indicates that G and T have pK's which are nearly equal, and that no other groups are being titrated in this region.

C. Neutral pH, $0.1\,\mu$.

A series of samples of DNA of varying composition were heated at $0.1\,\mu$ in $NaClO_4$, buffered at pH 7. The results were essentially the same as those of Marmur and Doty (1959) at $0.15\,\mu$, thereby establishing that the method of denaturation used was reliable and the compositions of the samples prepared were the expected ones. A linear relationship between T_m and X_{GC} can be found. These thermal denaturation curves are presented in figure 8.

The greater transition breadth observed for calf thymus DNA is not unexpected. As was seen in our studies of acid denaturation, samples rich in AT seem to show more hyperchromicity on denaturation than do samples rich in GC. The reason for this is unknown (Tinoco, 1961).

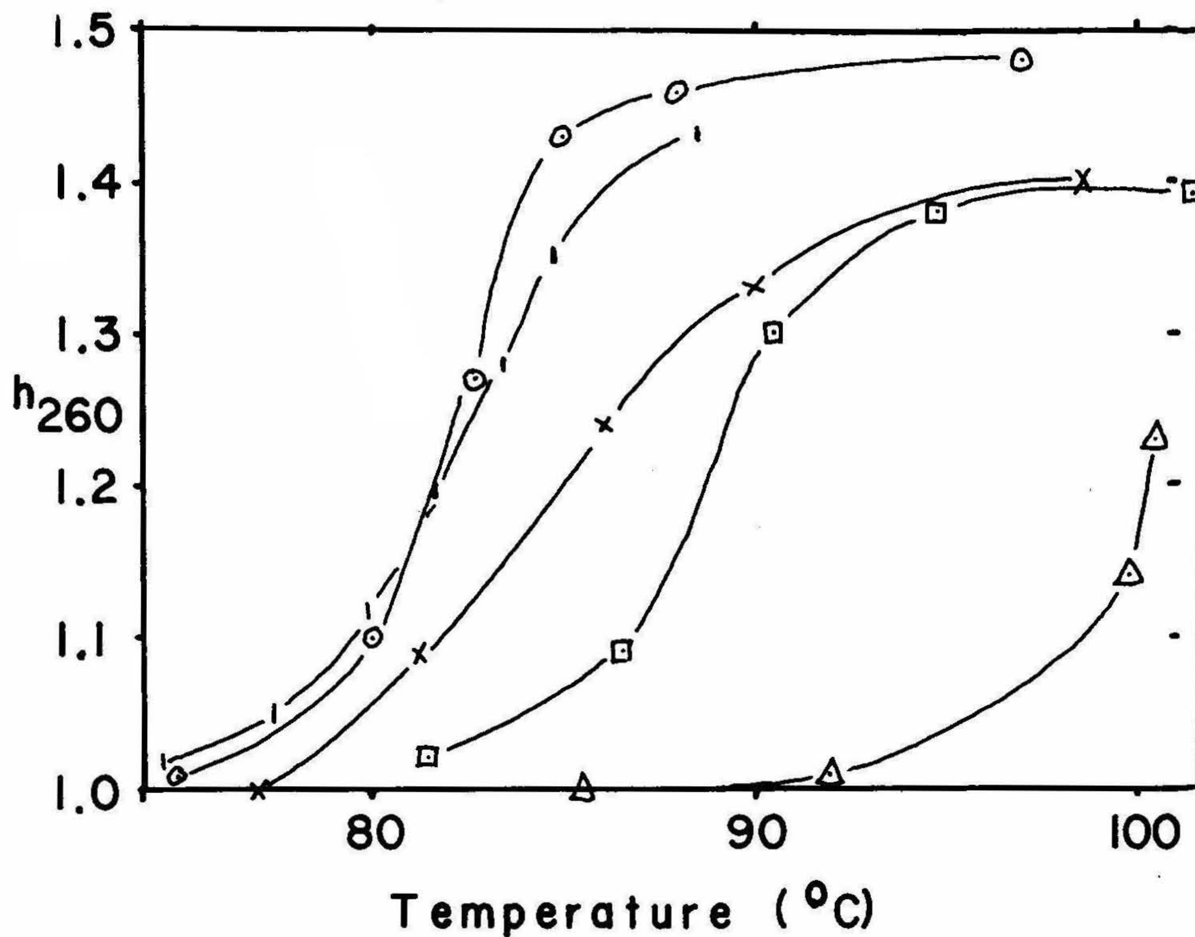
D. Neutral pH, low ionic strength.

1. Ionic strength dependence of denaturation.

It has been reported that DNA denatures more easily at low ionic strength than at high (Cavalieri, Rosoff, and Rosenberg,

	X_{GC}	T_m	σ_T
—⊙—⊙— B. megatherium	0.38	82.3	2.6
—ı—ı— D. pneumoniae	0.39	82.2	3.4
—×—×— Calf thymus	0.42	84.8	4.9
—⊡—⊡— E. coli	0.50	88.6	2.9
—△—△— M. lysodeikticus	0.72	100.2	1.2

Figure 8. Thermal denaturation of DNAs at 0.1 μ, pH 7.



1956). A heating curve published by Doty (1958) indicates a noticeably broader transition profile at 10^{-3} μ than at 0.15 μ. One would also expect the binding of positive ions to be greater at low ionic strength than at high, since the phosphate groups would be less shielded at low sodium ion concentrations. For these reasons, we have systematically studied denaturation at low ionic strengths in sodium ion medium.

A sample of B. megatherium DNA (38% GC) was studied at pH 7 (cacodylate) in a series of concentrations of $NaClO_4$. The heating curves in figure 9 indicate that the melting temperature is closely a linear function of pNa, and that the transition breadth more than doubles in going from $\mu = 0.1$ to $\mu = 3 \times 10^{-4}$.

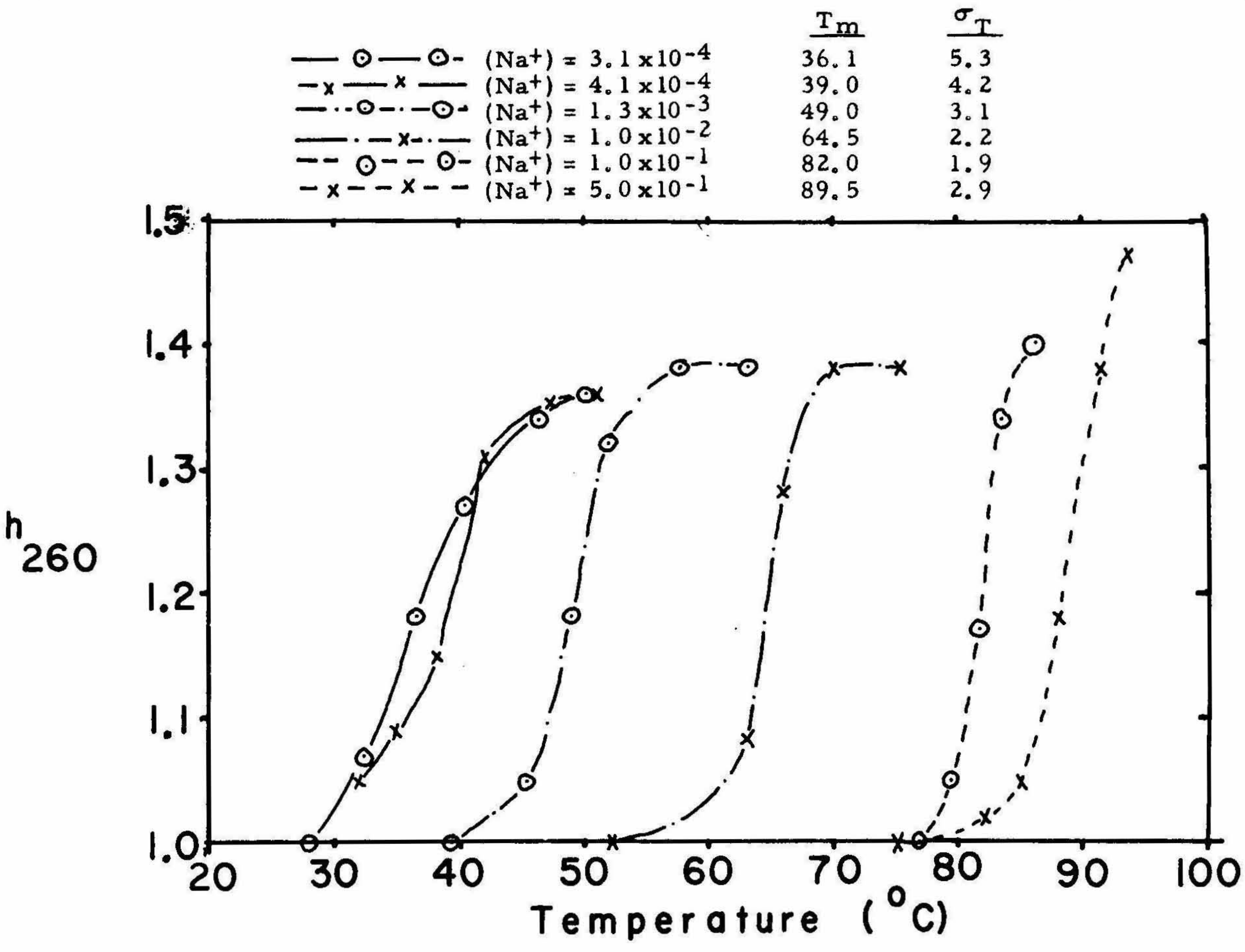
Experiments by Cavalieri, Rosoff, and Rosenberg (1956) indicate that pH may be an important factor in these low ionic strength experiments. However, from their results, one would expect no protonation of DNA at pH 7 at 3×10^{-4} μ. Furthermore, the results of Oth (1959) on changes in sedimentation coefficient on acidification at low ionic strength (1×10^{-3}) indicate that the transition observed takes place between pH 5 and pH 4. Calculation of $\frac{E260}{E280}$ at 0.1 μ gave the same value as at 3×10^{-4} μ, so that there is no spectral evidence for protonation.

It could be argued that the increased breadth at low ionic strength is due simply to the fact that DNA denatures at a lower rate at low temperatures. We normally have heated samples at the rate of 0.5° per minute, at all temperatures. To rule out this possible lag effect, samples of <u>E. coli</u> DNA were heated at the normal heating rate and at 0.1° per minute at low ionic strength. The transition profiles obtained were identical, within the customary experimental reproducibility.

We now see that at low ionic strengths equilibrium heating curves show significantly broader transition profiles than at high ionic strengths. This can be accounted for by several hypotheses.

1. The AT pair is stabilized selectively by ionic strength, for example by ionic interaction with the charge distributions

Figure 9. Thermal denaturation of B. Megatherium DNA at various ionic strengths in $NaClO_4$, pH 7.



on the adenine or thymine heterocycles. At low ionic strength, then, AT would be destabilized with respect to GC more than at high ionic strength, and $\sigma_T = \sigma_{GC} \frac{dT}{d(X_{GC})}$ would be greater.

2. The intrinsic breadth of the helix-coil transition is increased at low ionic strength either by a change in σ_o or in σ_j. If electrostatic forces would decrease the stacking energy e which determines σ_o, then σ_o would approach 1 at low ionic strengths, and the transition would be come broad. Alternatively, entropic cooperative factors would be lost if at low ionic strength the entropy of free ends were reduced with respect to the entropy of loops. Thus, σ_j would be changed in a way that would produce broadening.

3. There is selective binding of counterions to native DNA compared to denatured DNA.

The electrostatic binding of counterions decreases the free energy of the helical conformation more than it does that of the coil. This is the reason for the increase of melting temperature with increasing ionic strength. If also the binding of counterions is stronger for native DNA than for denatured, a partially denatured sample will have a predominance of sodium ions around still-native regions rather than denatured regions. These native regions would be further protected, and there would result a broad transition. This hypothesis is plausible in light of the results of Shack and Bynum (1959), that Mg^{++} is bound more strongly by native DNA than by denatured. Ascoli *et al* (1961) report similar results for Na^+ ion.

2. Compositional selectivity at neutral pH, 3×10^{-4} μ.

It is clear that we can decide on the merits of hypothesis 1 by measuring $\frac{dT}{dX_{GC}}$ at high and at low ionic strength. The 0.1 μ experiments were discussed above. At 3×10^{-4} μ, pH 7, we have performed heating curves on samples of various compositions. Exper-

iments at low ionic strength are reproducible only to ±2°, as has been discussed, but even with this experimental uncertainty, we can see in table 4 that there is not sufficient change in $\frac{dT}{dX_{GC}}$ from 0.1 μ to 3×10^{-4} μ to account for the change in σ_T. Thus, we are required to conclude that it is a change in the intrinsic transition breadth, by either hypothesis 2 or 3, which accounts for the change in total transition breadth. The biological experiments to be discussed later will bear on this point also. We shall also discuss the lack of plausibility of hypothesis 3.

E. The interaction of Mg^{++} with DNA.

1. Stoichiometry and strength of binding.

Conductometric measurements at low ionic strength by Doty and Zubay (1958) and by Felsenfeld and Huang (1959) indicate that Mg^{++} is strongly bound to DNA. The stoichiometry and relative strengths of binding to native and to denatured DNA, when determined in this way, are unreliable (Dekker, 1960). Experiments by Shack and Bynum (1959) at pH 9 to 10, using eriochrome black T as a Mg^{++} indicator, show that Mg^{++} is strongly bound up to one equivalent per phosphate, that it is displaced by excess Na^+, and that it is bound more strongly by native than by denatured DNA.

The effect of the binding of Mg^{++} on the denaturation of DNA was first noticed by R. Thomas (1955). He observed that calf thymus DNA denatured at room temperature in the presence of 5×10^{-3} M sodium ion, but that it was stable in the presence of concentrations of magnesium ion down to 5×10^{-5} M. We have studied this phenomenon to gain information about the stoichiometry of the binding, the competition with Na^+, the relative binding affinities of Mg^{++} for native and denatured DNA, and to see if Mg^{++} exhibits any compositional selectivity.

At $\mu = 3 \times 10^{-4}$, we can vary the concentration of Mg^{++} from 0 to 1×10^{-4} M, and find the temperature of melting as a function of

Table 4. Composition dependence of thermal denaturation.

		Tm						
Sample	X_{GC}	$0.1\,\mu$	σ_T	$3 \times 10^{-4}\,\mu$	σ_T	$r=1\,Mg^{++}$	$r=.2\,Ag^{+}$	σ_T
B. megatherium	0.38	82.3	2.6	35.2	5.3	78.9	72.9	10.3
Calf thymus	0.42	84.8	4.9	38.5	-	-	-	-
E. coli	0.50	88.6	2.9	42.6	-	82.6	82.2	11.0
M. lysodeikticus	0.72	100.2	1.2	52.7	-	87.6	92.5	11.3
	$\left\langle \frac{dT_m}{dX_{GC}} \right\rangle$	52		54			61	

r, the ratio of equivalents of Mg^{++} to equivalents of phosphate. In figure 9 are shown these heating curves for B. megatherium DNA; they indicate that there is about one equivalent of Mg^{++} bound per phosphate. The binding is strong, since at $r = 2$ the molecule has little more stability than at $r = 1$, as seen in figures 10 and 11.

It is interesting also to note the pronounced broadening of the transition at $r = 0.5$. The only reasonable explanation for this broadening which comes to mind is discussed as hypothesis 3 above: that native DNA binds Mg^{++} more strongly than does denatured, and that when a partially protected molecule does denature, its bound Mg^{++} ions tend to migrate to still-native molecules and enhance their stability.

2. Competition between Mg^{++} and Na^{+}.

It is secondly of interest to study the relative binding strengths of Na^{+} and Mg^{++} by competition, to check the magnitude of the ratio given by relative stabilization abilities, which was 500 at $r = 1$. An $r = 1$, Mg^{++}-DNA solution was made up, increments of $NaClO_4$ were added, and T_m determined. The results, presented in figure 12, indicate that Na^{+} ion competes more effectively than previously calculated, but that its effectiveness in stabilizing the helix per bound charge is less than that of Mg^{++}. In short, two Na^{+} ions can replace one Mg^{++}, but cannot stabilize the native structure as well. It seems, then, that although the effectiveness of Mg^{++} in stabilizing DNA stems primarily from its stronger electrostatic binding, its steric properties also contribute to the stabilization. Mg^{++} seems to be sterically suited to interaction with the charge configuration in native DNA.

F. The interaction of Co^{++} with DNA.

Experiments by P. Brooks (1960) indicate that Co^{++} confers stability upon DNA in much the same way as we have shown Mg^{++} ion does. Experiments were performed with our samples using our

Figure 10. Effect of Mg^{++} on thermal denaturation of B. megatherium DNA at 3×10^{-4} μ, pH 7.

		T_m	σ_T
—○—○	r = 0	35.0	6.3
—x—x—	r = 0.5 equiv. Mg^{++}/phosphate.	58.0	8.2
—□—□	r = 1.0	78.5	2.6
—△—△—	r = 2.0, DNA = 5×10^{-5} M (P)	81.1	1.4

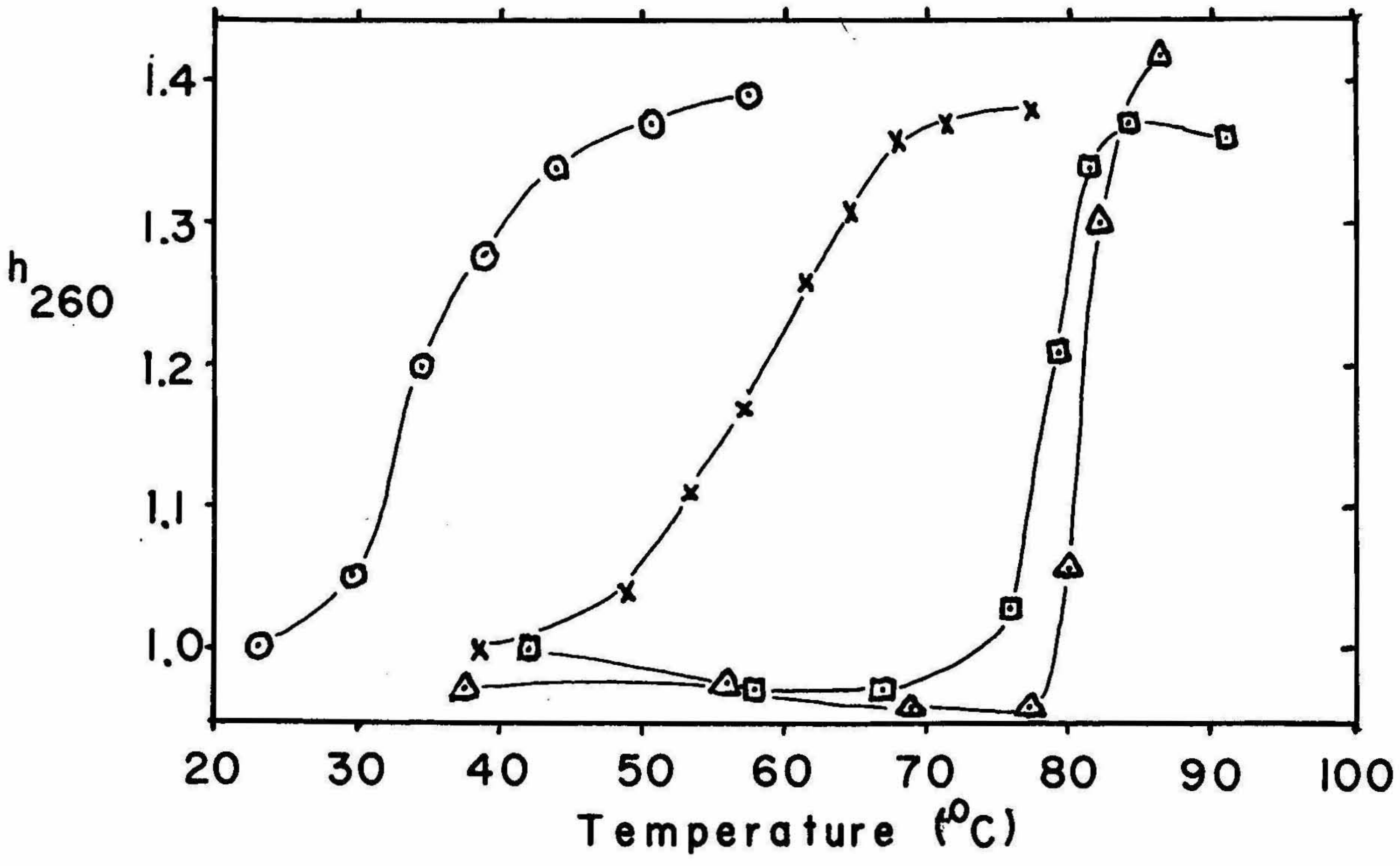
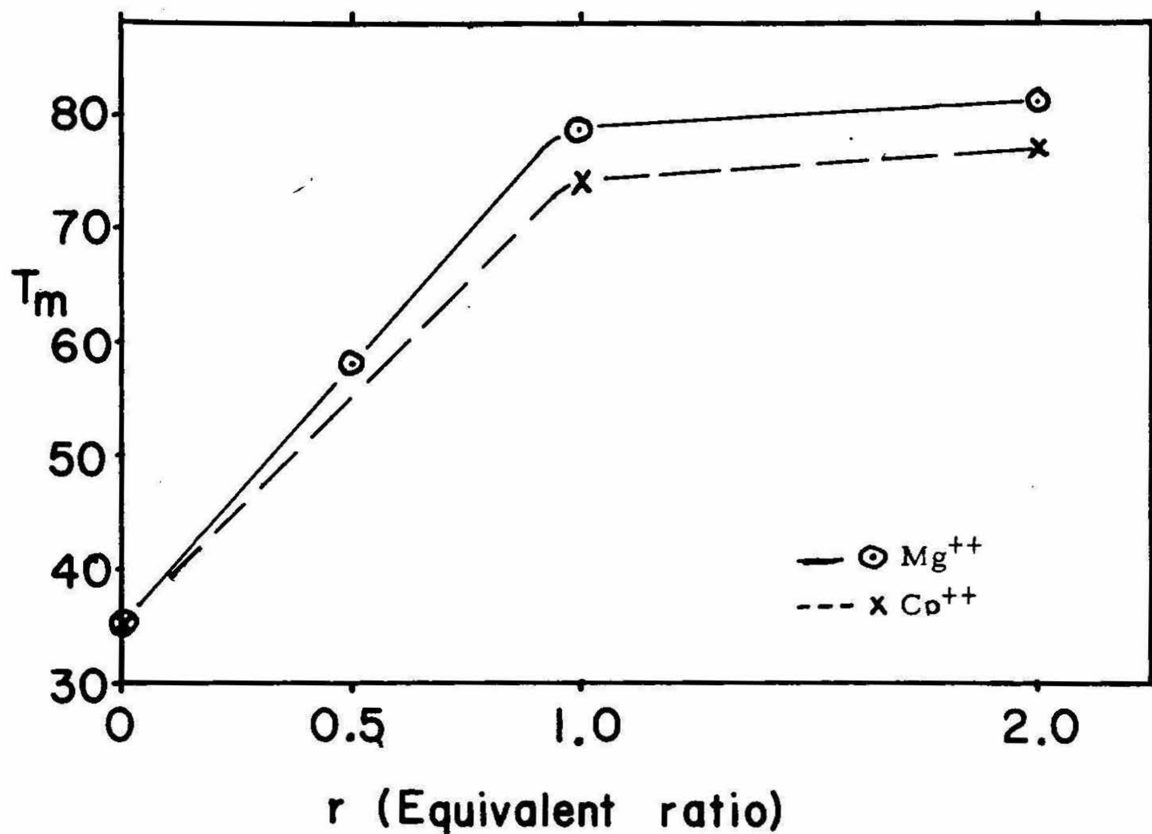


Figure 11. Transition midpoint for the denaturation of B. megatherium DNA in the presence of Co^{++} and Mg^{++}, $u = 3 \times 10^{-4}$, pH 7.



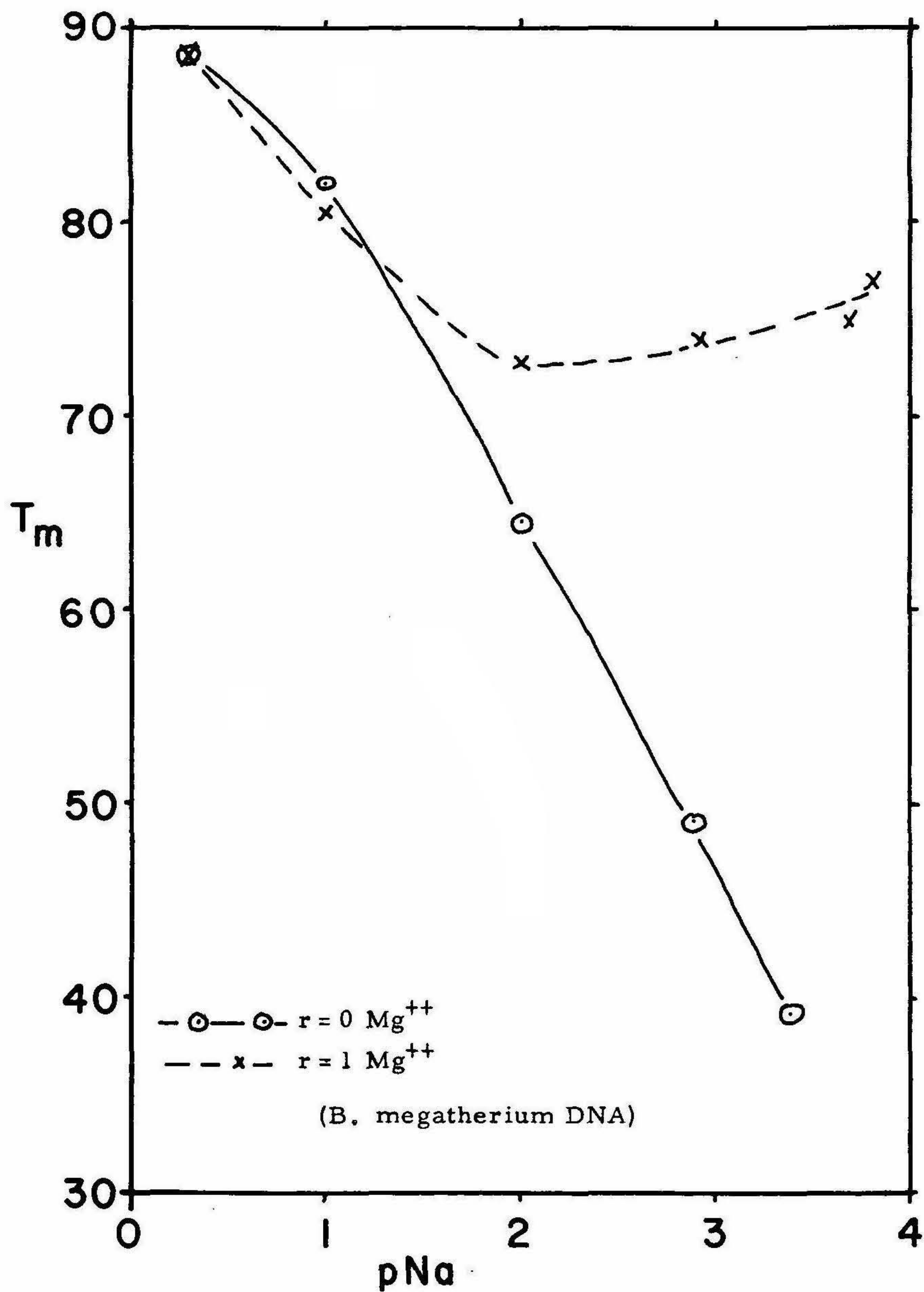


Figure 12. Competition between Na^+ and Mg^{++}.
T_m vs. pNa at r = 0 Mg^{++} and r = 1 Mg^{++}.

denaturation technique in order to compare the action of Co^{++} more closely with that of Mg^{++}. In the case of Co^{++}, as shown in figure 11, the binding is also strong and saturated at r = 1, but the protection conferred is somewhat less than that of Mg^{++}. Assuming there are no steric differences in the actions of Mg^{++} and Co^{++}, the binding of Co^{++} is not as strong as that of Mg^{++}.

G.. Compositional selectivity of Mg^{++} and Co^{++}.

We are finally interested in determining whether Mg^{++} or Co^{++} display any compositional selectivity in stabilizing DNA. The stabilization of _B. megatherium_, _E. coli_, and _M. lysodeikticus_ DNAs by r = 1 Mg^{++} was seen to decrease significantly with GC content. Similar results for two of these samples were observed with Co^{++}. On the other hand, we have seen that the stabilization given by increasing the sodium ion concentration from 3×10^{-4} to 10^{-1} is independent of composition. These results are shown in figure 13.

This compositional selectivity in the stabilization of DNA by Mg^{++} and Co^{++} is marked and certainly experimentally significant. There are two conceivable sources of this effect.

1. Selective binding. Either AT pairs in native DNA bind these divalent ions more strongly than do GC pairs in native DNA, or else GC pairs in denatured DNA bind these ions more strongly than do AT pairs in denatured DNA.

Zubay (1959) presents evidence for the binding of Mg^{++} to the heterocyclic bases in DNA. Conductometric measurements indicate more extensive binding to denatured DNA than to native. Also, hydroxymethylation of DNA with formaldehyde decreases the binding of Mg^{++}, also measured conductometrically. Zubay likens the binding of Mg^{++} by 8-hydroxyquinoline to the interaction he would expect between Mg^{++} and guanine. This would require guanine to exist in its enol tautomer, and could therefore only take place in denatured DNA. Zubay also considers a chelate between adenine and Mg^{++} involving

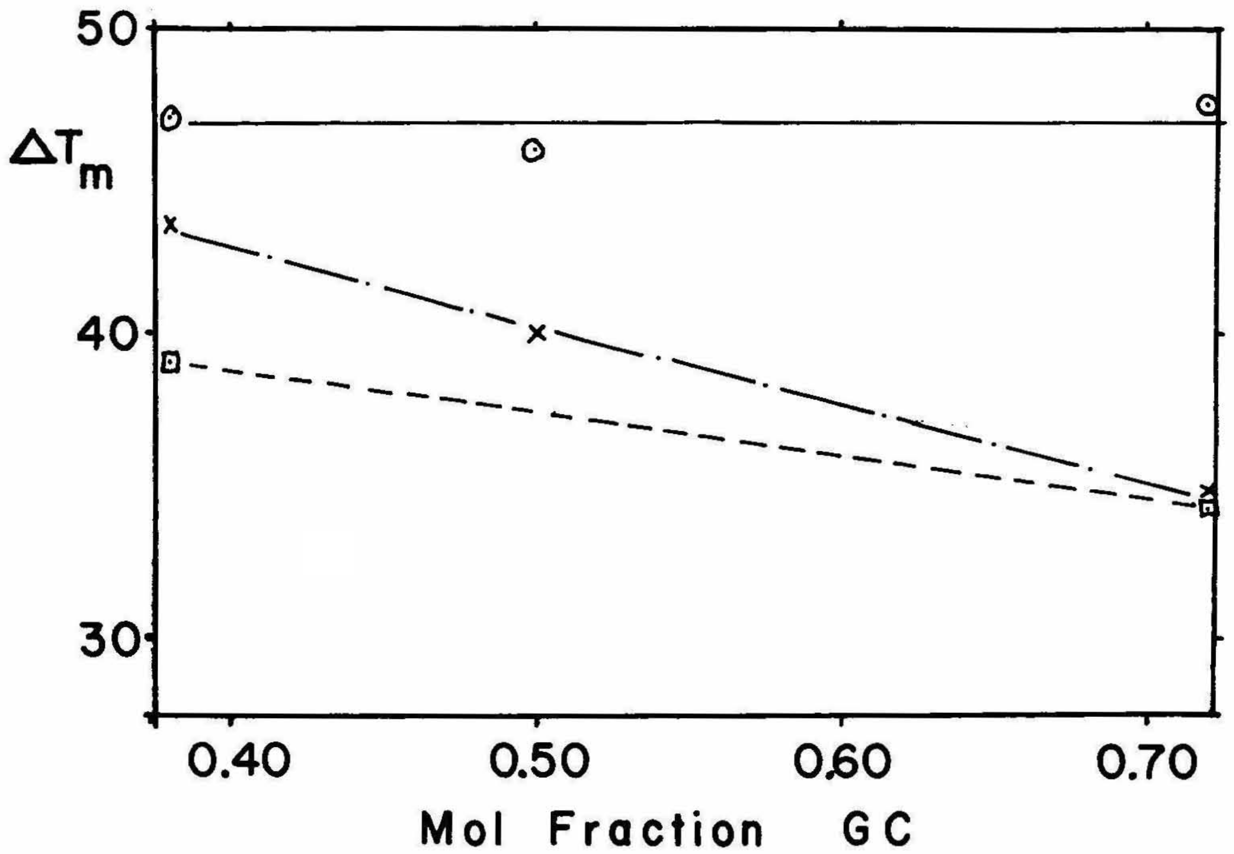


Figure 13. Stabilization of DNAs, compared to 3×10^{-4} μ Na^{+}, by addition of (i) 0.1 μ $NaClO_4$ —⊙——⊙—
(ii) r = 1 Mg^{++}, 3×10^{-4} μ —·—x—·—
(iii) r = 1 Co^{++}, 3×10^{-4} μ. -⊡---⊡--

the electrons of N-7 and the electrons of N-10 in forming a five-membered ring with Mg^{++}. This requires that the adenine amino group be non-coplanar with the adenine ring, and therefore this interaction also could only take place in denatured DNA.

The interpretations of Zubay could account for our result. The observation that Mg^{++} is bound more strongly by denatured DNA than by native is probably incorrect (Shack and Bynum, 1959, and Dekker, 1960). Nevertheless, there may still be significant binding in denatured DNA such as a complex between guanine and Mg^{++}. The strong complexing of Mg^{++} by native DNA may include a chelate between adenine and Mg^{++}, not like the chelate proposed by Zubay, but rather with a proton displaced from the N-10 amino group. This chelate enables the hydrogen bonding to be maintained.

Thus, on the selective binding hypothesis, we could account for the greater stabilization of AT rich molecules either by a complex of Mg^{++} with adenine in native DNA, or a complex between Mg^{++} and guanine in denatured DNA. Both of these binding modes predict the liberation of protons on the binding of Mg^{++} by either native or denatured DNA. These models, however, are both somewhat unlikely because there is no large difference between the action of Mg^{++} and that of Co^{++}. The binding constant of Co^{++} for 8-hydroxyquinoline is greater than that of Mg^{++} by a factor of 10^6 (Bjerrum, et al, 1957).

2. Temperature dependence of the binding. The extent of binding decreases at high temperatures. Since GC rich DNAs denature at higher temperatures than do AT rich DNAs, they are less protected by Mg^{++} at their denaturation temperatures than are the lower melting AT rich DNAs.

The binding of Mg^{++} by phosphate has been shown to have positive enthalpy change in the temperature region 20° to 50° (Clarke, et al, 1954). If this is true in the binding by DNA, then the binding will increase with increasing temperature.

However, if the DNA case is different from that of the small ion pair formation, and exhibits a negative enthalpy change in the

region of 60° to 100°, the binding of Mg^{++} and Co^{++} will decrease with increasing temperature. This second hypothesis can be experimentally evaluated only by measuring the temperature dependence of binding at high temperature.

We are not able to decide on the basis of the available evidence whether either of these hypotheses are acceptable in explaining the selective stabilization by Mg^{++} and Co^{++} of AT rich DNAs. There are drawbacks to each hypothesis, but no strong evidence for or against either one.

H. The influence of Ag^{+} ion on the denaturation of DNA.

Experiments by T. Yamane (1961) indicate that Ag^{+} forms complexes with the heterocyclic bases in native DNA without irreversible denaturation. The first complex is saturated at $r = 0.2\ Ag^{+}$, and a second complex is saturated at $r = 0.5$. Moreover, Ag^{+} seems to bind GC rich DNAs more strongly than AT rich molecules. If Ag^{+} stabilizes native DNA, then one might expect a broadening of the transition due to its selective stabilization of already-stable GC rich regions.

It was found that at $r = 0.2$, Ag^{+} does indeed stabilize DNA at low ionic strength, as shown in figure 14. What is more, these are extremely broad transitions, with $\sigma_T = 11^{o}$, and our hope of finding broadening due to selective stabilization seems to be well founded. However, comparison of T_m at $r = 0.2\ Ag^{+}$ for different samples gives no significant change in $\frac{dT}{dX_{GC}}$, and the broadening must be due to some other cause. This will be discussed more with regard to our biological experiments, and certainly the nature of the interaction of DNA with Ag^{+} remains intriguing and incompletely studied.

I. Summary and discussion of denaturation results.

We have studied the denaturation of DNA samples in a number of systems, observing the relative stabilities of helix and coil and the breadth of the transition. Use of samples of different nucleotide

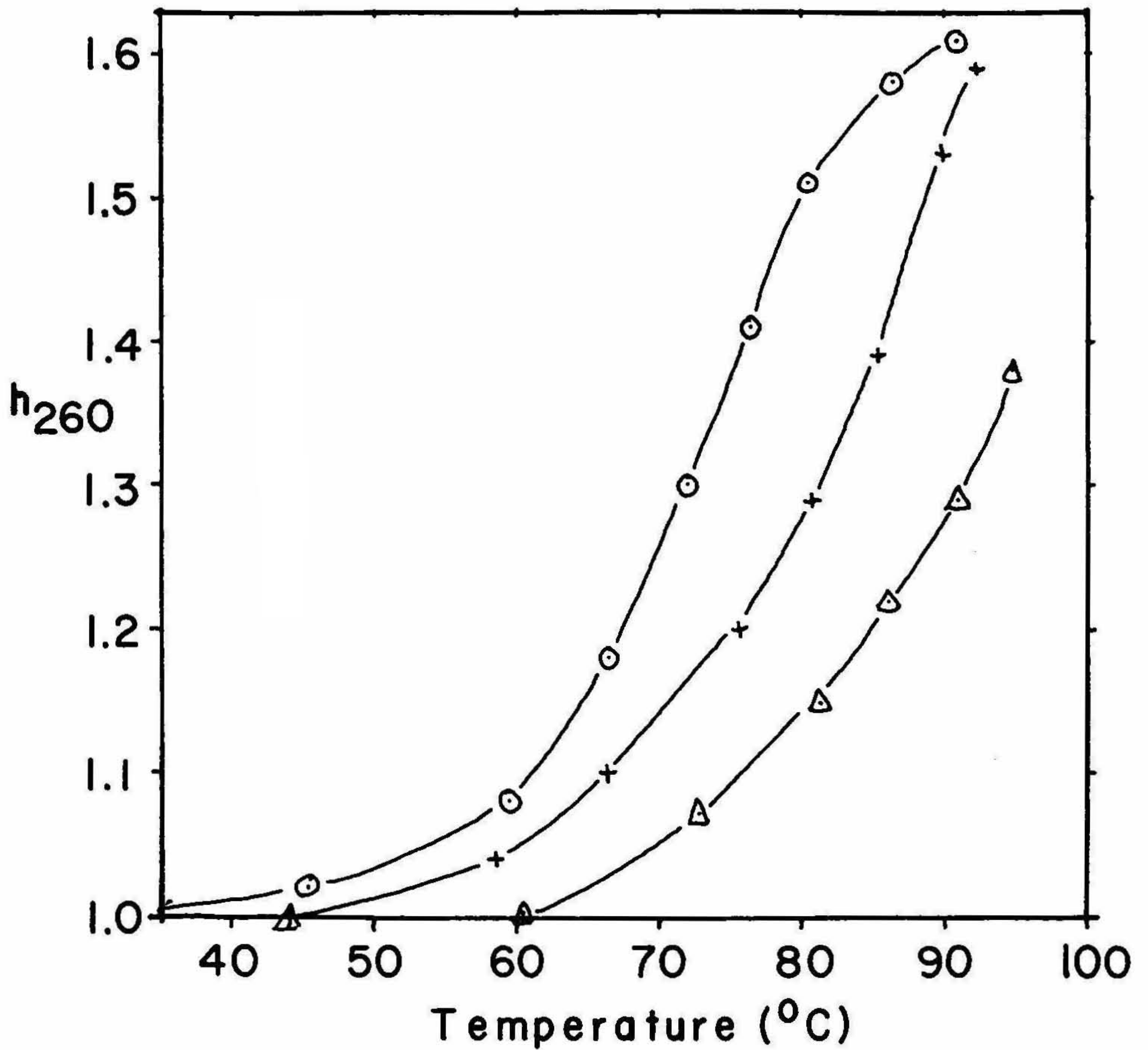


Figure 14. Thermal denaturation of DNAs of different composition at $3 \times 10^{-4} \mu$, r = 0.2 Ag^+, pH 7.

	X_{GC}	T_m	σ_T
⊙——⊙ B. megatherium	0.38	72.9	10.3
—+— E. coli	0.50	82.2	11.0
△——△ M. lysodeikticus	0.72	92.5	11.3

composition has enabled us to look for effects on the stability of DNA specific for base composition.

In pH denaturation, we have found no marked differences in sensitivity as a function of composition. We have found a change in $\frac{dpH}{dX_{GC}}$ for acid denaturation in going from 25° to 0°, and attribute this to participation of guanine protonation in denaturation -- participation which is more significant at 0° than at 25°.

Thermal denaturations at $0.1\ \mu$, at $3 \times 10^{-4}\ \mu$, and in the presence of $r = 0.2\ Ag^{+}$ all exhibit the same composition dependence. However, at $r = 1\ Mg^{++}$ or Co^{++}, AT rich DNAs seem to be stabilized more than GC rich DNAs. We have mentioned two sorts of explanation for this observation.

By none of these techniques, then, has there been a magnification of the difference in stability of AT and GC base pairs, and therefore none of them seems to be a promising attack on the molecular heterogeneity problem.

We have, however, found interesting properties of the intrinsic transition breadth. At low ionic strength in Na^{+} ion, at $r = 0.5\ Mg^{++}$ or Co^{++}, and at $r = 0.2\ Ag^{+}$, samples of bacterial DNA show broad transitions, which must be due to large intrinsic breadths. We have discussed two alternative explanations for this increased breadth at low ionic strength. One is a change in σ_{o} or σ_{j}, making the transition less cooperative. This allows more states of intermediate denaturation, thereby bringing internal heterogeneity into play. This internal heterogeneity may be in sequence, in completeness of pairing, or in the presence of single-strand breaks in the samples as they are prepared.

Secondly, the increased broadening may be due to the fact that native DNA electrostatically binds ions more strongly than does denatured. This is known to be true for Mg^{++} and Na^{+}, and is presumably true also for Co^{++}. However, recent experiments by Yamane (1961) indicate that this is not true for Ag^{+}, at least at $0.1\ \mu$. The Ag^{+} case may therefore be different.

We can make some calculations to decide on the plausibility of each hypothesis we have proposed to account for the increased transition breadth at low ionic strength. For the transition broadening to be due to a change in σ_o, we require a value of 0.1 for σ_o at low ionic strength, or 1 kcal/base pair for e. At high ionic strength, where we neglect electrostatic repulsions, e = 2 kcal/base pair. Therefore, we require a repulsive energy at low ionic strengths of about 1 kcal/base pair. In figure 3, if we assume the dielectric medium is not significantly changed in going from the loop to the free ends structure, we see that this energy arises only from repulsion between diagonal members of base pairs 2 and 3, since the distance between phosphate groups on the same chain does not change on denaturation. There are, of course, repulsions between such charges as on d and d' in structure II, and neglecting these makes our estimate of the repulsive energy difference too high.

We calculate, on the basis of the Watson-Crick model, that

$e_{rep} = \frac{q^2}{4 x 10 x 20 Å} = 0.4$ kcal/base pair, taking the phosphate groups as half shielded by sodium counterions (Inman and Jordan, 1960) and a dielectric constant as low as 10 for the inside of the molecule. Thus, even this generous estimate of the repulsive energies could account for only a part of the broadening observed at low ionic strength.

On the other hand, we find also that the broadening to be expected from the selective binding mechanism is also not nearly large enough to account for the observed effect.

The experiments on the denaturation of B. megatherium DNA at 0.1μ and at $3 x 10^{-4} \mu$ indicate that at low ionic strength the mid-point is 5° higher than it would have been had the denaturation begun at the same temperature as was observed at low ionic strength, but had the transition been as sharp as that at high ionic strength.

The experiments of Ascoli, et al (1961) indicate that the value of pNa changes by 0.3 upon thermal denaturation of sodium DNA by heat at a DNA concentration of $6.7 x 10^{-3}$. From the charge-fraction

data of Inman and Jordan, one can calculate binding constants for sodium ion which predict this result of Ascoli, et al. For native DNA, $K_f = 5 \times 10^3\ M^{-1}$; for denatured, $K_f = 5.6 \times 10^2\ M^{-1}$. Using these binding constants, one can see that for a DNA concentration of 1×10^{-4} and a sodium ion concentration of 3×10^{-4}, the free Na^+ ion concentration changes by about 5×10^{-5} on half-denaturation under these conditions. This change in free sodium ion concentration would produce a shift of the midpoint of only 1 or 2 degrees, compared to the 5 degree shift observed.

Thus, we cannot quantitatively account for the broadening of the denaturation transition by either of these two mechanisms by which the intrinsic transition breadth would be affected. In fact, the sum of the two effects is about half as great in magnitude as the effect observed.

It is possible that our approximations in calculating σ_o for the low ionic strength transition are entirely incorrect in that we have not considered the structure of the dielectric medium and the counter-ion distribution in detail around the loop and free-ends structure. To do this would be very difficult. In light of this, we cannot rule out the σ_o hypothesis in accounting for the broadening of the transition. We can, however, quite reasonably dismiss the selective binding hypothesis to account for the broadening in low concentrations of sodium ion.

It is more likely that it is a change in σ_j which accounts for the broadening observed at low ionic strength. As one lowers the ionic strength, denatured DNA becomes highly extended, and therefore this component decreases in entropy. Zimm calculates σ_j by the model of Jacobsen and Stockmayer, which compares the entropy of a loop to that of a random flight chain. This may become a poor model at low ionic strengths. We would expect σ_j to change in the direction which would account for the broader transition observed.

The broadening of the thermal denaturation transition begins to occur even at 10^{-2} ionic strength. This indicates that the broadening mechanism is indeed an electrostatic interaction affecting σ_o or σ_j.

In a homogeneous electrolyte solution, interactions extend over distances of the order of the Debye length, given by $K = 0.33\ \mu^{1/2} (\mathring{A})^{-1}$, at room temperature, in aqueous media. Thus, for $\mu = 1$, $K^{-1} = 3\ \mathring{A}$; for $\mu = 10^{-2}$, $K^{-1} = 30\ \mathring{A}$; and for $\mu = 10^{-4}$, $K^{-1} = 300\ \mathring{A}$. These Debye lengths are overestimated for the interactions within the polyelectrolyte, since there is binding of counterions to the macromolecule. In any case, we would not expect large electrostatic interactions between charges on a chain at $\mu = 1$, since the charges are separated by more than $3.4\ \mathring{A}$. We would expect charge interactions to become significant when the ionic strength becomes as low as 10^{-2}.

Our chemical experiments have therefore established that none of the systems studied enhanced the difference in stability between AT rich and GC rich DNAs. We have established that in systems containing non-saturating concentrations of interacting cations, very broad intrinsic transitions are observed. These broad transitions may be due to the selective binding of counterions by native DNA compared to denatured in the cases of non-saturating concentrations of Mg^{++} and Co^{++}, but at low concentrations of sodium ion, some other mechanism produces broadening; perhaps it is an effect upon Zimm's cooperative parameters σ_o or σ_j.

In looking for approaches to the problem of the heterogeneity of a single preparation of DNA, then, the most interesting systems to look at to date are those which produce broad intrinsic transitions. Even though no specificities based on gross composition are involved, these broad intrinsic transitions may elucidate features of the internal heterogeneity of individual molecules.

The means of observing individual molecules in a single preparation of DNA is to study the transforming activity of DNA prepared from a strain of D. pneumoniae which has several distinguishable genetic determinants. We shall discuss this technique and the experiments we have performed by means of it.

V. Results and Discussion: Biological experiments.

A. The assay.

Microbial transformation is a phenomenon whereby genetic information is transferred from one mutant of a species to another by purified DNA. It has been developed to date in four organisms, D. pneumoniae, B. subtilis, H. influenzae, and rhizobium. DNA is isolated from cells of genetic characteristic A. Cells lacking characteristic A are grown to a physiological state called competence in which they can irreversibly incorporate DNA molecules. DNA from A cells is added, and one finds a certain fraction of the recipient cells display characteristic A within a few generations. These transformed cells can pass on this characteristic to their progeny, and are hence genetically transformed.

By means of this phenomenon, one can assay a sample of DNA for its content of biologically active molecules. The interpretation of this assay rests on a number of experiments, and is discussed thoroughly in Appendix III. From our analysis of this assay, we must say that the current models of reaction are insufficient to account for the several experimental facts on the behavior of the assay curve and the binding of DNA. It is difficult to construct a more satisfactory model because of the lack of decisive experiments. For these reasons, we feel that we cannot draw any conclusions about the type of changes in the active molecules which accompany a loss in activity of a sample of DNA. We cannot decide whether the activity of a denatured sample consists of a fraction of molecules which have renatured, or instead, of all marked molecules. In this latter case, all the marked molecules would have either a reduced binding efficiency or a reduced efficiency of genetic incorporation once bound.

B. Transformation results.

Assays in the linear region of the response curve (ca. 0.07 μg/ml) were used to measure biological activities. Slow renaturation

processes did not seem to be taking place, since assays thirteen days apart gave results agreeing to within ±10% for samples stored at 4° in 0.2 M phosphate, pH 7.

1. $0.1\,\mu$ inactivation.

The experiments of Ginoza and Zimm (1961) indicate that the fast component of denaturation is essentially complete in fifteen minutes, and that the slow component has progressed very little in that time. Therefore, samples were placed in a constant temperature bath for fifteen minutes, and then aliquots were withdrawn and diluted into phosphate buffer. The inactivation curves in figure 15 were thereby obtained.

Sharp inactivation is observed for all three markers, with the average $\sigma_T = 0.8^{o}$. The inactivations of Roger and Hotchkiss indicated σ_T less than 0.4^{o} (the total uncertainty in temperature in their experiments), while those of Marmur and Lane have $\sigma_T = 1.5^{o}$, and Ginoza and Zimm have $\sigma_T = 0.6^{o}$.

2. Low μ inactivation.

The kinetics of inactivation at $3\times10^{-4}\,\mu$ at 40°, 60°, and 70° indicated that the slow component was negligible even at 70° over periods up to four hours, and so one can either withdraw samples from a constant temperature bath, or from a sample being taken through a heating curve. Both methods were used, and the inactivation curves in figure 16 were obtained, to be compared to the hyperchromicity curve also in figure 16.

The breadth of the inactivation curves is markedly increased over that at $0.1\,\mu$, with the average $\sigma_T = 11.3^{o}$. Apparently the separation between the midpoints of markers is also increased.

If we take the breadth of the inactivation of a single marker as a measure of the intrinsic breadth, then we can calculate the heterogeneity breadth of a particular sample. If this heterogeneity breadth

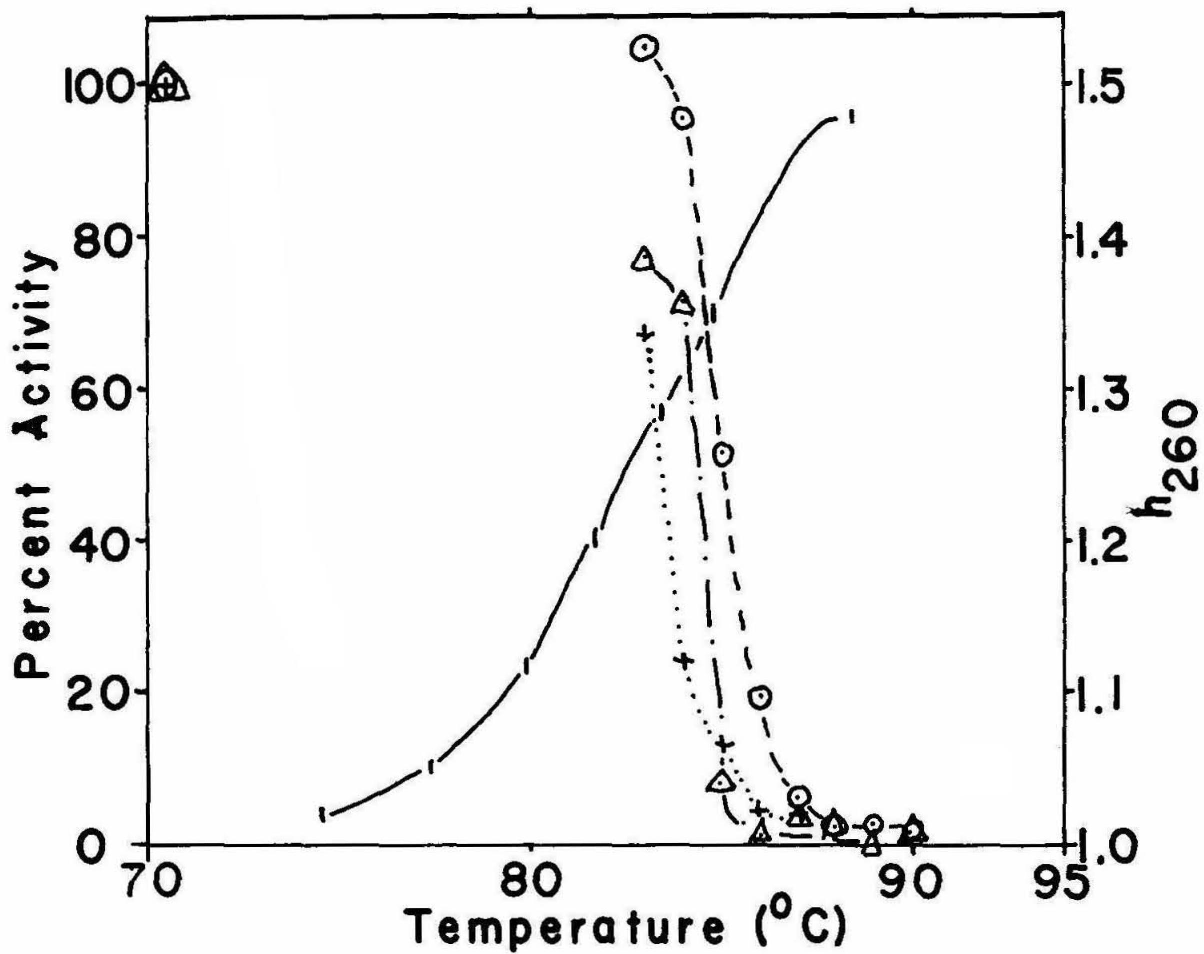


Figure 15. Inactivation of $S^rQ^bB^m$ transforming DNA at 0.1 μ, pH 7, compared to hyperchromicity.

	T_m	σ_T
—⊙- - -⊙ S^r	85.0	0.9
·····+···· Q^b	83.4	0.9
—△-·—△·- B^m	84.4	0.7
—ı——ı h_{260}	82.0	3.4

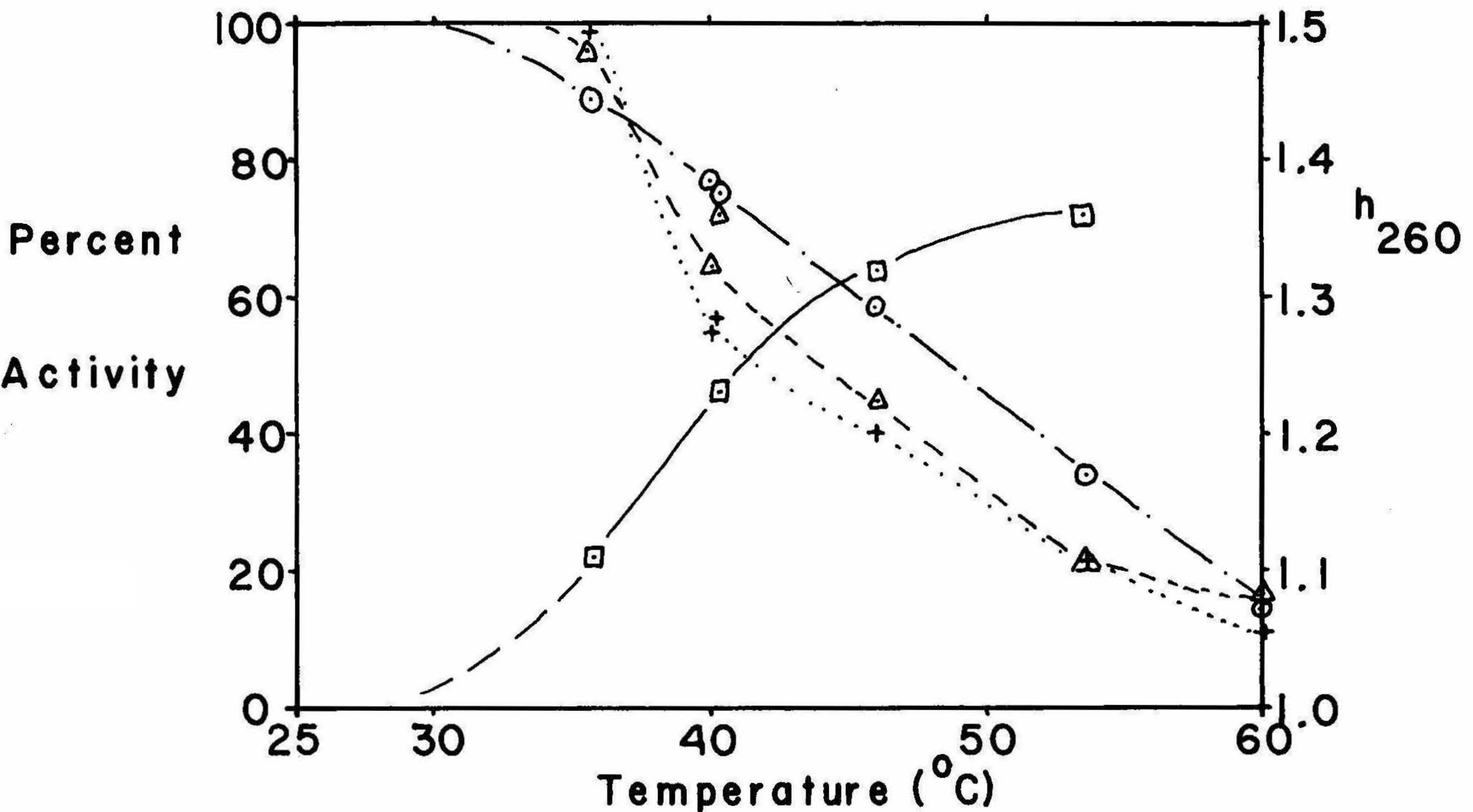


Figure 16. Inactivation of $S^rQ^bB^m$ DNA at $3 \times 10^{-4} \mu$, pH 7, compared to hyperchromicity.

	T_m	σ_T
S^r	47.5	12.1
Q^b	41.8	10.9
B^m	43.4	10.9
h_{260}	38.6	6.4

is due only to base composition, it should be independent of ionic strength by the results reported above.

Taking the high ionic strength result that $\sigma_{T, intr} = 0.8^o$ and $\sigma_{T, tot} = 2.8^o$, we calculate $\sigma_{T, het} = 2.7^o$. Here we have used the gaussian approximation. Taking this value for $\sigma_{T, het}$, and the low ionic strength result $\sigma_{T, tot} = 6.4^o$, we would expect that at low ionic strength we would find $\sigma_{T, intr} = 5.8^o$. The observed transition breadth for the inactivation of a single marker at low ionic strength is $\sigma_{T, intr} = 11.3^o$. This inactivation breadth is therefore unexpectedly large, and we must conclude that the loss of biological activity in low ionic strength denaturation is a different function of the total hyperchromicity profile than at high ionic strength. Reasons for this will be discussed presently.

3. Inactivation at low μ in the presence of $r = 0.2$ Ag^+.

The kinetics of inactivation in the presence of Ag^+ indicate a rather rapid slow component, precluding reliance on the technique of withdrawing aliquots during heating curves. Analysis of the fast component of the inactivation reaction shows that there is no magnification of differences between markers, and that the transition is rather broad. Thus, again we must conclude that Ag^+ produces broad transitions without any selectivity in stabilization.

The fact that the slow inactivation component is rather serious in the presence of Ag^+ suggests that the breadth observed may be, in part, due to this degradation. It is known that protonated DNA undergoes depurination more rapidly than does DNA in neutral solution (Thomas and Doty, 1957), and it may be that Ag^+ binds guanine and/or adenine in a way similar to protonation, thereby enhancing depurination by reducing the electron density of N-9. Experiments to test this hypothesis can be done.

4. Kinetics.

The kinetics of inactivation of DNA have been thoroughly

discussed by Guild and Ginoza (1961) and by Ginoza and Zimm. The slow inactivation seems to depend strongly on the mode of preparation, as DNA deproteinized by the Sevag treatment exhibits an early slow component compared to DNA deproteinized by phenol. Extrapolation of these results to 75° indicates that the inactivation observed with $r = 0.2$ Ag^+ is severe, but the proper control has not been done in this laboratory.

The kinetics of loss of activity under various conditions are shown in figure 17.

C. Discussion: Three antibiotic resistance markers in pneumococcus.

To relate the inactivation of the genetic markers in a sample with the hyperchromicity of the sample involves the question of recovery upon recooling. This recovery seems to be partially determined by the rate of cooling, by the concentration of the DNA, and by the ionic strength of the medium in which the cooling is done. In all our biological experiments, we have used a quenching technique whereby we dilute a small aliquot of heated sample in a large volume of room temperature phosphate buffer. The experiments of Doty, et al (1960) indicate that renaturation does not take place extensively at this temperature for samples heated far above the denaturation transition at high ionic strength.

Experiments by Marmur and Lane (1960) show that pneumococcal DNA behaves on fast cooling at 0.15 μ as shown in figure 18. Curve a represents the hyperchromicity measured at temperature T. Curve b represents the hyperchromicity measured at room temperature for a sample heated to T and then rapidly recooled. The midpoints of these two curves are different, with $T_m(a)$ 85° and $T_m(b)$ 88°.

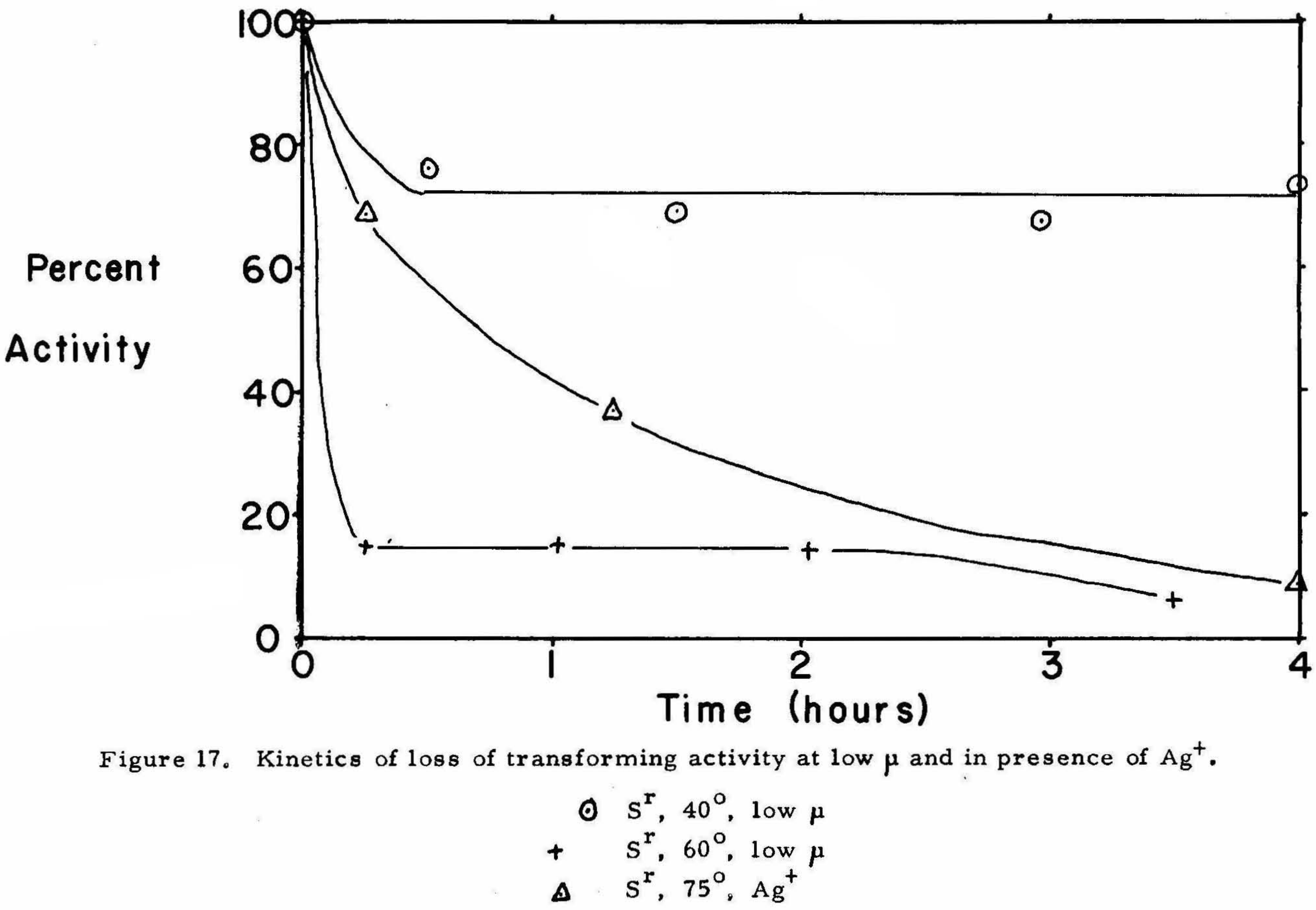


Figure 17. Kinetics of loss of transforming activity at low μ and in presence of Ag^+.

⊙ S^r, 40°, low μ

+ S^r, 60°, low μ

Δ S^r, 75°, Ag^+

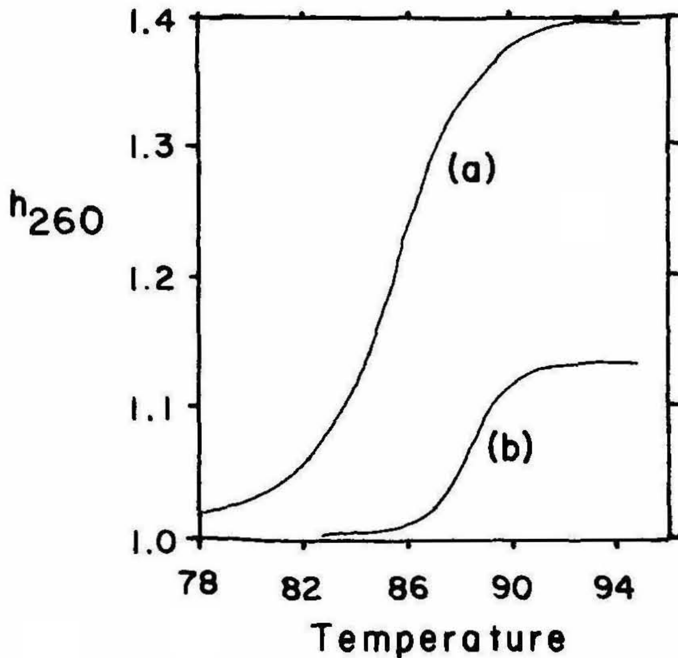


Figure 18. Hyperchromicity of pneumococcal DNA (Marmur and Lane);
(a) at elevated temperature T;
(b) at room temperature after recooling from T.

Since we have performed our high ionic strength experiments by fast cooling, it is reasonable to refer our denaturation results to the midpoint which would be observed for the recooled sample. The only difference between our recooling procedure and that of Marmur and Lane is that we withdraw an aliquot into a room temperature micropipet and dilute into buffer, whereas Marmur and Lane cool at high concentration by immersing the hot cell in water. We therefore take a T_m 3° above the midpoint observed in the heating curve as a reference for our inactivation experiments.

Experiments in this laboratory by Mr. T. T. Bopp and Mr. R. Jensen indicate, furthermore, that fast or slow cooling of samples denatured at low ionic strength results in very little recovery by optical

criteria. However, when concentrated $NaClO_4$ or r = 1 Mg^{++} is added to these irreversibly denatured samples, complete optical recovery is observed overnight. These recovered samples do not display the denaturation curves typical of native samples, showing some denaturation at temperatures below the expected transition region.

Thus, we might expect to find some sort of renaturation phenomenon in phosphate buffer even for samples fully denatured by optical criteria at low ionic strength.

The results presented in figures 15 and 16 are summarized in table 5. They indicate that there is a marked difference between the inactivation at high ionic strength and that at low ionic strength. At high ionic strength, the midpoint of each marker lies near the center of the irreversible hyperchromicity transition, and the breadths of inactivation are smaller than the total breadth measured optically. On the other hand, at low ionic strength the midpoint of the inactivation of each marker lies far above the midpoint of the hyperchromicity curve which would be obtained by heating to a given temperature and then recooling at low ionic strength. Furthermore, the breadths of inactivation are markedly larger than the total transition breadth measured optically. We shall discuss this further after discussing the differences observed between markers in their inactivation.

There is general agreement from denaturation data on the relative stabilities of the S^r and B^m markers. The density and stability of the Q^b marker, however, do not agree quantitatively, nor do they agree relative to other markers. We cannot press this point because we have no assurance that the B^d and B^m markers are the same, although they have the same antibiotic resistance threshold. The result could be accounted for, however, by stating that the Q^b marker has a larger cross-section for thermal inactivation than do the other markers. This explanation rests on the idea that states of intermediate denaturation exist, whereby some regions of molecules are melted and others are native. A marker would behave in trans-

Table 5. Observed denaturation differences between markers.

Marker	Reported here				Marmur & Lane	Ginoza & Zimm
	0.1 μ		3×10^{-4} μ		0.15 μ	0.15 μ
	T_m	σ_T	T_m	σ_T	T_m	T_m
S^r	85.0	0.9	47.5	12.1	89.0	90.5
Q^b	83.4	0.9	41.8	10.9	-	-
B^m	84.4	0.7	43.4	10.9	87.5	88.5
B^d	-	-	-	-	-	-
Hyperchr. (ambient)	82.2	3.4	38.6	6.4	85.5	-
Hyperchr. (recooled)	~85	-	~39	-	88.5	-

Table 6. Calculated compositional differences, $X_{GC} - \overline{X_{GC}}$, between markers.

Marker	Rolfe & Ephrussi-Taylor Molec. Density	vs. Recooled h. 0.1μ	vs. Recooled h. 3×10^{-4}μ	vs. Ambient h. 0.1 μ	vs. Ambient h. 3×10^{-4}μ
S^r	+0.016	0.000	+0.17	+0.053	+0.169
Q^b	+0.012	-0.030	+0.06	+0.023	+0.061
B^m	-	-0.011	+0.09	+0.042	+0.091
B^d	-	-	-	-	-

formation much less efficiently if a melted region were near it. It is known from ultraviolet inactivation data (Litman and Ephrussi-Taylor, 1961) that the cross-section for inactivation of Q^b is ten times that for S^r in this strain. Therefore, this explanation may be sensible.

Ignoring these possible size effects, we calculate the compositional differences tabulated in table 6. For high ionic strength denaturations, using the hyperchromicity for recooled samples as a reference, we find all markers denature with a midpoint at or below the mean. On the other hand, Rolfe and Ephrussi-Taylor find these markers all to have gross densities above the mean. A gross interpretation of this result is that the genetically active portion of each of these molecules is rich in AT. Another interpretation is that the genetically active molecules renature less readily than do the rest of the population renature by optical criteria. This second explanation seems plausible when we realize that optical renaturation may involve non-specific and also complementary, but denatured, unions (NCUD and CUD states), whereas genetic renaturation may require N states.

The compositional differences calculated from low ionic strength denaturations are far greater than those determined by molecular density and by high ionic strength denaturation. The compositions calculated are all far to the GC side of the total composition. This result as well as the marked broadening observed at low ionic strength will now be discussed in terms of renaturation.

At low ionic strength the broadening mechanism allows small helical regions to exist, regions which may be small enough to be very rich in GC. These small, GC rich regions remain helical at temperatures far above the midpoint of the transition and are nearly undetectable in their effect on the hyperchromicity of the sample. However, they are important in allowing extensive regions, if not whole molecules, to renature at room temperature when 0.2 F phosphate buffer is added.

We are thus measuring, by this interpretation, the melting out of the small, most stable regions of the molecules carrying each marker.

Quite understandably, the midpoints of the inactivation are far on the GC side.

We are thus postulating that, at low ionic strength, the reaction N ⟶ CUD may go at low temperatures, but the reaction CUD ⟶ SS requires much higher temperatures, because of the stabilization of the small helical regions in the CUD molecules, small regions which are very rich in GC and which are allowed to exist as helices by the mechanism which broadens the intrinsic transition.

This point of view accounts quite reasonably for the marked shift in the midpoint of the low ionic strength inactivation to much higher temperatures than is observed optically. The very marked breadth of the loss of transforming activity must be attributed, however, to some source of heterogeneity in this effect. This heterogeneity must be internal. It could consist of several regions which maintain helicity to different high temperatures. The eventual loss of one of these regions prevents the renaturation of one portion of the molecule, and hence the reduced biological activity of the entire molecule. A second possible cause of internal heterogeneity would be the existence of single strand breaks. The first type of internal heterogeneity would operate only if there exist renatured states in which part of a molecule is helical and part still denatured. The second type of internal heterogeneity results in "strand separation" for small regions, and does not have this requirement.

It seems likely, then, that the broad inactivation curves observed at low ionic strength are due to a renaturation phenomenon dependent on the presence of small regions which maintain their helicity to high temperatures. This interpretation rests on the statement that renaturation can take place extensively in our experiments if the strands have not separated.

Critical experiments to test these interpretations of the low ionic strength inactivation of markers do not readily suggest themselves. Both sources of heterogeneity in renaturability would predict that a recooled sample will contain molecules of varying content of denatured or of "stripped" regions, and that the transforming ability

of a molecule would decrease as its content of such regions increased. This prediction could be experimentally investigated, for example, by determining the transformation activity of a partially active sample as a function of density.

VI. Summary.

We have studied the denaturation of DNA in the presence of ions which interact with the macromolecule in different ways. We have studied the compositional specificity of these denaturations. We have then investigated the inactivation of three antibiotic resistance markers in pneumococcal transforming principle in some of these systems.

The binding of ions.

1. Cytosine is protonated extensively in the acid titration of DNA before irreversible denaturation occurs. The site of protonation, probably N-3, requires the breakage of hydrogen bonds, and yet no hyperchromicity is observed until irreversible changes take place.

2. The effect of increased Na^+ concentration on the acid denaturation of DNA is primarily to reduce the extent of protonation at any pH, rather than to stabilize the molecule at a given extent of protonation.

3. Magnesium, cobalt (II) and silver (I) ions are strongly bound to DNA. Magnesium and cobalt binding is saturated at one equivalent per phosphate. All three ions produce marked stabilization of the native conformation at low ionic strengths.

4. Sodium ion competes weakly with magnesium ion for binding to DNA, and is not quite as effective in stabilizing the native conformation once it is bound.

The transition midpoint.

1. There is cooperation between thermal energies and effects of titration in both the alkaline and acid regions in bringing about denaturation. Lower temperatures require more extensive protonation or more extensive deprotonation to produce denaturation.

2. There is cooperation between thermal energies and electrostatic energies in producing denaturation. These electrostatic energies are

affected by the binding of cations to DNA. The order of effectiveness in stabilizing DNA is $Na^+ \ll Co^{++} < Mg^{++} < Ag^+$.

The transition breadth.

1. Acid and alkaline denaturations have transition breadths of about 0.2 pH units under all conditions studied.

2. In the presence of limited concentrations of ions which are bound by DNA, broad transitions are observed. Compared to the transition breadth at 0.1 ionic strength, the breadths are twice as great at 3×10^{-4} μ in sodium ion, and at r = 0.5 Co^{++} or Mg^{++}. The breadths are four times as great at r = 0.2 Ag^+. These increased breadths are not attributable to an increased dependence of the denaturation temperature on base composition in these media. The broadening at low ionic strength in sodium ion is probably due to a change in the energetic or entropic cooperative factors σ_o or σ_j.

Compositional dependence of denaturation.

1. Alkaline denaturation at 2° and 20° and acid denaturation at 25° display the same composition dependence of the pH of denaturation. In all these cases, GC rich DNAs are more stable than AT rich DNAs. At 0°, the composition dependence of acid denaturation is reduced, and this is attributed to the increased importance of guanine protonation in the acid denaturation at 0°.

2. Thermal denaturation in the presence of sodium ion, either at high or low ionic strength, has the composition dependence reported by Marmur and Doty.

3. Thermal denaturation in the presence of r = 0.2 Ag^+ has essentially this same composition dependence.

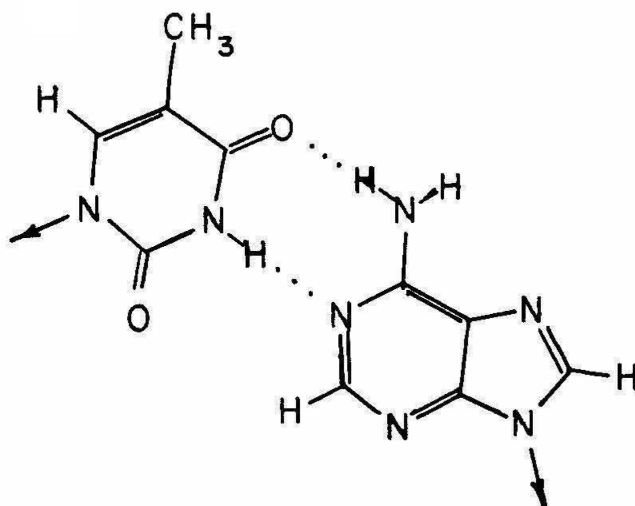
4. The stabilization of DNA by Mg^{++} and by Co^{++} at r = 1 at low ionic strength is greater for AT rich DNAs than for GC rich DNAs. This could be attributed either to selective binding or to a decrease in bind-

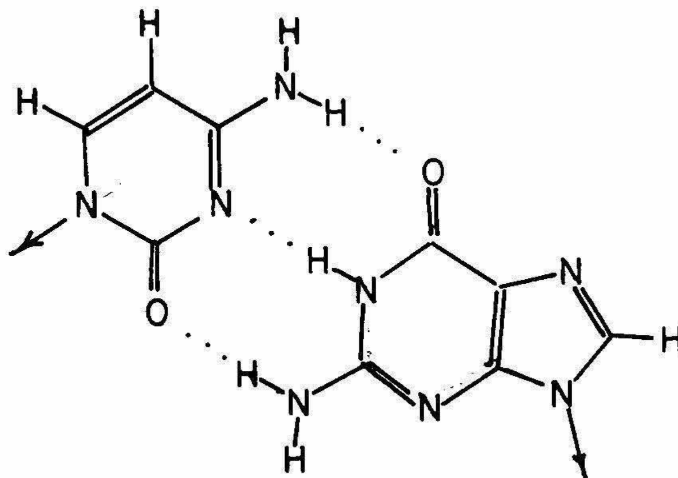
ing at higher temperatures.

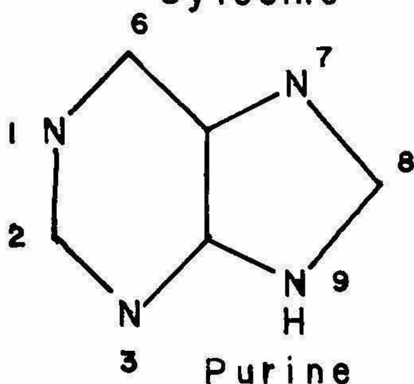
The inactivation of three markers in pneumococcal transforming principle.

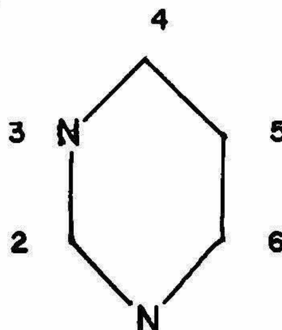
1. The inactivation temperatures of the S^r and B^m markers at 0.1 μ qualitatively agree with those reported by Marmur and Lane and by Ginoza and Zimm. With the inactivation temperature of the Q^b marker, they predict compositional differences between the molecules carrying these markers which are of the same magnitude as those determined by density-gradient techniques by Rolfe and Ephrussi-Taylor. The compositions determined by denaturation do not quantitatively agree, however, with the gross compositions. The relation between the inactivation of these markers and the optical changes in the entire sample indicates either that the genetically active portion of each molecule is rich in AT, or else that renaturation is more particular for genetic molecules than for others.

2. The low ionic strength inactivation of these markers displays a marked breadth and a shift to higher temperatures relative to the optical transition. This can most reasonably be interpreted as due to renaturation of molecules which have not completely denatured or strand-separated. The difference between high and low ionic strength results would then depend upon the operation of a transition broadening mechanism which allows small helical regions to exist at low ionic strength. The heterogeneity observed at low ionic strength inactivations may be either in the gradual loss of these persistent helical regions along a molecule, or else in the presence of single-strand breaks surrounding regions of varying ultimate stability.









Appendix I. The Watson-Crick base pairing, and the nucleotide numbering system.

APPENDIX II. Media used in transformation.

Agar medium.

Difco neopeptone	30 g.
Difco yeast extract	12 g.
NaCl (Reagent)	25.5g.

Dissolve in two liters of distilled water, neutralize with NaOH to pH 7.0 to 7.4, simmer, filter off precipitate of phosphates, and add one liter more distilled water. Make 1.5% bactoagar, autoclave 20 min at 120°. For use, make 0.375% glucose.

Culture of large batches. Same composition and preparation as above (without agar), but with twice as much yeast extract. Supplement with 1:100 fresh yeast extract (*vide infra*).

P medium: For culture of strains and for precultures in experiments.

Difco neopeptone	5. g.
NaCl (Reagent)	4.2g.

Dissolve in 500 ml distilled water, autoclave at 120° for 20 minutes. For use, to each 100 ml add 0.1 ml 1 F NaOH, 0.125 ml 10% glucose, and 8 ml sterile charcoal-absorbed yeast extract (*vide infra*). For culture of strains, use 5 ml in tubes with 0.1 ml sterile rabbit blood*, incubate 1:20 inoculum about 2 hours.

NS medium: For competence cultures and DNA reaction solutions.

Difco neopeptone	30 g.
Na_2HPO_4 (Reagent)	19.9 g.
$CaCl_2$ (Reagent)	100 mg.
KCl (Reagent)	9 g.

Dissolve in 2.5 liters distilled water, acidify with HCl to pH 3.4, and add 15 g. Norite A in five portions, stirring with each addition. Allow to stand in the cold for 2 hours, then filter to remove charcoal. Reneutralize to pH 7.8 with NaOH, remove the precipitate by filtration, autoclave for 10 minutes and filter the new precipitate immediately. Complete the volume to 3 liters and sterilize

*The blood is sterile, not the rabbit.

at 120° for 20 minutes.

For use, to each 300 ml add 24 ml charcoal-absorbed yeast extract and 0.75 ml 10% glucose. Supplement with 1:100 fresh yeast extract.

Albumin solutions: To add to competence cultures.

Make up 4% solutions of Armour Bovine Albumin, Fraction 5, List 2290. Bring to pH 2.5. Pipette into a sterile flask, put in 100° water bath for 18 minutes, then cool and store. For use, adjust pH to 7.4.

Fresh yeast extract: To supplement cultures, especially those of aminopterin resistant mutant.

Boil 1.5 liters of distilled water, pour over 1 lb Baker's yeast, heat for 5 minutes, then cool rapidly. Centrifuge and filter to separate residue, then sterilize by filtration.

Difco yeast extract, absorbed on charcoal: For P and NS media.

Difco yeast extract 50 g.

Dissolve in 450 ml distilled water, bring pH to 3.0 to 3.2 with HCl, add 5 g. Norite A in 3 portions, filter. Neutralize to pH 7.8 with NaOH, remove precipitate by filtration, bring to 500 ml volume, and sterilize by filtration.

APPENDIX III. The Michaelis-Menton model applied to the transformation assay.

We should like to discuss accurately the assay for the concentration of biologically active DNA by transformation. If one has a sample of DNA isolated from A, called DNA (A), and performs the transformation experiment with varying concentrations of DNA (A) at constant bacterial concentration, he finds typically that at low concentrations of DNA, the number of transformants is proportional to the concentration of DNA (A). However, at high DNA concentrations, the number of transformants reaches a limiting value independent of the DNA concentration. Hotchkiss (1957) found that addition of inactive thymus DNA lowered the number of transformants at this plateau, and that the plateau seemed therefore to be due to a competition for bacterial binding sites by all DNA molecules. At lower DNA concentrations, the sites are not saturated and active molecules are not excluded by competition.

When transforming DNA is inactivated by such treatment as thermal denaturation, it is found to have reduced activity in both the linear and plateau regions (Roger and Hotchkiss, 1961), and also to be bound less extensively by the cell at plateau concentrations (Lerman and Tolmach, 1958). Both of these reports contradict the model of the transformation system proposed by Hotchkiss, as we shall show.

Fox (1957) determined that in his most active preparations of pneumococcal DNA, 2.1×10^{-15} g. of DNA was irreversibly fixed for each transformant obtained. Less active samples required fixation of as much as 1.2×10^{-14} g. to give one transformant. Since the DNA content of this strain of pneumococcus is 1.5×10^{-15} g., and we assume that a particular determinant occurs only once per cell, then at maximum efficiency a marker gives rise to a transformant more than 50% of the times that it is fixed to a cell. Goodgal and Herriott (1956) found similar efficiency of transformation in _H. influenzae_.

Lerman and Tolmach (1957) determined that the number of cells transformed is proportional to the amount of DNA irreversibly bound.

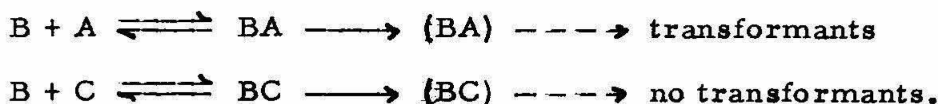
Therefore, it may well be that inactivation results in reduced activity simply because molecules can be fixed by cells less efficiently. Lerman and Tolmach (1958) have measured both P^{32} incorporation and transforming activity allegedly at plateau concentrations (however, vide infra) for pneumococcal DNA. At temperatures just below T_m for marker inactivation, loss in activity is several times as great as loss in incorporation. Above T_m, the losses in activity and incorporation are comparable -- at least for the fast component of the reaction.

Thus, it seems that the reduced activity of heated DNA is in part due to reduced binding efficiency and also due to reduced efficiency in producing a transformant once bound. Just before T_m, reduction in genetic efficiency must be the important factor, while above T_m, both genetic and binding efficiency are reduced. Our interpretation of Lerman and Tolmach's experiments rests on the hypothesis that competition occurs only in binding, and not in genetic incorporation. This seems likely when we remember Fox's result that the binding of an entire bacterial complement of DNA to a cell usually results in a transformant.

Cavalieri and Rosenberg (1958) have written out a complete analysis of the transformation assay on the model proposed by Hotchkiss -- a model similar to that of Michaelis-Menton kinetics. These authors include differences in binding ability between active and competing molecules, and the molecular weight dependence of binding, which goes to zero as the molecular weight approaches 1×10^6 (Litt, Marmur, Ephrussi-Taylor, and Doty, 1958).

We should like to discuss the Hotchkiss model in simple detail for inactivation of DNA, ignoring molecular weight effects. Consider a sample of transforming DNA, homogeneous in molecular weight and in binding ability. Let X_A be the mol fraction of genetically marked DNA, X_C the mol fraction of competing DNA, and $X_I = 1 - X_A - X_C$ the mol fraction of inert material. D is the total DNA concentration. Take g_A to be the probability that an A molecule will give a transform-

ant once irreversibly fixed to the cell, with $g_A = 1$ for native samples. Take E_A and E_C to be the binding efficiencies of A molecules and competing molecules, respectively, with $E_A = E_C = 1$ for native samples. The reaction scheme is



$$B + A \rightleftharpoons BA \longrightarrow (BA) \dashrightarrow \text{transformants}$$

$$B + C \rightleftharpoons BC \longrightarrow (BC) \dashrightarrow \text{no transformants.}$$

If the number of transformants is proportional to the velocity of the reactions producing (BA), since each assay is run for a fixed time, then the number of transformants is given by

$$T = \frac{g_A X_A E_A (D)}{1 + E_A X_A (D) + E_C X_C (D)} ,$$

and the incorporation of P^{32} is given by

$$I = \frac{(D)(X_A E_A + X_C E_C)}{1 + (D)(X_A E_A + X_C E_C)} .$$

Remembering that X_A is much greater than X_C for an unfractionated sample, we can write the low DNA concentration (linear region) and high DNA concentration (plateau region) limits for transformants and for incorporation of P^{32}.

Linear region	Plateau region
$T = g_A E_A (A) = g_A E_A X_A (D)$	$T = \frac{g_A E_A X_A}{E_C X_C}$
$I = E_C (C) = E_C X_C (D)$	$I = 1$

Inspection of the behavior of these equations indicates that the following conditions must be true if this model is applicable:

1. If the ratio of activities in two samples is the same in plateau and in linear regions (as reported for heat inactivation by Roger and Hotchkiss, 1961), then there must be no change in incorpora-

tion of P^{32} in the linear region. This ratio is $\frac{1}{G_C X_C} = \frac{(D)}{I}$.

2. The converse of 1 is true.

3. There is never a change in incorporation of P^{32} in the plateau region.

4. One cannot distinguish in the inactivation of a marker between
   (a) decrease in g_A alone
   (b) selective decrease in X_A alone
   (c) selective decrease in E_A alone;

and these are the only changes which will allow the result of Roger and Hotchkiss to hold.

5. If we accept Lerman and Tolmach's data on binding of P^{32} we must first state that it was really obtained away from the plateau region (the DNA concentrations used are not stated explicitly), and then we must say that there is no way to account for Roger and Hotchkiss' results. As pointed out by Roger and Hotchkiss, we cannot say that the residually active DNA is renatured.

We must then say that the transformation assay does not allow us to make any conclusions about the type of changes in the active molecules which have altered the activity of the sample. It is probable that the model discussed here is incomplete or incorrect, and it is difficult to construct a more satisfactory one because of the lack of decisive experiments.

APPENDIX IV. Glossary of terms.

Abbreviations:

pNa	$-\log_{10}(Na^+)$
pH_F	$-\log_{10}(H^+)_F$
T_m	temperature of the midpoint of the transition
X_{GC}	mol fraction (guanine and cytosine).

AT polymer: a high molecular weight polymer synthesized by the DNA polymerase of Kornberg in the absence of primer. Though to have the Watson-Crick structure, with sequence A-T-A-T-A- - -.

Autolysis: the lysis of a culture of bacteria at a certain state of growth without any known outside influences.

Bacteriophage: a nucleoprotein particle which can attack susceptible bacteria, multiply in the bacterial cell, and cause its lysis; that is, a bacterial virus.

Biological activity: for transforming DNA, the number of transformants produced per unit weight of DNA in the linear region of the assay.

Boltzman factor: the Boltzman factor of an energy e is the exponential of $\frac{e}{kT}$, where k is the Boltzman constant.

Coil (random): a macromolecule consisting of many end-to-end segments whose joints are universal.

Competence: the transient physiological state of transformable bacteria in which they can incorporate DNA and be transformed.

Complementarity: the perfect pairing between two polynucleotide chains satisfying the Watson-Crick base pairing system.

Conformation: one of the secondary structures of a macromolecule.

Cooperative phenomenon: a process in which interaction energies between molecules are more important than the self energies of molecules.

Depurination: the loss of purines from D NA by breakage of the N-9 gly-

cosidic bond. Polymeric apurinic acid is formed.

Ecteola cellulose: cellulose esterified with weakly basic groups which can be changed positively for chromatography of the DNA polyanion.

Gene: in the simple view, the segment of DNA carrying the information required by a cell for a particular function; a marker.

Histone: a protein found predominantly in nucleoproteins containing large amounts of the dibasic amino acid lysine.

Hypochromicity: the reduction of formal extinction coefficient of a substance without a large shift in wavelength of maximum absorption or in spectral form.

Hyperchromicity: increase of formal extinction coefficient.

Marker: see gene.

Normal probability integral: the integral of the normal probability curve, having values from 0 to 1.

Phenotypic expression: the transfer of genetic information in a cell into the operation of the functions requiring it.

Protoplast: A cell which has lost its cell wall and is contained only by a cytoplasmic membrane.

Sevag treatment: deproteinization of a preparation of DNA by shaking with chloroform and amyl alcohol.

Titer: the concentration of organisms in a culture, per ml.

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IX. Propositions.

I should like to point out that many of the ideas contained in these propositions cannot be simply attributed to me, because they were developed in discussions with several people, including N. Davidson, J. Vinograd, P. Ts'o, J. D. Smith, A. Roller, J. Weigle, D. Wulff, R. Stewart, and R. Jensen.

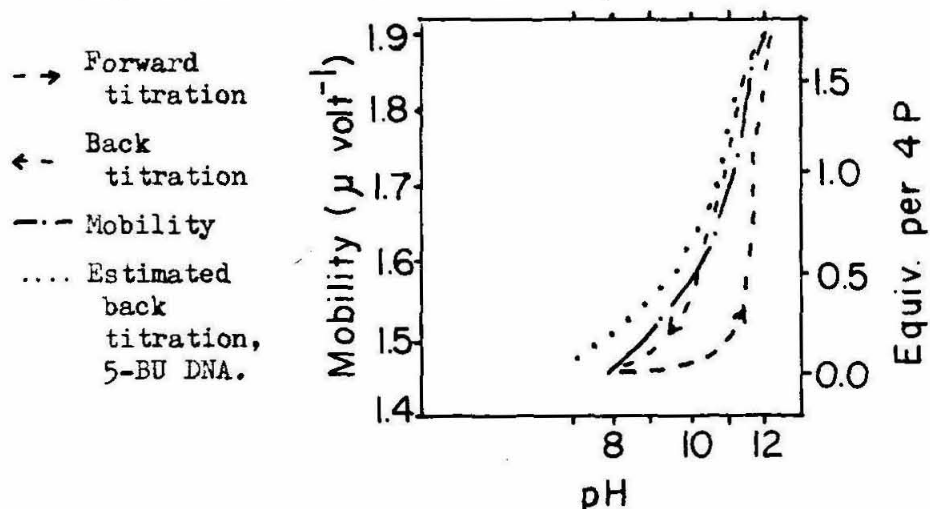
<u>Proposition 1</u>. The separation of single-stranded polynucleotides by electrophoretic mobility in the alkaline region should permit: (i) the isolation of the complementary strands of ØX DNA and its replicating factor, and any other pair of complementary strands suitably biased in thymine or guanine content; (ii) the rapid separation of 5-bromouracil labelled subunits of hybrid DNA, enabling one to approach the analysis of the kinetics of subunit (strand) separation.

DNA is a polyanion at neutral pH, carrying one negative charge on each phosphate group and about 10^4 phosphate groups per molecule. The negative charges are about half neutralized at 0.1 ionic strength, according to Jordan (1960). DNA also contains two nucleotides out of every four which are titrable in the alkaline region, namely thymine and guanine. In the monomeric state, these nucleotides have pK_a's near 10. We see in figure A (Creeth, et al., 1949) that the effective pK_a of the polymer is near 11. The experiments on the alkaline denaturation of DNA indicate that above pH 11 the secondary bonding between the strands of the double helix is irreversibly broken, and presumably the strands separate in the time required for them to unwind. This time is a sensitive function of the length of the strands, and may be as long as five

minutes for viral DNAs. (Longuet-Higgins and Zimm, 1960.)

In the alkaline region, we see in figure A that DNA has a particularly high mobility. Furthermore, it has been reported that monomeric 5-bromouracil has a pK_a 2 units below that of thymine. This analogue may be completely substituted for thymine in some viral DNAs. (Meselson, unpublished work.) We estimate then that DNA (denatured) containing 5-bromouracil instead of thymine would show the dependence of electrophoretic mobility on pH sketched in figure A.

Figure A. Titration and mobility of DNA in alkaline region.



We propose to adapt the DNA electrophoretic technique which is being developed in this laboratory, and has been investigated by others. (See Jordan, 1960.) We want to attack two problems:

(i). <u>The separation of complementary strands</u>. In a duplex DNA molecule, the Watson-Crick requirement does not set the mol fraction of one particular nucleotide in one strand equal to the mol fraction of that nucleotide in the other. Indeed in the one known case where one can determine the base compositions of strands 1 and 2, there is marked asymmetry. This case is that of the bacteriophage ØX174, which contains a single-stranded DNA molecule whose complement seems to exist in the

bacterial cell which replicates the phage. (Sinsheimer, 1959, and unpublished work.) The composition of ØX strands allows us to calculate the composition of its complement, replicating factor (RF).

	Adenine	Thymine	Guanine	Cytosine
ØX	1.00	1.33	0.98	0.75
RF	1.33	1.00	0.75	0.98

We see that for ØX, Thymine + Guanine = 2.31, while for RF, Thymine + Guanine = 1.75. Thus, at full alkaline titration, we predict a difference in mobility of 0.11 μ/sec//volt/cm, a difference which allows easy separation. It may well be that there exist other DNAs of similar bias in thymine or guanine content. For such cases, the experiment proposed here is at an advantage compared to other ways which might be suggested for separating the complements. Complement separation would allow us to answer several questions of current interest in molecular biology, but we shall not dwell on them here.

(ii). Subunit separation by bromouracil substitution. In an experiment where one changes the isotopic composition of the medium in which bacteria are dividing, one finds after one generation that all the molecules of DNA are isotopic hybrids - with one subunit old and one new. (Meselson and Stahl, 1958.) These subunits may well be the single strands of the Watson-Crick double helix. One can also make a hybrid with bromouracil in one subunit and thymine in the other. Degradation of such substituted DNAs has been reported for thermal denaturation, but does not occur for formamide denaturation.

We propose to separate the subunits after such a denaturation by electrophoresis around pH 10. From the estimated behavior of substituted DNA in figure A, we predict a difference of 10% in the mobilities of the two subunits. If we can resolve bands separated by 2 mm., we calculate

that we can observe separation in 1 minute at a field of 100 volts/cm. To achieve a field this high at 10^{-2} ionic strength, allowing the cell to rise only 1 degree above the cooling bath, we calculate that we require an electrophoresis cell tube-shaped and of the order of 30 μ in diameter.

Thus, by electrophoresis in the alkaline region, we can easily separate the complementary strands of DNAs biased in thymine or guanine content, and we can, with difficulty, approach the kinetics of the separation of the subunits of a thymine-bromouracil hybrid molecule.

Proposition 2. A model is proposed for the pneumococcal transformation system, maintaining the kinetic model of Hotchkiss (1957) but introducing the hypothesis that the number of binding sites available to denatured DNA is less than the number available to native.

An experimental approach is suggested for the evaluation of this model, an approach which allows one to determine changes in binding efficiency, in number of available sites, and in efficiency of genetic incorporation.

With this information available, it is proposed furthermore to determine the efficiency of genetic incorporation for DNA molecules whose strands have been covalently linked together.

In Appendix III of this thesis we have given a discussion of the transformation assay system, indicating the dilemmas we feel to be unresolved in this field. The discussion here must hinge on that in the interests of brevity.

An interpretation of the transformation assay system. We begin by

accepting two unconfirmed experimental facts: that plateau binding is less for denatured DNA than for native (Lerman and Tolmach, 1959); and that the relative activity T for a sample is the same in linear and plateau regions (Roger and Hotchkiss, 1961). Letuus also use the kinetic scheme proposed by Hotchkiss (1957), modifying it only by considering differences in available binding sites.

We first postulate that, if S_n is the number of sites available to native DNA in a competence culture, then denatured DNA of relative activity T can be bound by only $S(T) = K_b S_n T$ sites. For example, a sample with only 10% activity may have only 10% as many available binding sites, with $K_b = 1$. Perhaps it has 20% as many binding sites, with $K_b=2$. In this case, it is only active at 10% because $g = 0.5$, where g is the efficiency of genetic incorporation once irreversibly bound. Clearly, $K_b = g^{-1}$.

Secondly, we postulate that the binding efficiency for denatured DNA (its Michaelis constant in Hotchkiss' model) is lower than that for native, accounting for the reduced activity in the region of low DNA concentrations where the number of available sites is unimportant. We write for a sample of activity T that the binding efficiency is given by $E(T) = LE_n T$, where E_n is the binding efficiency for native molecules. Again, a sample with 10% activity may be only 10% as efficient at irreversibly binding to a cell, with $L = 1$; or, it may have a binding efficiency of 20% and $g = 0.5$. Here $L = g^{-1}$.

Our model is testable on two grounds. First, it predicts that S(T) and E(T) decrease monotonically as T decreases. Secondly, it predicts that $L = K_b$. This second prediction would not hold if there were competition on the level of genetic incorporation, competition which

would make g in the linear region unequal to g in the plateau region.

The experimental testing of this model. Recent experiments by Goodgal and Herriott (1961) on the transformation of hemophilus offer some evidence that not all the cells in a competence culture irreversibly bind DNA. They calculate that between 2% and 75% of the cells bind DNA irreversibly, on the basis of the discrepancy between the predicted and observed numbers of double transformants. To determine the distribution of bound molecules over the population of cells would be difficult if not prohibitive. We can, however, evaluate our model by measuring changes in binding at low and at high DNA concentrations, as begun by Lerman and Tolmach. From the low concentration result, one can then unambiguously determine g.

The determination of g for strand-linked DNA. We know now how to determine g, where g is the relative probablity that a molecule irreversibly bound to the cell will produce a transformant. It would be very interesting to determine this probability for a DNA molecule whose complementary strands were linked together by some strong bond, thereby preventing strand separation which may be a necessary part of replication. Genetic expression from this molecule would have to take place either by breakage or else by replication not involving strand separation.

Marmur and Grossman (1961) have evidence that extensive ultraviolet irradiation reduces the transforming activity of DNA to 1% and produces strong links between the subunits of hybrid DNA. Geiduschek (1961) also reports the formation of cross-links, with nitrous acid treatment or by reaction with a bifunctional mustard "HN-2." His nitrous acid treatment corresponds to 1% transforming activity also (Litman and Ephrussi-Taylor, 1959). The 1% activity in such samples may reside in molecules

which have not been strand-linked. We propose to determine g for strand-linked molecules in the following way:

One thermally denatures a sample of transforming DNA, bands it in the density gradient, and measures its specific g as a function of density. One then treats a sample of transforming DNA with a cross-linking agent. This sample is thermally denatured in the same way as in the control, and also banded in the density gradient. Again one determines g as a function of density. Molecules which are strand-linked have been shown to renature readily, and so one can assign an increase in activity in the native density region to strand-linked molecules.

In summary, we have proposed an analysis of the transformation assay, and an experimental evaluation of this analysis. If one can understand the assay in sufficient detail, he can determine the efficiency of genetic incorporation for strand-linked DNA, an experiment that may be difficult but is also interesting.

<u>Proposition 3</u>. Forbes and Templeton (1957, 1958) have reported that the 2300 Å absorption band of benzoic acid does not obey Beer's law in cyclohexane solution. The formal extinction coefficient increases with increasing concentration with a small red shift in the absorption maximum. These workers attribute this effect to dimer formation.

We propose that this is so and that it can be explained on the coupled oscillator model of Tinoco (1960, 1961). We predict that this effect should be peculiar to dimers which are oriented in the rigid way required in benzoic acid dimers. Order-of-magnitude calculations indicate that this is weak to intermediate coupling by the criterion of Simpson and Peterson (1957) and that a 50% change in formal extinction

coefficient is to be expected on dimerization. It is suggested that the existence of this coupling and the long life of the dimer in solution make this system an interesting and fairly simple system for the study of energy transfer between molecules in solution.

The data of Forbes and Templeton does not include the precise concentrations of benzoic acid for the dilution series they report. Furthermore, their reported formal extinction coefficients do not agree with that reported in Landolt and Bornstein (1951). We can only estimate the reference concentration within a factor of two from their data. From this estimate, we can use the equilibrium constant for dimer formation measured in CCl_4 by Maier and Rudolph (1957): $K_f = 1 \times 10^5\ M^{-1}$. We calculate the concentrations of monomer and dimer in the measurements of Forbes and Templeton.

conc. (F)	ϵ_F(obs)$\times 10^{-3}$	λ(max) Å	(Dimer)	(Monomer)
1×10^{-5}	11.9	2280	3.3×10^{-6}M	3.3×10^{-6} M
4×10^{-5}	12.9	2300	1.8×10^{-5}	4.4×10^{-6}
4×10^{-4}	13.7	2310-20	2.0×10^{-4}	4.0×10^{-6}

Taking 13.7×10^3 as the formal extinction coefficient for the dimer, we calculate from the low concentration result that the monomer has an extinction coefficient of 8.3×10^3. This of course rests on the assumptions that we have chosen the correct equilibrium constant and estimated the reference concentration well.

Tinoco has shown how the weak coupling of oriented oscillators can produce changes in intensity of absorption in electronic bands without large shifts in the energy of the transition. Tinoco uses a point dipole approximation in calculating the interaction between two chromophores. We have decided that one cannot place this point-dipole at the center

of gravity of the electronic charge distribution, since the two chromophores are separated by a distance less than the dimensions of their π-electron systems. We have arbitrarily taken the equivalent point dipoles at the positions shown in figure B, 5 Å apart. This step must be considered quite approximate but necessary at this extent of our knowledge.

To calculate the expected change in intensity of absorption for the benzoic acid dimer, we first note that the resonance between the carboxyl group and the aromatic ring prevents rotation about the ring-to-carboxyl bond, and the tandem hydrogen bond formation shown to exist in the crystal structure of benzoic acid prevents rotation of one monomer with respect to the other. Secondly, we state that the 2300 Å transition in benzoic acid is a π-π^* transition, the counterpart of the 2040 Å transition in benzene. We expect a second π-π^* transition with a maximum at 2040 Å in benzoic acid, the counterpart of the strong 1840 Å band in benzene. It is common to find the transition directions of two such bands not only in the plane of the ring but also perpendicular to one another. We have taken the transition direction of the 2300 Å band along the axis of the dimer, and that of the 2040 Å band perpendicular to that axis. Integration of the absorption spectrum of benzoic acid in Landolt and Bornstein gives an f-number for the 2300 Å band of 0.33. On the basis of a series of benzene derivatives studied by Platt and Klevens (1948) we take an f-number for the 2040 Å band of 1.0.

We can calculate the dipole-dipole interaction energy for our 5 Å separation (Pitzer, 1953). This gives a value of 2400 cm^{-1}, somewhat less than the vibronic halfwidth of the 2300 Å band, which is 3500 cm^{-1}. This result, by the criterion of Simpson and Peterson, assures us that

the coupling is weak or intermediate, and that we can use Tinoco's treatment for the change in intensity.

The magnitude of the change in intensity due to the weak coupling of oscillators is given by

$$\frac{F_{oa}}{f_{oa}} = 1 - 4K\lambda_{oa}^2 \frac{G_{ij}e_i \circ e_j f_{oa'}\lambda_{oa'}^2}{\lambda_{oa}^2 - \lambda_{oa'}^2} ,$$

where K involves universal constants, F_{oa} is the oscillator strength for the dimer, f_{oa} that for the monomer, oa is the 2300 Å transition, oa' the 2040 Å transition, and G_{ij} the dipole interaction quantity given by

$$G_{ij} = \left[\hat{e}_i \circ \hat{e}_j - \frac{3(\hat{e}_i \circ \vec{r}_{ij})(\hat{e}_j \circ \vec{r}_{ij})}{r_{ij}^3} \right] \frac{1}{r_{ij}^3}$$

, where i and j refer to the two chromophores and the ê's are unit vectors in the direction of the transition moments.

We calculate a value of $\frac{F_{oa}}{f_{oa}} = 1.22$ on our model. From our analysis of the data of Forbes and Templeton, we have a value of 1.65, and so we are encouraged that Tinoco's treatment can account for the order of magnitude of the increase in formal extinction coefficient of benzoic acid on dimerization.

The apparent existence of coupling between the two chromophores in this dimer suggests that it may be possible to find efficient energy transfer in solution when dimers are formed between benzoic acid and another aromatic carboxylic acid. The rate of dissociation of the dimer in CCl_4 at 20° is 5×10^6 sec^{-1} (Maier and Rudolph). The low-lying triplet states of benzoic acid have been reported by Porter and Windsor (1958).

In summary, we propose that the change in formal extinction coefficient on dimerization of benzoic acid can be explained by weak coupling between the chromophores. This system might offer a simple model for such coupling and for the study of the energy transfer it permits.

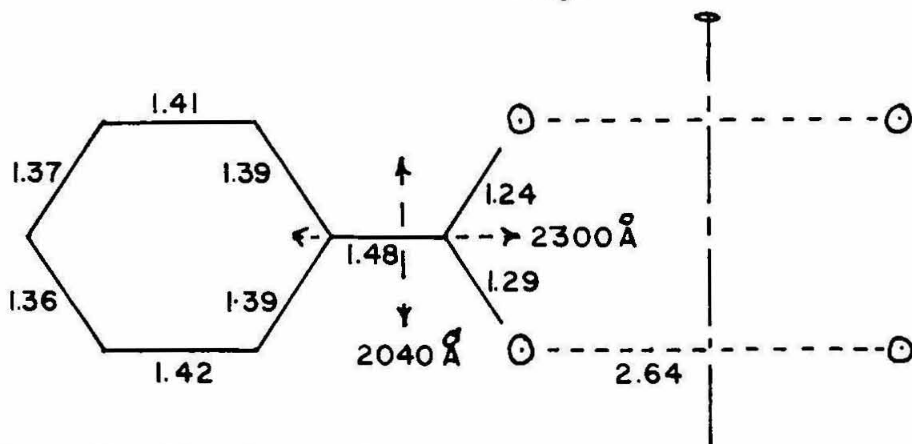


Figure B. Structure and assumed equivalent point dipoles for electronic transitions in benzoic acid dimer.

Proposition 4. An experiment is suggested whereby one can determine whether a polynucleotide molecule is single-stranded, on the basis of the assumption that such a molecule will always give two particles when a ^{32}P decays in its phosphodiester linkage. This assumption predicts the rate of change of the number of particles for a sample lightly doped with ^{32}P if one knows the absolute activity of the ^{32}P. The observation of this rate of change in particle number is not straightforward, and electron microscopy techniques are probably not yet suitable. An autoradiographic technique for particle counting is suggested, a technique which differs from those in use by employing a metal ion autoradiographic stain rather than incorporated label which would interfere with the experiment proposed. This suggested technique has several difficult aspects.

Criterion for single-strandedness in polynucleotides. In the absence of aggregation between molecules and internal hydrogen bonding, a single-stranded polynucleotide will split every time a ^{32}P in its backbone decays. Biological evidence indicates that the efficiency for splitting a double-stranded molecule may be around 0.1.

Let us assume we have a method for counting particles, and that the aggregation problems and internal hydrogen bonding problems have been accounted for. Then we can perform the following experiment. We lightly label one subunit of a density hybrid of DNA with ^{32}P. We isolate this subunit after a mild denaturation treatment, and at time t = 0 we measure the absolute activity of ^{32}P in the sample. Knowing the half-life of ^{32}P, we then know the activity at any time. The number of particles in unit weight of the sample at time t is equal to the number of particles at t = 0 plus the number of ^{32}P decays between times t = 0 and t, if the subunit is indeed single-stranded. If the molecule is multi-stranded, the rate of change in the number of particles will be much smaller than this. It is clear that this analysis will not only depend on development of the experimental approach to counting particles, but also is limited by the correction to the statistics because there is a minimum-sized piece which can be detected. The autoradiographic technique of Levinthal and Thomas (1957), for example, has only been able to detect pieces larger than 10^7 in molecular weight.

The importance of this experiment arises from the controversy over the strandedness of the replication subunit. The work of Cavalieri, et al. (1961) indicates that the subunit is not a single strand, but is a Watson-Crick double helix! There is evidence against this point-of-view (Schildkraut, et al., 1961). Experiments to determine the strandedness by the kinetics of enzymatic hydrolysis suffer from many drawbacks, and experiments to measure the mass-to-length ratio by electron microscopy not only suffer from the pitfalls of this technique, but also can only be applied to molecules in their two- (or four-)stranded stage. Our proposed experiment enables one to look at the subunits after they are

separated, so that one is not confronted with the possibility that the subunits separate during the observation.

Technical problems. The analysis outlined depends first upon abolition of aggregation and intramolecular hydrogen bonding. We propose to attack this problem in our procedure by using very dilute solutions (ca. 10^{-10} F (P)) and we can also work in the alkaline region and at low ionic strength until the particles are plated. After particles are fixed for observation, whether by electron microscopy or by autoradiography, we need not worry about these effects.

The use of electron microscopy for observation of these particles is limited by the sampling artifacts. Denatured DNA appears as small spheres, and to count these requires very clean preparation. Furthermore, both the streaking method of Beer (1961) and the selection of fields for observation seem to involve bias in favor of native molecules. Development may remove these objections, but we alternatively suggest an autoradiographic method.

Briefly, one would take a dilute solution of the particles to be counted, spread it on a very thin cellulose sheet, wash with an autoradiographic staining solution of β-emitting cations which are strongly bound to DNA, wash away the unbound cations, and then make an emulsion sandwich. After exposure and development, one would then count the number of stars in the emulsion. Sampling errors could be eliminated by using polystyrene latex spheres (10 μ) for reference (Levinthal and Thomas).

We can estimate the volume of resolution from the volume reported by Levinthal and Thomas, and calculate a volume 10 μ x 50 μ x 50 μ. To have an average of $\frac{1}{4}$ molecule per volume of resolution, we need a DNA

concentration of ca. 10^{-10} F (P). This concentration reduces the probability of having two particles of molecular weight 10^7 in one volume of resolution to 2.4%. However, the binding constants for ions such as Ag^+ are of the order of 10^7 M^{-1} at 0.1 ionic strength. To achieve significant ion-binding, one may need to work at very low ionic strengths or perhaps stain with an ethanol solution of a cation such as Ag^+.

One can obtain ^{111}Ag carrier-free, for $5.00 per millicurie, at activities above 0.05 mc/ml as $AgNO_3$. It has a 7.5 d half-life and emits primarily a 1.06 Mev β-ray. These characteristics are all quite suitable.

If one counts only stars with 100 or more rays to avoid background artifacts, and has 10% labelling of a DNA particle, then he can detect a piece as small as 6 x 10^5 molecular weight in 7.5 days. This minimum size is 2% of the predicted single-strand size for DNA from phage, and so only 4% of the ^{32}P decays in strands of this size will fail to give observable pieces. We dope at a concentration of 4 ^{32}P atoms per molecule.

We finally mention that development of an autoradiographic technique such as that outlined here will enable one to determine number-average molecular weights for samples of molecular weight above 10^6.

We have proposed an experiment which critically tests whether a polynucleotide molecule is single-stranded. In lieu of electron microscope observation of the number of particles, we have proposed an autoradiographic technique for counting particles. This suggested technique may encounter difficulty in avoiding aggregation and in getting extensive ion binding at very low DNA concentrations.

Proposition 5. Techniques are being developed for the microtome slicing of long DNA molecules. To reduce the statistical degeneracy of the segments obtained, one not only must align the molecules parallel to one another but also with their ends in register. It has been suggested that the placing of their ends in register might be accomplished by attaching a lipid group on the ends of the DNA molecules and aligning them at an aqueous-organic interface. We propose that the DNA polymerase of Adler, et al. (1958) be used to attach a nucleotide analogue to the ends of DNA molecules. This analogue will permit the subsequent selective attachment of a group such as the stearyl group. We propose that such a method of attack will enable one to eliminate molecules which have not been put in register, and that one can subsequently determine in the slicing step how efficient this means of aligning molecules has been.

The microtome slicing of macromolecules is a problem being attacked in this laboratory, and certainly is far from being solved. In this sense, this proposal is precocious. It is suggested in order to be able to reduce the statistical degeneracy of slicing to two or one, depending upon whether the ends of the DNA molecules are distinguishable.

Let us assume that we can place a lipid group on one or both ends of this polyelectrolyte. Such a molecule will migrate to the interface between its aqueous solution and an oil layer above. The lipid group will tend to lie in the organic phase, and the polyelectrolyte in the aqueous phase. If the lipid group, such as the stearyl group, has a solubility ratio of 10^7 between these phases, we calculate that the orientation energy is of the order of 20kT. Such a situation not only places the ends of the DNA molecules in register, it also allows one to orient the macromole-

-cules parallel to one another by reducing the area of the interface. Stearic acid has a cross-section of about 20 Å^2 in a Langmuir experiment (Harkins, 1952), while that of DNA should be at least 300 Å^2. If lipid groups are attached to either end indiscriminately, molecules will be oriented in either direction. A few will have lipid groups on both ends and be oriented hair-pin fashion. Both of these situations produce a two-fold degeneracy when we proceed to slice segments.

This approach enables one to eliminate molecules which have not reacted by ultracentrifugation. Molecules with stearyl groups on them at the interface have a net force up, even if they weigh 10^7 and are centrifuged at 40,000 rpm. This force, due to the rapid change in chemical potential felt by the stearyl group as it passes through the interface, is an order of magnitude larger than the gravitational force for a solubility ratio of 10^7 and an interface thickness of 100 Å. Thus, we can eliminate molecules which have no end-group simply by placing this interface system in the ultracentrifuge.

The specific attachment of a lipid group to the ends must rest on the specificity of the reaction reported by Adler, et al. (1958). They found that native DNA in the presence of one nucleotide triphosphate, the DNA polymerase, and Mg^{++}, incorporated 1 labelled nucleotide per thousand in the molecule. Analysis of the nearest-neighbors of these added nucleotides showed that sometimes a few nucleotides were added, especially in the case of cytidine triphosphate. It seems then that this enzyme offers high specificity for the ends of DNA molecules.

The range of nucleotides which can undergo this reaction is not known. One must find an analogue of a naturally occurring nucleotide to maintain the specificity established by the enzymatic reaction. We sug-

-gest that a very suitable intermediate would be a thymine or cytosine nucleotide triphosphate substituted in the 5-position by an aliphatic amino group. The acylation of free nucleotides takes place only at very high temperatures (Ts'o, unpublished work), while the acylation of aliphatic amino groups, such as ϵ-amino lysyl groups in proteins, takes place in aqueous solution at low temperatures. This procedure would seem therefore to be able to maintain the specificity established by the DNA polymerase. Whether an analogue such as this could be incorporated is not known. If not, then one must find a simpler analogue containing a substituent which allows the introduction of this aliphatic amino group. We lack information on these sorts of syntheses in pyrimidines.

The use of this reaction of the DNA polymerase for end-group attachment allows us to test the efficiency of aligning molecules during the slicing step. One can incorporate labelled nucleotides, and then monitor the appearance of label in the slice segments.

We then have proposed the use of the DNA polymerase for establishment of specific end-group attachment to DNA molecules. We have shown that the ultimate attachment of a stearyl group allows one to eliminate unreacted molecules by ultracentrifugation. We have mentioned a type of analogue which will permit attachment of a stearyl group through acylation of aliphatic amino groups. We have indicated how one can evaluate the efficiency of orientation of these molecules at an aqueous-organic interface.

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