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ANALYSIS OF CIRCULAR COVALENTLY CLOSED DEOXYRIBONUCLEIC ACID BY AGAROSE GEL ELECTROPHORESIS

by
Robert John DeLeys

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Microbiology)
in The University of Michigan
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LIST OF ABBREVIATIONS

A-T	adenine-thymine base pair
ATP	adenosine triphosphate
BSA	bovine serum albumin
cal	calorie
Ci	curie
cpm	counts per minute
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
G-C	guanine-cytosine base pair
kcal	kilocalorie
krpm	thousand revolutions per minute
pfu	plaque forming unit
pH	negative logarithm of the hydrogen ion concentration
RNA	ribonucleic acid
V	volt

LIST OF SYMBOLS

A	coefficient relating free energy of superhelix formation to the number of superhelical turns.
α	topological winding number
β	duplex winging number
C	gel concentration
c'	critical free dye concentration
c_f	free dye concentration
ε	twist angle required to bring functional groups at a nick into alignment for joining
g_i	degeneracy factor
G_τ	Molar free energy of superhelix formation
g_τ	Molar free energy of superhelix formation per base pair
k	constant relating free energy of superhelix formation to the number of nucleotides in a DNA and the number of superhelical turns
K_a	association constant
K_R	retardation coefficient
M	molecular weight
μ	electrophoretic mobility
N	number of nucleotides
N_i	number of molecules in the ith energy state
N_o	number of molecules in the ground state

ν	molar ratio of bound dye to nucleotide
ν_c	molar ratio of bound dye to nucleotide at the critical free dye concentration
ν_m	molar ratio of bound dye to nucleotide at saturation
π	pi (3.14)
ϕ	duplex unwinding angle
R	universal gas constant
σ	superhelix density; number of superhelical turns per 10 base pairs
S	sedimentation coefficient
τ	superhelix winding number
T	absolute temperature

INTRODUCTION

Circular covalently closed duplex DNA has been found widely distributed in nature. Although first observed from the DNA tumor viruses polyoma (1) and SV40 (2), the list has now been extended to include such diverse DNAs as mitochondrial DNA (3), the intracellular replicative forms of most DNA bacterial viruses such as of ØX174 (4), lambda (5), and all bacterial plasmids such as colicinogenic factor E1 (6), and the sex factor F (7).

It is now clear that in the prokaryotic kingdom, most DNA molecules exist as covalently closed circles during at least some part of their replicative cycle. A significant amount of attention has been focused on the detailed structure and special chemical properties of covalently closed DNA in an effort to gain an understanding of the biological significance of covalent closure. In spite of the fact that many of the properties of DNA in general are unaltered by covalent closure, such closure does impart some special properties to the DNA molecules. In order to predict, at least in qualitative terms, the behavior of a covalently closed molecule under a particular set of conditions, it is first necessary to appreciate the unique topology that covalent closure confers upon the molecule.

Definitions and Topological Considerations

In 1965 Vinograd, Lebowitz, Radloff, Watson, and Laipis (8) proposed that naturally occurring closed circular duplex DNA molecules contain superhelical turns which are maintained by the chemical

forces of the Watson-Crick secondary structure of DNA. Superhelicity is a property which is absolutely dependent on covalent closure. Since the two strands of the Watson-Crick duplex are intertwined, superhelical turns cannot be removed or generated without simultaneously changing the average pitch (base pairs per turn) of the duplex.

Superhelicity may be defined in terms of three winding numbers. The superhelix winding number, τ, is defined as the number of turns the duplex makes about the axis of the superhelix. The topological winding number, α, is defined as the number of revolutions made by one strand about the duplex axis when the molecule is constrained to lie in a plane, and the duplex winding number, β, is the number of revolutions a strand makes about the duplex axis in the unconstrained molecule. These three winding numbers are linearly related by the relationship (9)

$$\tau = \alpha - \beta \qquad (1)$$

By convention, right-handed duplex turns are considered positive and the quantities α and β are taken to be positive in native DNA. The sign convention applied to superhelical turns depends on the nature of the turn. If the superhelical turns are interwound, then turns which appear left handed are considered positive. For non-interwound superhelical turns such as toroidal turns, the sign convention is reversed due to a 90° rotation in the viewing axis (9). Superhelicity is most commonly thought of in terms of interwound turns although the light scattering evidence presented by Campbell and

Jolly (10) suggests that DNA with few superhelical turns per unit length may adopt a toroidal conformation in solution.

When the magnitude of τ is significant compared to α, equation 1 becomes inexact. Maestre and Wang (11) and Campbell and Lochhead (12) have shown by circular dichroism measurements that, under these conditions, there is some deformation of the primary helix. This finding indicates that the actual number of superhelical turns in a highly supercoiled molecule will be somewhat less than predicted from equation 1. If, however, τ is redefined as titratable superhelical turns, the equation becomes exact under all conditions.

The three winding numbers, τ, α, and β, are clearly extensive properties. The magnitude of each will depend on the length of the DNA molecule under consideration. It is frequently useful to express the degree of supercoiling in terms of superhelical density, σ. The superhelix density is an intensive quantity independent of DNA length and is defined as the number of superhelical turns per ten base pairs. The equation relating τ to σ is

$$\sigma \equiv \tau/\beta^{\circ} \qquad (2)$$

where β° is equal to one-tenth the number of base pairs in the molecule (9). The superhelix density is perhaps the most important parameter since it is the one which is measured experimentally.

From equation 1, it is evident that τ is a function of the two quantities α and β. Because the strands of the DNA are intertwined. the magnitude of α can be altered only by interrupting the continuity of the strands and allowing them to rotate with respect to each other.

In the absence of strand scissions, changes in τ will be equal in magnitude to changes in β. Under these conditions, equation 1 reduces to

$$\Delta\tau = -\Delta\beta \qquad (3)$$

Factors which influence the magnitude of β change the number of superhelical turns and are measurable as differences in the superhelix density.

Two environmental factors which can alter the pitch of the duplex are temperature and ionic strength (13,14). The manner in which the effect of these factors can be determined are discussed in the next section. However, in qualitative terms the effects of changes in ambient conditions can be predicted. The effect of increasing temperature has long been known to unwind the primary helix slowly until, at the melting temperature, a large cooperative strand separation occurs. Thus, the influence of increasing temperature on the tertiary coiling of a closed circular DNA molecule will be to introduce positive superhelical turns. Likewise, a reduction in the ionic strength of the solvent will tend to increase the degree of electrostatic repulsion between adjacent phosphates, thereby causing an ionic strength-dependent unwinding of the primary helix with the same effect as increasing the temperature.

The pitch of the duplex can also be altered by a variety of compounds capable of intercalating between base pairs. These agents have proven useful for studying the tertiary structure of DNA and include compounds such as the acridines, phenanthridinium derivatives,

and the antibiotic actinomycin D (9). A feature shared by all intercalating compounds is a planar ring system. Insertion of the planar compound between base pairs causes the primary helix to unwind (15). Of all the reagents available, the most commonly used is the compound ethidium bromide (EtBr, 3,8-diamino-6-phenyl-5-ethylphenanthridinium bromide).

In principle, other reagents may be used but ethidium and related phenanthridinium compounds have proven most useful for several reasons. One requirement which must be met if a compound is to be useful for the study of DNA structure is adequate binding at high salt concentrations. Since many of the techniques used to study DNA rely on the use of a high ionic strength solvent, the association constant for the binding reaction must be relatively high in order to achieve adequate binding ratios. The amino-acridines are not as useful as ethidium because of their lower affinity for DNA in high salt media (16). A second requirement which is fulfilled by the phenanthridium dyes is sufficient maximum site occupancy (15,17). Actinomycin D which also unwinds the primary helix fails in this respect because of its specificity for adjacent cytosine and guanine (18).

In addition to its supercoiled form, commonly termed Form I, a DNA molecule may exist in two alternative forms which differ substantially in their properties from the covalently closed species (see Figure 1). The first alternative, the nicked circular form, or Form II, is generated following cleavage of a single phosphodiester bond in the supercoiled molecule. Generation of a related

species, the linear or Form III species, requires phosphodiester bond breakage in both strands either at points opposite each other or in such close proximity that there is insufficient hydrogen bonding in the overlap region to maintain circularity. It is the loss of strand continuity in Form II and linear molecules which distinguishes these forms from the covalently closed species. Since free rotation of the strands with respect to each other is possible when the covalent continuity of the strands is lost, the quantities τ and α are meaningless when applied to nicked circular and linear DNA molecules.

Properties of Closed Circular DNA and Evaluation of Topological Parameters

Hydrodynamic Properties and Methods of Analysis

The hydrodynamic properties of supercoiled as well as the nicked circular and linear forms of DNA have been investigated extensively and are well characterized. The technique most frequently employed to investigate these properties is ultracentrifugation. Measurements of sedimentation rates may be made either in an analytical ultracentrifuge in solutions of neutral salts or in a preparative ultracentrifuge by zonal sedimentation through preformed sucrose gradients.

Qualitatively, the sedimentation behavior of a DNA in its three forms is predictable. The radius of gyration of a molecule will depend on its conformation. The native supercoiled form which is tightly wound upon itself is significantly more compact

than either the nicked circular or linear forms. The nicked circular form, although more open than the Form I, is still more compact than the linear molecule because it is not free to extend fully. In light of this, the DNA expected to have the greatest sedimentation coefficient is Form I while linear molecules are expected to have the least. This behavior is clearly demonstrable in the ultracentrifuge. Based on sedimentation data, empirical formulas have been derived which relate the observed sedimentation coefficient to molecular weight for each of the three conformational possibilities (19,20).

$$S_{(L)} = 2.8 + 0.00834\ M^{0.479} \qquad (4)$$

$$S_{(II)} = 2.7 + 0.01759\ M^{0.445} \qquad (5)$$

$$S_{(I)} = 7.44 + 0.00243\ M^{0.58} \qquad (6)$$

The quantity denoted by S is the sedimentation coefficient, M is the molecular weight of the DNA and the subscript refers to the molecular form of the DNA. Two conclusions may be drawn by inspection of these equations. First, the sedimentation rate of the supercoiled DNA will always be significantly greater than that for either of the two other forms for a given molecular weight and secondly, the ratios of the sedimentation coefficients will vary with molecular weight.

Equation 6 was derived for native supercoiled DNAs which all have similar superhelical densities (21). Upholt *et al.* (22), using a series of SV40 DNAs having different superhelical densities, have shown that the sedimentation coefficient of a closed circular DNA molecule is dependent upon α in a complex way. Using values of

α calculated with a 12° duplex unwinding angle for ethidium,[1] they found that as σ increases from zero to -0.02, the sedimentation coefficient rises sharply and then decreases somewhat to a local minimum at $\sigma = -0.04$. As the superhelix density is further increased, the sedimentation coefficient rises steadily. This non-monotonic variation in S-value as a function of σ indicates that as σ is increased, the molecule goes through at least three types of supercoiled conformations. Upholt et al. (22) have attributed the initial increase in sedimentation coefficient as α increases from zero to -0.02 to the progressive reduction in the radius of gyration of an essentially random-coil structure. As the superhelix density continues to increase, the covalently closed molecule becomes more rigid and rod-like as a tight interwound structure is formed. This change is reflected as a decrease in the sedimentation coefficient to a local minimum at $\sigma = -0.04$. By further increasing the magnitude of σ, rigid branched structures develop which have a decreased radius of gyration and a correspondingly higher sedimentation rate.

These experiments designed to study sedimentation behavior as a function of α depended upon making in vitro a series of SV40 DNA molecules differing in superhelical density. This was accomplished by closure of nicked circular SV40 DNA in the presence of the

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[1] The duplex unwinding angle of ethidium bromide (ϕ) was long considered to be 12°, based on the model building studies of Fuller and Waring (23). Recently, however, this value has been challenged (24,25,26). The widely accepted value of ϕ is now 26°. The experiments leading to this revised value are discussed in detail in the subsection entitled The Duplex Unwinding Angle of Ethidium Bromide.

intercalative dye ethidium bromide followed by extraction of the dye at the completion of the reaction. The superhelix density of the product is directly proportional to the amount of ethidium bound by the DNA at the time of ligation. The characteristic sedimentation coefficient versus σ curve may be essentially duplicated by titrating a highly negatively supercoiled DNA with ethidium bromide (22). The amount of ethidium bound will determine both the sign and the magnitude of σ and will influence the sedimentation behavior accordingly. Sedimentation values pass through the same local minimum and local maximum at appropriate values of σ until a minimum is reached when the superhelix density reaches zero. At this point, the covalently closed molecules are fully relaxed and co-sediment with nicked circles. The addition of more ethidium results in further unwinding of the duplex. This, because of the topological constraints, causes positive superhelical turns to be introduced into the molecule and the sedimentation coefficient increases.

The curve relating sedimentation coefficient to σ forms the basis for the widely used sedimentation velocity-dye titration technique which is employed to determine the superhelix density of a DNA in the absence of dye (9). The sedimentation rate of a DNA is measured at a series of ethidium concentrations until the minimum in the curve where σ equals zero is found. Both the nicked circular and covalently closed forms of the DNA are assumed to have the same affinity for ethidium bromide at this point. The

free dye concentration at which co-sedimentation of Form I and Form II occurs (c') may be used with the association constant (K_a) for the binding reaction to determine the ratio of moles of ethidium bound per mole of nucleotide (ν_c) by use of the Scatchard equation (27).

$$\nu_c = \frac{\nu_m K_a c'}{1 + K_a c'} \qquad (7)$$

The quantity ν_m represents the maximum ratio of moles dye bound per mole nucleotide. Experimentally, this has been found to be approximately 0.18 for ethidium which is somewhat lower than the value predicted by Bauer and Vinograd (28) for their excluded site model for binding. The excluded site model was proposed to explain the dye-binding data that by extrapolation indicates a maximum binding ratio which is considerably less than 0.5, the expected value if ethidium could intercalate between each base pair at saturation. The model states simply that the binding of a dye molecule precludes the binding of a second dye molecule at an adjacent site on the DNA duplex. Hinton and Bode (29) have pointed out that even if the excluded site model is correct, the predicted ν_m of 0.25 might not be observed. When an ethidium molecule intercalates an odd number of base pairs away from its neighbor, at least one-half of an intercalation site is lost. Therefore, a ν_m of 0.25 would require a special type of order at equilibrium which would limit the number of possible states.

The quantity ν_c can be related to the more useful quantities τ_o and σ_o by the following relationships (9).

$$\tau = \tau_o + \left(\frac{\phi}{2\pi}\right) 10\ \beta^o\ (2\nu) \qquad (8)$$

$$\sigma = \sigma_o + \frac{10\phi}{\pi}\nu \qquad (9)$$

The subscript zero refers to the initial condition in the absence of dye and the quantity ϕ refers to the magnitude of the duplex unwinding angle of the intercalative dye expressed in radians. When $\nu = \nu_c$, τ and σ are equal to zero and the equations are solved for τ_o and σ_o respectively. Clearly, meaningful information can be obtained only when ϕ is known with a reasonable degree of accuracy.

Many of the circular covalently closed DNAs available have been titrated in the ultracentrifuge. Vinograd and co-workers, using a duplex unwinding angle of $\pi/15$ for ethidium, reported values of -0.039 and -0.033 respectively for the superhelix densities of SV40 and polyoma in 2.83M CsCl. Bujard (30) has reported that the superhelix density of the animal virus papilloma is the same as that of polyoma. Wang (21) has titrated a variety of closed circular DNAs harbored by Escherichia coli and reported that although the molecular weights and number of superhelical turns measured covered a twenty-fold range, the superhelical densities were constant within a factor of about 1.5. The native DNA having the greatest superhelix density of any DNA so far discovered is that of the enveloped bacteriophage PM2 (31). The

superhelix density of this DNA was determined by sedimentation velocity-dye titration to be -0.053. In spite of the wide range of molecular weights, the similarity in the superhelix density of these DNAs is striking.

Other techniques have been employed to monitor hydrodynamic properties as a function of superhelix density. A technique which provides a direct measure of the hydrodynamic characteristics of a DNA molecule is viscometry (32,33). The behavior of nicked circular and supercoiled DNA closely resembles the behavior observed in the ultracentrifuge. The more compact supercoiled DNA has a lower intrinsic viscosity than does the nicked circular molecule. Smit and Borst (32) have used viscometry to follow the course of dye titrations of supercoiled DNA and noted a rapid increase in the viscosity as σ approached zero followed by a rapid reduction in the viscosity as more ethidium was added. Similarly, Revet et al. (33) have used this method to titrate PM2 DNA. One of the particularly attractive advantages of using viscometry as opposed to sedimentation analysis is that the viscometric method can be employed with any solvent system and can be used even when the DNA is in an opaque medium.

A technique which has been only recently applied to circular DNAs is gel electrophoresis. For relatively large molecular weight DNA, the medium of choice is either agarose or a combination of agarose and polyacrylamide. Fisher and Dingman (34) have made a comprehensive study of the composite agarose-polyacrylamide system

and have shown that native supercoiled molecules of SV40 DNA can easily be distinguished on the basis of electrophoretic mobility relative to the nicked circular and linear counterparts. However, the relative mobilities of the three species are strongly dependent upon the agarose concentration used. It is clear from the data that Fisher and Dingham presented that it is difficult to correlate electrophoretic mobility with hydrodynamic characteristics. Unlike velocity sedimentation or viscometry which do not require a supporting medium, the agarose and agarose-polyacrylamide gel systems rely on the presence of such a supporting material to affect separations. As a result, the mobilities of the sample components are strongly influenced by the physical nature of the support and their interaction with it. Linear DNA in the composite system always migrated at least as rapidly as its nicked circular counterpart, a result which would not have been predicted from behavior in the ultracentrifuge.

Dingman et al. (35) offered an explanation for the peculiar behavior of linear DNA molecules on gels (35). They proposed that double stranded linear DNA molecules migrate through the supporting matrix end-on. On the basis of the measured retardation coefficients for linear molecules, it was determined that linear molecules behave on gels as though they had a molecular radius of about 35Å cross-sectional radius of DNA in solution as determined from the low angle X-ray scattering measurements obtained by Bram and Beeman (36). Circular molecules had retardation coefficients which indicated a

significantly larger molecular radius but the actual values obtained were greatly influenced by the magnitude of the electric field.

The most commonly used matrix for electrophoretic separation of DNA is agarose (37). It has proven to be extremely useful for molecular weight determinations and as a preparative technique for purifying specific DNA fragments from mixed populations. Thorne (37) and Sharp et al. (38) have observed that supercoiled DNA migrates more rapidly than nicked circular or linear DNA molecules of the same molecular weight. As in the composite system, the mobility of linear molecules relative to the circular forms is dependent upon the agarose concentration.

Native supercoiled DNA migrates on agarose gels as a single species. Keller (39), however, has found that covalently closed DNA with superhelix densities intermediate between zero and that of the native DNA can be resolved as discrete bands with electrophoretic mobilities intermediate between those of Form I and Form II DNAs. Similar observations have been made by Pulleyblank et al. (40) and Depew and Wang (41). Work performed concurrently with the work presented in this thesis also demonstrates the utility of agarose gel electrophoresis as a tool for performing dye titrations (42). Since this is the subject of this thesis, the characteristics of the agarose gel system and its use in evaluating the conformational properties of supercoiled DNA will be presented more completely in the sections headed RESULTS and DISCUSSION.

Restricted Dye Uptake and Buoyant Density

In the absence of intercalative dyes, little information can be obtained about the tertiary structure of covalently closed DNA by isopycnic banding in equilibrium density gradients. All forms of a DNA will have the same density regardless of the topology. In the presence of a suitable intercalative dye such as ethidium bromide or propidium diiodide, the buoyant differences between covalently closed and covalently open DNA are dramatic (9).

As mentioned previously, ethidium binds by inserting between base pairs. In so doing, the dye is able to effect a slight unwinding of the primary helix. The superhelical density and the extent to which the duplex is unwound will depend on the number of dye molecules bound. Since ethidium bromide has a lower buoyant density than that of the DNA, the buoyant density of DNA into which ethidium has intercalated will be less than that of the same DNA in the absence of dye. At high dye concentrations, the dye will be maximally bound to the nicked circular and linear forms of the DNA. However, because of the unique topology of covalently closed DNA, the effect of duplex unwinding must be taken into account when intercalating dyes bind to it. As demonstrated in the velocity sedimentation-dye titration experiments, as the amount of ethidium bound progressively increases, the superhelix density of covalently closed native DNA first decreases to a minimum at ν_c and then increases as supercoils of the positive sense are introduced (9). Eventually a point is reached when the free energy of binding is

exactly counterbalanced by the free energy increase required to introduce an additional small degree of torsion. For EtBr, this point will be reached, as long as ν_m is not first attained. On the other hand, unlike covalently closed DNA in which free rotation of the strands is restricted, nicked circular or linear DNA is free to bind dye until ν_m is attained. Since the uptake of dye by the covalently closed DNA is restricted by the topological and hence the thermodynamic properties of the molecule, it will have bound less dye at high dye concentrations than nicked circular or linear molecules and will have as a result a higher buoyant density. This property forms the basis of the CsCl-dye equilibrium density gradient technique which is one of the primary steps in the purification of closed DNA.

Bauer and Vinograd (9,16) have employed the technique of equilibrium centrifugation to study the tertiary conformation of covalently closed DNA more completely. At low ethidium concentrations, Form I DNA binds more ethidium than nicked circular DNA because the free energy of superhelix formation in the negatively supercoiled molecule favors any process which leads to unwinding of the duplex. This will be considered in greater detail in the section entitled The Free Energy of Superhelix Formation. In the negatively supercoiled molecule, this effectively increases the affinity of Form I DNA for the dye relative to Form II. At low ethidium concentrations, Form I DNA will bind more dye than nicked circular DNA and will band at a lower density than Form II when a

mixture of the two species are centrifuged to equilibrium. When the critical free dye concentration is reached (c'), the covalently closed molecule is fully relaxed. In general, the assumption is made that at this point in the titration both the covalently closed and nicked circular DNAs will have the same affinity for the dye. This allows the Scatchard equation (equation 7) (27) to be employed to calculate ν_c. Addition of more ethidium result in a further reduction in buoyant density of both forms although the nicked circular DNA will have a greater affinity for the dye than Form I because torsion is not generated.

The calculations of Bauer and Vinograd (16) lead to the prediction that the relative buoyant density separations between the nicked and closed forms of a DNA should be linearly related to the superhelix density of the closed DNA in the absence of dye. The binding reaction in the presence of excess dye may be best illustrated if the binding is considered in two stages. In the first stage, the negatively supercoiled molecule relaxes when ν_c dye molecules per residue are bound. In the second stage, additional dye molecules are bound and positive superhelical turns develop. Positive turns will be introduced until the free energy of binding is effectively counterbalanced by the free energy of superhelix formation. If the amount of dye bound from ν_c to the balance point is termed ν_s, then the total amount of dye bound in excess dye is $\nu_c + \nu_s$. Since ν_c is directly proportional to the magnitude of σ_o, the total amount of dye bound at an arbitrarily high dye

concentration will also be directly proportional to σ_o and the buoyant separation between the nicked and closed forms of the DNA will decrease as σ_o increases. When σ_o of the supercoiled DNA is such that $\nu_c + \nu_s = \nu_m$, the two forms will band together.

Hudson et al. (43) have demonstrated in a qualitative fashion the predicted buoyant density behavior of closed DNAs relative to nicked circles. Gray et al. (31) have taken advantage of this phenomenon to devise an analytical method for determining superhelical densities on the basis of the difference in relative separation of the closed and nicked forms of any DNA with respect to SV40 DNA isolated from virions. Virion DNA was used as a reference in the system since Eason and Vinograd (44) have reported that SV40 DNA isolated from the intracellular pool was more heterogeneous in terms of its superhelix density than DNA isolated from virions. Espejo et al. (45) have reported similar results for PM2. Other investigators, however, have failed to detect this (42).

Although ethidium bromide is commonly used in the aforementioned technique, Hudson et al. (43) have found that the use of propidium diiodide results in a much greater buoyant density shift for a given difference in σ. The relative separations are greater by a factor of about 1.8 and therefore the use of this dye lends a greater degree of precision to the technique.

The Free Energy of Superhelix Formation

The free energy of superhelix formation is clearly a positive quantity and will favor any process which tends to lessen the

torsion and relax the molecule. This free energy can be considered to be the sum of an enthalpic term which arises as a result of distortion of the primary helix and an entropic term which results from the more highly ordered conformation of the supercoiled molecule. The most obvious proof of the assertion that the free energy of superhelix formation is positive is the consequence of introducing a single strand scission into the molecule. By breaking a phosphodiester bond, the topological constraints within the molecule are released and the initially supercoiled molecule relaxes spontaneously. The free energy associated with the native supercoiled molecule becomes apparent in other ways as well. As was mentioned earlier, a negatively supercoiled molecule has a higher affinity for intercalative dyes than does a nicked molecule. This may be restated somewhat more precisely by saying that the removal of superhelical turns is accompanied by a negative free energy change associated with the binding of the dye. When ν is greater than ν_c and the molecule possesses positive turns, the free energy change is positive and tends to lower the free energy change associated with the binding reaction.

The free energy of superhelix formation also becomes apparent during thermal and alkaline denaturation of covalently closed molecules (46). When negatively supercoiled DNA is titrated with hydroxyl ions, a small but detectable melting of the primary helix occurs prior to the onset of the titration of Form II. Vinograd *et al.* (46) correlated pH with the buoyant density and the fraction

of bases titrated in alkaline CsCl equilibrium gradients and found that as the pH was raised, Form I DNA underwent a small buoyant density shift before there was any change in the buoyant density of Form II which indicates that a portion of the duplex of the Form I molecules had been melted. The early melting, or early helix-coil transition, was complete when the supercoiled molecule was fully relaxed. Above the pH at which the early transition occurs, nicked circular DNA denatured very rapidly with a concomittant increase in the buoyant density. The covalently closed DNA, however, continued to be titrated slowly at pH ranges well above that required to completely denature the Form II. This broad late helix-coil transition has been attributed to stabilization of the primary helix as a result of the inability of the molecule to unwind. Since unwinding of the duplex requires, from topological considerations, that positive superhelical turns be introduced, the duplex is stabilized by the unfavorable free energy of superhelix formation. Conversely, at the beginning of the titration curve, the positive free energy of superhelix formation has a destabilizing influence and promotes early melting of the DNA. The regions of a DNA molecule most susceptible to this early melting effect are the A-T rich regions while the titration of G-C rich regions occur predominantly in the late helix-coil transition portion of the curve.

Vinograd et al (46) also examined the thermal stability of covalently closed molecules relative to their nicked circular

counterparts. Although they could detect no hyperchromic effect which could be attributed to an early helix-coil transition, the thermal melting curve of the closed DNA was much broader than that of the open molecule and exhibited a much higher T_m. The interpretation of these results is the same as that offered for the alkaline denaturation data. Bauer and Vinograd (28) in an elegant series of calculations were able to arrive at an estimate of the free energy of superhelix formation for SV40 DNA at 25°C in 5.8M CsCl. Their approach involved the derivation of statistical mechanical expressions describing the binding isotherms of ethidium to both nicked circular and native closed circular SV40 DNA. The expressions for the closed DNA included terms to account for the free energy of superhelix formation as a function of the number of superhelical turns. The calculations were based on their excluded site model for ethidium binding and the assumption that the average number of dye molecules bound is approximately equal to the most probable binding number. The coefficients which describe the free energy as a function of the number of superhelical turns were determined from the best least-squares fit of their statistical mechanical expressions to the experimental binding data. The authors pointed out that the excluded site model adequately describes the binding of ethidium to DNA and indicate that the difference in the binding isotherms of closed circular and nicked circular DNA can be attributed to the free energy of superhelix formation rather than differences in the intrinsic

binding constant or the number of available binding sites. Their equation which relates the free energy of superhelix formation to the number of superhelical turns is

$$\Delta G_\tau/RT = 0.88\ \tau^2 - 0.0038\ \tau^3 \qquad (10)$$

The quantities R and T have their usual meaning. A plot of ΔG_τ versus τ reveals that the curve is approximately parabolic with a slight asymmetry due to the cubic term. At small values of τ the cubic term is negligible. Using a value of 12° for the unwinding angle of ethidium and a value of τ for SV40 DNA of -13, the free energy of superhelix formation for native SV40 DNA was calculated to be 84 kcal per mole or 18 cal per base pair. Bauer and Vinograd (28) have indicated, however, that the numerical values obtained for the free energy of superhelix formation are dependent upon the angle of duplex unwinding due to the binding of ethidium and as such is subject to revision if the unwinding angle deviates from 12°.

Wang has taken a somewhat different approach to the determination of the free energy of superhelix formation (47). The method he employed also relied on determining the binding isotherms for ethidium:DNA binding for both supercoiled and nicked circular DNA. Wang plotted the quantity $\xi = \nu/c_f$ versus ν where c_f refers to the free ethidium concentration and obtained typical Scatchard plots. From these, he was able to plot $\ln (\xi_I/\xi_{II})$ as a function of ν and showed that within experimental error the relationship was linear. This linear relationship could be adequately described

by the function $\ln(\xi_I/\xi_{II}) = a_1(\nu - \nu_c)$ where a_1 is a proportionality constant. This led to an expression for the free energy of superhelix formation in the absence of ethidium in terms of ν_c

$$G_\tau = \frac{-a_1 NRT\nu_c^2}{2} \qquad (11)$$

Appropriate manipulation of this relationship leads to the prediction that at a given superhelical density, the free energy per 10 base pairs is insensitive to molecular weight, a conclusion which was also reached by Bauer and Vinograd (28) and Davidson (48). The error in Wang's measurements were such that he could not evaluate enthalpic or entropic contributions to the free energy. The advantage to his method, however, is that it involves no assumptions concerning the mode of ethidium binding and does not require knowledge of the duplex unwinding angle for ethidium. The results obtained by Wang were lower than those obtained by Bauer and Vinograd by a factor of about 1.5. Wang has indicated that any difference in molecular parameters related to bending or twisting of the DNA between the DNA and the ethidium:DNA complex will introduce a source of error into his method. In light of more recent findings concerning the unwinding angle of ethidium, the results of Bauer and Vinograd are subject to revision (25). Pulleyblank et al. (40) and Depew and Wang (41) have observed that, on agarose gels, covalently relaxed DNA can be resolved into a set of bands containing species differing by a single superhelical turn. Since this is the central topic of this thesis, a

detailed discussion of these papers will be presented in the discussion of the experimental results reported herein.

Environmental Effects and Superhelicity

Covalent closure has proven to be a useful feature in that minor changes occurring at the level of the base pair are magnified when summed over all base pairs in the molecule and become apparent as changes in the tertiary conformation which is an easily evaluated property. Thus, small changes in the primary helix occurring as a result of a change in the environment can be readily quantified by the appropriate application of one of the aforementioned techniques.

The two most important environmental parameters which effect the extent of duplex winding are ionic strength and temperature. The first to use covalently closed DNA to attempt to evaluate the duplex changes which occur as a result of changing the environment of the DNA was Wang (14). Wang and co-workers used in their experiments λb2b5c DNA isolated from virions and converted to a covalently closed form with T4 ligase. They observed only two sedimenting boundaries when the sedimentation medium was the reaction mixture in which the DNA had been closed. If CsCl was added to a concentration of 3M, three boundaries were observed which, in order of increasing sedimentation were linear, nicked circular, and covalently closed molecules. Similarly, closure of the molecules at different temperatures led to measureable differences in the observed rate of sedimentation. Wang, in a later report, presented

a more systematic study of the consequences of ionic strength and temperature shifts (13). In this study, Wang correlated the relative sedimentation coefficients of closed and nicked circular λb2b5c DNA with the number of superhelical turns calculated on the basis of a 12° unwinding angle for ethidium bromide. By closing the DNA at a fixed temperature and observing the change in sedimentation ratio as a function of the temperature at which the velocity sedimentation was performed, Wang concluded that the duplex unwinds slightly by a factor of 4-5 x 10^{-3}° per base pair per degree Centigrade as the temperature is increased.

Agarose gel electrophoresis has been used recently by Depew and Wang (41) and Pulleyblank et al. (40) to evaluate temperature effects. These reports will be discussed in detail with the results presented in this thesis.

Wang, in this same report, attempted to correlate the change in base rotation angle with ionic strength and the counterion. Previously, other independent investigators had reported ionic strength and counterion effects (49,50). Wang undertook a systematic study of these environmental factors and found that as the ionic strength decreases, the primary helix unwinds slightly and is dependent upon the magnitude of the ionic strength shift and is somewhat dependent on the nature of the counterion. Similar results have been reported by Upholt et al. (22) and by Hinton and Bode (29).

Shure and Vinograd (51) have used agarose gel electrophoresis to correlate the relative values of the duplex winding number under

two sets of environmental conditions. Since this technique offers far greater sensitivity than any other technique currently in use, it was possible to determine these differences with a significant degree of accuracy. The two conditions they investigated were the conditions for the ligase reaction (1 mM $MgCl_2$) and the conditions for the relaxation enzyme reaction (200 mM NaCl). The results of their comparison indicate that the duplex is slightly overwound in the ligation buffer relative to the relaxation buffer. This result is somewhat surprising since on the basis of ionic strength considerations alone it would seem as though the opposite should be the case. This unexpected result indicates that the difference may lie in the mode of interaction of the cation with the DNA. A possible explanation could be the predominant formation of Debye ion atmospheres with sodium ions as opposed to site binding by the divalent magnesium ion (52).

The Duplex Unwinding Angle of Ethidium Bromide

Clearly, from the previous discussion, many of the techniques used to evaluate the tertiary structure of covalently closed DNA rely on the precise knowledge of the duplex unwinding angle of ethidium bromide. Most of the calculations presented incorporate a duplex unwinding angle for ethidium of $\pi/15$ or 12°. Unfortunately, there has been a considerable amount of uncertainty in this value. The model building studies of Fuller and Waring (23) led them to conclude that a duplex unwinding angle of $\pi/15$ was sufficient to accommodate a bound ethidium. They note, however, that other models

could also be built which would adequately represent the intercalation of ethidium and cite as a possible alternative a model which postulates an unwinding angle of $\pi/5$. Paoletti and LePeqc (53) proposed an alternative model based on their studies of fluorescence depolarization due to resonance energy transfer between ethidium cations bound at nearby sites on the DNA. They suggest that the distortion due to intercalation extends over a four base pair region and results in a 13° overwinding of the duplex. Although attractive because it offers an explanation for the excluded site behavior of ethidium binding to DNA (53), there is now a preponderance of evidence which demonstrates that both the model of Fuller and Waring and the Paoletti and LePeqc model are incorrect. Schmir et al. (54) have ruled out the possibility that ethidium causes an overwinding of the DNA duplex in a series of ethidium bromide titrations of partially replicated mitochondrial DNA before and after the removal of the progeny strands. In each case it required more ethidium to fully relax the molecules once the progeny strands had been removed, thereby showing that intercalated ethidium has the same effect on a DNA duplex as a displacing strand and that ethidium must, therefore, unwind the duplex.

A different approach to this problem which attempted to shed light on both the magnitude and direction of the duplex change caused by ethidium binding was taken by Pulleyblank and Morgan (24). Their approach involved partial derivatization of supercoiled DNA with N-cyclohexyl-N'-β(4-methylmorpholinium)-ethyl carbodiimide

which combines specifically and reversibly with thymine and guanine residues (55). The reaction of DNA with this water soluble carbodiimide causes strand separation. The partially derivatized molecules were relaxed and reclosed by the combined action of a nuclease and polynucleotide ligase and were then de-derivatized by mild alkali treatment, thus allowing the denatured regions of the DNA to assume the normal Watson-Crick structure. The resulting DNA was shown to have the same sense of supercoiling as the native DNA, thereby establishing the sense of naturally occurring superhelices. When the sedimentation coefficient of PM2 DNA was plotted versus the fraction of bases derivatized, a minimum is apparent at 16 percent reaction. If it can be assumed that the derivatized regions of the DNA are completely unwound at the time of final ring closure, then the superhelix density of the de-derivatized product should be equal to the fraction of bases derivatized at the time of ligation. Values for the unwinding angle due to bound ethidium were found to be 2 to 3 times greater than the 12° value suggested by Fuller and Waring. A problem inherent in the approach taken by Pulleyblank and Morgan is that little is known about the nature of the derivatized regions of the DNA. Since the water soluble carbodiimide is positively charged, it is conceivable that there could exist weak but significant ionic interactions between the derivatized strands and would most likely lead to overestimation of the superhelix density. Nevertheless, their result gives a very good first approximation of the duplex unwinding angle for ethidium.

Wang (25) has addressed himself to this problem as well. He noted that in a restricted pH range close to the melting pH of a DNA in the absence of topological constraints, the difference in the fraction of bases titrated at a particular pH between two covalently closed DNAs having differing degrees of superhelicity is directly proportional to the difference in the molar ratio of ethidium bound per mole nucleotide required to relax the two DNAs fully. The proportionality constant relating these two measurable quantities incorporates the unwinding angle of ethidium which is easily calculated with an estimated uncertainty of less than 10 percent. According to Wang, the duplex unwinding angle of ethidium is 26°. This result clearly supports the results obtained by Pulleyblank and Morgan (24). Although the alkaline buoyant density results obtained by Vinograd et al. (46) tend to support the 12° value for duplex unwinding, Wang has indicated that an inherent difficulty in the method used by Vinograd and co-workers is the precise determination of pH. This is not a critical consideration in the approach taken by Wang. This corrected value for the duplex unwinding angle introduces a simple multiplicative error into previous calculations in which a 12° unwinding angle was used, and results calculated using a 12° unwinding angle must be corrected accordingly.

Keller (26) presented additional evidence which supports Wang's conclusions. Keller correlated the relative buoyant density difference between native and covalently closed relaxed SV40 DNA with

the difference in the number of superhelical turns between the two samples. The approach taken by Keller involved agarose gel electrophoresis which permitted an unambiguous determination of the difference in superhelicity between the two samples by a direct band counting procedure which does not require a knowledge of the duplex unwinding angle of ethidium. The results of Keller's calculations indicate the primary helix is unwound by 27.7 ± 5° upon binding one molecule of ethidium. A similar approach was taken by Shure and Vinograd (51) who arrived at a value of 23 ± 3° for the duplex unwinding angle of ethidium.

Enzymatic Alteration of the Topological Winding Number

The discussion of the characteristics of superhelical DNA has, to this point, been concerned primarily with the influence of agents affecting the primary helix and the use of these agents to evaluate topological parameters. Clearly, these agents can alter only two of the three winding numbers which describe the tertiary coiling of a covalently closed DNA. By definition, since these agents do not cause breakage and reformation of phosphodiester bonds, they cannot alter the topological winding number.

There presently exist four biological means for altering the topological winding number. These are illustrated schematically in Figure 1. This discussion will be limited to these four which are enzyme-mediated and are likely to represent probable mechanisms for altering the topological winding number *in vivo*.

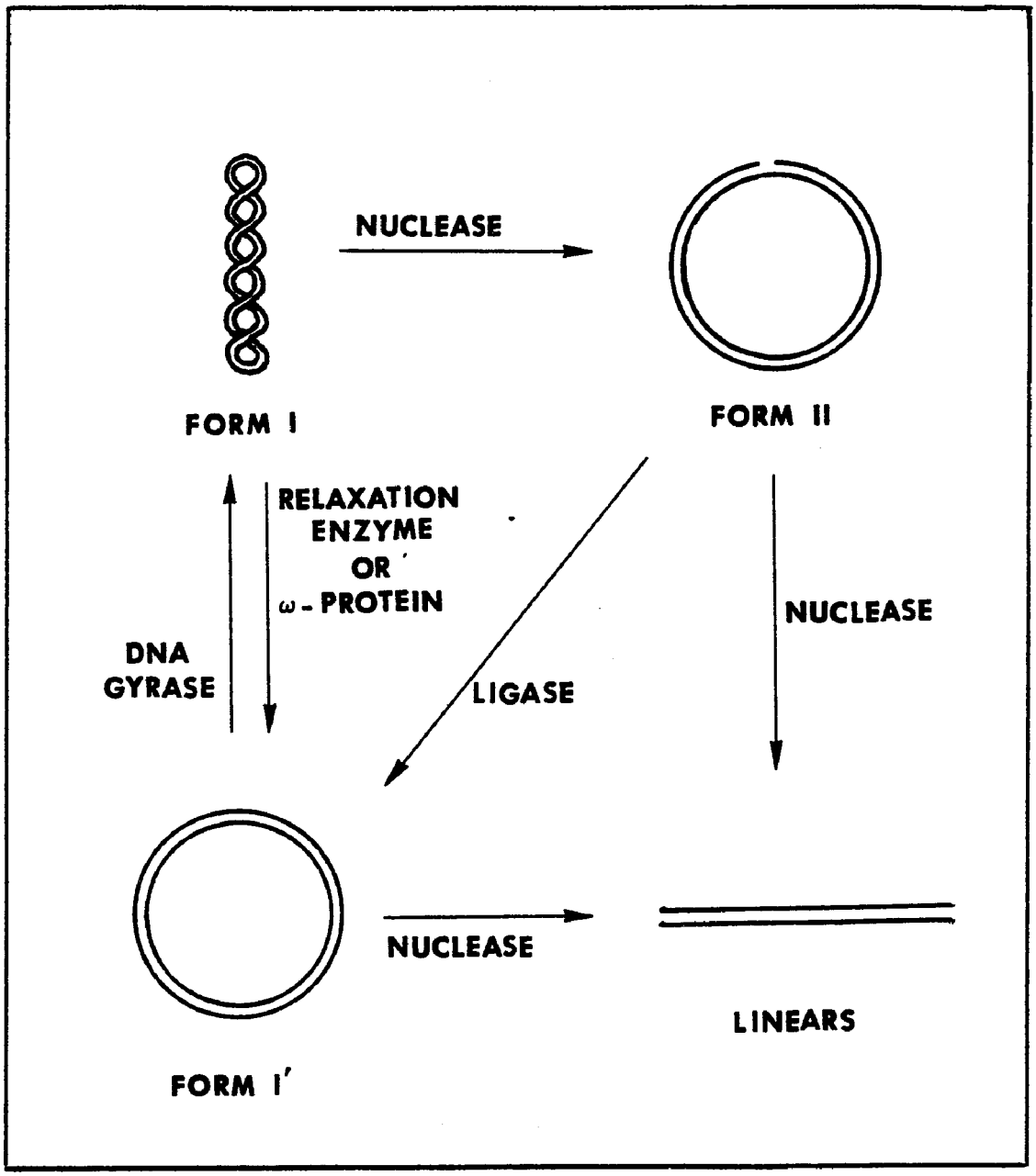


Figure 1. Forms of Circular DNA and Enzymatic Means of Interconversion

Nuclease and Ligase

The first method by which topological alteration can occur is the sequential use of an endonuclease and a DNA ligase. Hudson et al. (43) have used this method to prepare a series of covalently closed DNA molecules having various degrees of superhelicity. Limited treatment of a covalently closed supercoiled molecule with DNase I, for example, introduces a single strand break into the molecule and allows it to relax. Subsequent ligation of the nicked circular molecule restores strand continuity and renders the tertiary structure sensitive to environmental changes. As long as the environment is not altered, the topological winding number will be equal to the duplex winding number.

Relaxation Enzyme

Champoux and Dulbecco (56) originally reported the existence of an enzymatic activity from secondary mouse embryo cells which was capable of removing both positive and negative superhelical turns. Incubation of supercoiled polyoma DNA with nuclear extracts containing the activity resulted in the complete relaxation of the DNA. Control experiments ruled out the sequential action of a nuclease and a ligase since ligase activity could not be detected under the conditions of the relaxation assay. Furthermore, the relaxation activity did not require the presence of magnesium or ATP which ligase requires for activity.

The purification of this enzyme for further study was initially hampered by the lack of a suitable rapid assay system. Vosberg et al. (57) developed an assay based on the fluorescence enhancement of ethidium bromide upon binding the closed circular form of PM2 DNA. Since the amount of dye bound depends on the superhelix density of the DNA, the degree of fluorescence enhancement can be used as a measure of relaxation activity. Vosberg et al. (57) were able to purify the activity from LA9 mouse cells to homogeneity as determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis. Upon investigation of the activity, they found that the enzyme was active over a broad pH range and that it was unable to function as a ligase when supplied with a nicked circular substrate. The optimum salt conditions were in the vicinity of 0.2M NaCl but the sodium chloride could be replaced with 40 mM $MgCl_2$ with the retention of a significant degree of activity. The activity could be effectively inhibited by the addition of denatured salmon sperm DNA and to a lesser extent by undenatured DNA. The activity was also sensitive to the presence of p-hydroxymercuribenzoate which indicates that a sulfhydryl group is important for activity.

Keller and co-workers (39,58) have used agarose gel electrophoresis as an assay system for monitoring relaxation activity. They originally chose to employ this system because of the observation that their substrate for the enzyme, supercoiled SV40 DNA, migrated more rapidly on gels than did SV40 Form II. However, upon

developing the system, Keller made the surprising observation that intermediates in the relaxation reaction having superhelical densities between zero and that of the original substrate could be clearly resolved into discrete bands. Based on the number of bands observable and the estimated number of superhelical turns present in native SV40 DNA, Keller concluded that adjacent bands differed by a single superhelical turn. Thus, he was able to follow the progressive relaxation of the initial superhelical substrate during the course of the reaction. Additional studies by Keller (58) showed that the protein acts catalytically rather than stoichiometrically and therefore may be appropriately termed an enzyme. These results were later confirmed by Vosberg and Vinograd (59).

Champoux has recently looked for intermediates in the relaxation enzyme reaction containing single strand breaks (60). He was able to identify nicked intermediates by velocity sedimentation on alkaline gradients and was able to chase labeled DNA out of the nicked intermediate by addition of a large excess of unlabeled DNA. This demonstrates that the enzymatic reaction proceeds through a nicked intermediate which is rapidly sealed by the enzyme. The kinetics of the relaxation reaction are not one-hit which indicates that only a limited number of turns are released per nicking event. Champoux has suggested that the enzyme may become covalently linked to the DNA at the site of the nick thereby conserving the energy required for the closing step.

The ω-Protein

A seemingly related enzyme from E. coli was reported by Wang (61) to reduce the superhelicity of a negatively supercoiled substrate. In early investigations, Wang (14) noted that he occasionally observed the presence of a low superhelix density form of λb2b5c DNA in cell lysates. This prompted the search for a specific activity capable of altering the extent of supercoiling of a covalently closed molecule. Wang found such an activity and designated it ω. The purification of the activity was difficult because of the lack of a rapid assay system. Because at the time the work was done other techniques were not available, the assay consisted of measuring changes in the sedimentation coefficient of supercoiled λb2b5c DNA. This approach necessarily restricted the number of assays which could be reasonably and conveniently performed. Nevertheless, Wang was able to achieve a 1000-fold purification of the protein. The action of the protein caused a progressive reduction in the superhelix density of the negatively supercoiled substrate but had no effect if the substrate was positively supercoiled. Single stranded DNA was found to be an effective inhibitor of the reaction. The ω-protein seemed to function optimally in the presence of a divalent metal ion but the requirement was not absolute and activity could be detected when replaced by monovalent cations at a higher concentration. The ω-protein also had no apparent cofactor requirements.

DNA Gyrase

The unidirectional activity of the ω-protein was somewhat disturbing in terms of potential situations which might arise *in vivo* where relaxation would be required. Recently, however, Gellert *et al.* (62) have reported a new activity which they successfully isolated from *E. coli* and designated DNA gyrase. This enzyme is capable of introducing negative superhelical turns into a covalently closed relaxed substrate and has an absolute requirement for magnesium and ATP. Although the reaction is presumably driven by the hydrolysis of ATP, this has not as yet been demonstrated. The cofactor requirement is not surprising since the introduction of superhelical turns is thermodynamically unfavorable. In the presence of spermidine, the superhelix density of the product is about 1.5 times the superhelix density of native ColE1 DNA while in the absence of spermidine, the final superhelical density of the product is about equal to that of native closed DNA.

The Origin of Tertiary Coiling in vivo

Vinograd and Lebowitz (63) speculated shortly after the discovery of covalently closed DNA that one possible origin of superhelical turns was the coiling of the DNA into an ordered structure *in vivo*. Wang, in early experiments, speculated that tertiary coiling *in vitro* arises as a result of the differences in ionic strength, ionic composition, and temperature between the intracellular environment and the *in vitro* environment (14). Wang

did indicate, however, that the existence of an ordered tertiary structure *in vivo* could not be ruled out but that the mechanism responsible for the coiling must be such that the number of turns introduced must be proportional to the length of the DNA.

Fundamental Chromosome Structure in Eukaryotes

In most eukaryotic organisms, the association of DNA with histones has long been recognized. The five histones, F1, F2A1, F2A2, F2B, and F3 constitute the bulk of the protein which is intimately associated with the DNA. Early attempts to elucidate the precise structure of chromatin were frequently influenced by the mode of sample preparation and the results were variable. Nevertheless, it became apparent early-on that chromosomal DNA in eukaryotes must be highly condensed (64).

Some of the first evidence that the DNA was condensed into a regular ordered structure came from the work of Pardon and Wilkins (65) and Pardon *et al.* (66) which involved X-ray diffraction from films of oriented nucleohistone. Based on their data, they proposed a supercoil model to explain the condensation of DNA. Their model suggested that the DNA was wound into a continuous uninterrupted supercoil which was stabilized by bound proteins. Unfortunately, much of the X-ray data was also subject to the influences of ambient ionic conditions and the relative humidity.

Bram and Ris (67) observed that chromatin prepared for electron microscopy by the most gentle methods appeared as 100 Å fibrils

having many regularly spaced knobby protuberances. In light of the problems associated with sample preparation, however, this result could not be regarded as definitive.

Experiments which began to bear directly on the primary structure of chromatin were performed by Hewish and Burgoyne (68). Using intact nuclei which contain an endogenous divalent metal-requiring nuclease, they were able to show after limited digestion of the chromatin that DNA fragments were generated which were multiples of a unit length. Upon sizing of the fragments, the lower length limit was found to be on the order of 200 base pairs and seemed to represent a unit of repeating chromatin structure. Kornberg (69) proposed a model for the base subunit structure of chromatin based on these results, on known patterns of histone association, and on electron micrographic evidence. This model, in light of more recent results appears to be fundamentally correct. Kornberg proposed that the chromatin fiber is a flexible chain of repeating units 100 Å in length and that the basic chromatin unit with which the DNA is associated is an eight histone complex containing two molecules of each of the histones except F1. Olins and Olins (70) observed in chromatin from burst nuclei that appeared as a string of uniform beads regularly spaced along the chromatin fiber. More recently, Griffith observed that SV40 minichromosomes isolated from infected monkey cell cultures appear as a string of beads 100 Å in diameter and connected by short flexible bridges

corresponding to about 30 base pairs of DNA (71). Similarly, Oudet et al. (72) have also obtained electron micrographic evidence of this same fundamental structure and, in addition, showed that they could reconstitute a similar beaded structure from purified histones and DNA.

Other investigators, using staphylococcal nuclease, showed that the beads themselves could be isolated following limited nuclease treatment (73,74). The beads, also known as ν-bodies or nucleosomes, contained 150 to 200 base pairs of DNA and appeared to represent the fundamental repeating unit of chromatin. Bakeyev et al. (75) have shown that each nucleosome contains approximately equimolar quantities of each of the histones F2A1, F2A2, F2B, and F3 but that the amount of F1 observed is variable.

Considerable attention has been focused on the nature of the association of the DNA with the histones comprising the nucleosome. According to Kornberg's model (69) and the direct evidence obtained by Griffith (71), the DNA is condensed about 7-fold in the chromatin fiber. This implies that within each nucleosome the DNA is bent or coiled to achieve that degree of condensation. Pardon et al. (76) have examined low angle neutron scattering from chromatin subunits and have determined that the radius of gyration of the DNA within the subunit is approximately 50 Å while the radius of gyration of the protein is only 30 Å, suggesting that the DNA is situated on the outside surface of the nucleosome. Corroborating evidence was obtained from experiments performed by Noll (77) in

which he treated chromatin with DNase I. DNase I appeared to exhibit a preference for a limited number of cleavage sites within the subunit. Digestion of chromatin *in situ* with DNase I followed by denaturation and electrophoresis of the DNA reveal that the resulting fragments are multiples of a 10 base pair unit. The accessibility of the DNA to the enzyme suggests that the DNA is located at or near the outside surface of the nucleosome. This unusual result was subsequently confirmed by Camerini-Otero *et al.* (78) and Simpson and Whitlock (79) using staphylococcal nuclease. Extensive digestion decreases the size of the unit length DNA fragment reported by Hewish and Burgoyne from about 200 base pairs to about 140 to 160 base pairs. This unit fragment, when subjected to electrophoresis under denaturing conditions, can be resolved into a series of bands corresponding to multiples of 10 base pairs. This significance of the 10 base pair interval is poorly understood. Cantor (80) has offered a model to account for this unusual pattern of nuclease susceptibility. According to Cantor's preferred model, if DNA binds the histone core along a circularly re-entrant path, the location of the DNA entrance and exit can occur at any number of distinct sites. Assuming that the DNA is susceptible to cleavage at only one point on the nucleosome, the location of the DNA entrance and exit sites will determine the proximity of these sites to the cleavage site, thereby determining the length of the DNA fragment observed on denaturing gels. This, combined with the natural

10-fold periodicity of B DNA, can account for the observed cleavage pattern. As Cantor points out, the striking feature of the digestion pattern is that the cleavage occurs only at a small number of potentially susceptible sites.

The precise orientation of the histone monomers within the nucleosome and their interaction with the DNA remains undefined. Camerini-Otero et al. (78) examined the nuclease digestion patterns obtained by treatment of reconstituted complexes of DNA with virtually all possible combinations of purified histones. Their conclusions are based on the assumption that protection of DNA fragments of discrete length by a histone or histone combination is indicative of an intimate association with the DNA which is representative of the naturally occuring interaction. It was evident from their results that no single histone could protect DNA in such a way as to preserve fragments of a discrete length. The only subsets of histones capable of protecting fragments of a defined length during nuclease digestion were F2A2, F2B, and F2A1, and mixtures containing F2A1 and F3. No evidence of structure could be found in the absence of F2A1. However, only when all four major histones were present did the digestion pattern appear very similar to that of native chromatin. Of particular interest was the observation that the F2A1-F3 histone pair could weakly stabilize lengths of DNA of the size of the nucleosome core although the predominant intermediate was a fragment 70 base pairs in length. The authors

suggest that a dimer of F2A1 and F2 interacts strongly with a DNA segment 70 base pairs in length and that a pair of such dimers interact to form the 140 base pair structure characteristic of nucleosomes. Roark et al. (81) have shown that in solution an equilibrium exists between F2A1-F3 dimers and tetramers. In a subsequent report by Sollner-Webb et al. (82) analysis of reaction rate constants indicated that the DNA in F2A1-F3:DNA complexes is restricted in its accessibility to staphylococcal nuclease in much the same way as native chromatin. Additional evidence along this line was presented by Boseley et al. (83). They reported that each of the four major histones are necessary for proper reconstruction of chromatin as determined by X-ray diffraction although a partial reconstitution and development of higher order structure was possible using only F2A1 and F3.

Simpson (84) has recently shown that F2A1 and F3 interact with the ends of nucleosome DNA. Isolated nucleosomes were labeled at the 5'termini of their DNA with [^{32}P] ATP and polynucleotide kinase. The label was then cross-linked to a lysyl residue of a neighboring histone and the noncross-linked DNA removed by digestion with DNase I. The histones were then separated by polyacrylamide gel electrophoresis and the label located by autoradiography. The results indicated that F2A1 and F3 occur with equal frequency as nearest neighbors to the ends of nucleosome DNA.

The absolute arrangement of histones within the nucleosome has not been defined. A number of investigations have been

conducted to probe the internal arrangement of proteins within the nucleosome (85,86). Physical studies indicate that the four predominant histones have several features in common. The N-terminal region of the histones is basic and seems to be the region which interacts with the DNA. The histones also possess a hydrophobic region which extends to, or nearly to, the C-terminus. These regions have been shown in certain cases to have a strong tendency to self-associate (87,88).

Germond et al. (89) have presented the first strong evidence which confirms the hypothesis that the DNA associated with the nucleosome is coiled. Using purified histones and closed relaxed SV40 DNA, nucleosomes were reconstructed in vitro and examined by electron microscopy. The resulting structures appeared twisted and had nucleosomes spaced at regular intervals. Treatment of these structures with the relaxation enzyme from Krebs II ascites cells followed by removal of the protein resulted in the appearance of supercoiled DNA as determined by agarose gel electrophoresis. The amount of supercoiling was dependent on the histone to DNA ratio in the reconstruction mixtures and the association of histones with the DNA was found to be facilitated when native supercoiled SV40 DNA was used in place of covalently closed relaxed DNA. Crick (90) has addressed the problem of DNA coiling within the nucleosome and from his model-building studies has suggested that the DNA could be "kinked" rather than bent smoothly without invoking a large unfavorable free energy change. As yet there is no evidence which either supports or contradicts his model.

Weintraub et al. (91) have offered an attractive model for nucleosome structure which is quite detailed. Their model proposes that the nucleosome is composed of two symmetrically paired half-nucleosomes consisting of one molecule of each of the four major histones. The DNA is associated with the nucleosome as a single left-handed non-interwound coil about 95 base pairs in circumference. The basic N-terminal regions of the histones lie along the DNA and protect from nuclease digestion about 40 base pairs of DNA at each end of the 140 base pair coil. Their model is attractive because it can account for most experimental results and also provides a mechanism whereby transcription can take place without requiring complete dissociation of the protein. The authors propose that each half-nucleosome binds only to one strand of the DNA such that transient unpairing of the half-nucleosome would not cross-link the strands. It would then be possible for polymerases to read the sequence of the DNA without requiring total disruption of the nucleosome structure.

The precise amount of winding per nucleosome has yet to be established. From the work of Germond et al. (89) and from calculations involving the known superhelix densities of naturally occurring DNA and the nucleosome frequency per unit length, it seems reasonable to conclude that the amount of supercoiling generated per nucleosome is on the order of 1 to 1.25 turns. Any quaternary coiling of the nucleosomes is expected to contribute to this figure. Little is currently known about quaternary coiling

of chromatin but certain X-ray diffraction data seems to suggest the presence of an ordered quaternary structure. Carlson and Olins (92) have attempted to fit calculations for helices composed of various numbers of nucleosomes to their X-ray data. They suggest that a regular array of nucleosomes is responsible for the X-ray data but that none of the models for nucleosome coiling that they considered was uniquely consistent with their data. However, several of their models did seem preferable when such factors as the non-uniform electron density of the nucleosome as well as the solvent and background scatter are considered. Bram et al. (93) have proposed a model based on their neutron scattering experiments in which the 100 Å nucleosomes are coiled into a higher order structure with a major pitch of 500 Å and a radius of 130 Å. However, to date there is little supportive evidence for this model.

At the chromosomal level, Benyajati and Worcel (94) have found that the isolated folded interphase genome of Drosophila melanogaster is supercoiled. The intact DNA sediments very rapidly on neutral sucrose gradients with a sedimentation coefficient of about 5000S and represents DNA complexed with RNA and protein. The rapidly sedimenting DNA can be converted to a more slowly sedimenting form by intercalating ethidium or by limited treatment with DNase I. They have determined that the superhelix density of the DNA is about the same as that of other native closed circular DNAs and corresponds to about one turn per 200 base pairs. In addition, the DNA

appears to be subdivided into domains of supercoiling which are about 85,000 base pairs in length. The nature of the constraint which defines the domains has not been defined.

Chromosome Structure in Prokaryotes

Despite the fact that in general bacteria, and in particular E. coli, have been studied more extensively than eukaryotic cells, the fact remains that far less is certain about the organization of bacterial DNA. At least in the well known E. coli species, no proteins have been found which are analogous to eukaryotic histones. A specific search for such proteins failed to detect any DNA binding proteins present in sufficient quantities to serve as the functional equivalents of histones (95). However, a recent report by Griffith has provided the first clue to the fundamental structural organization of the bacterial genome (96). Griffith, by lysing bacteria directly on electron microscope grids, was able to visualize in the electron microscope beaded structures which appeared virtually identical to the nucleosomes of eukaryotic cells. These beads were highly unstable and could be seen only when the cells were lysed directly on carbon coated grids and the dehydration steps begun within one to two minutes of lysis. Although it is generally conceded that electron microscopy is a technique which is particularly susceptible to the generation of artifacts, the result is clearly consistent with, and offers an explanation for, the observed superhelix density of native prokaryotic DNAs if it is postulated that the DNA is coiled within

each prokaryotic bead as it is within eukaryotic nucleosomes. Griffith suggests that the beaded structure in prokaryotes may be stabilized in the cell by complex formation with magnesium, polyamines, and perhaps a heterogeneous class of weakly bound proteins.

At the chromosomal level, Worcel and Burgi (97) have found that the intact E. coli chromosome sediments at a rapid rate on neutral sucrose gradients. As with the Drosophila chromosome, the E. coli chromosome can be converted to a more slowly sedimenting form by intercalation of ethidium or by limited treatment with DNase I. The authors have, on the basis of a velocity sedimentation-dye titration, concluded that the chromosome has a superhelix density similar to that of the smaller circular plasmid DNAs isolated from E. coli and corresponds to about one turn per 200 base pairs. Furthermore, the overall chromosome appears to be subdivided into domains of supercoiling each consisting of about 85,000 base pairs.

The apparent similarity between the structural organization of eukaryotic and prokaryotic DNA is striking. Assuming Griffith's observations are correct, both eukaryotic and prokaryotic DNA seem to be similarly organized both at a fundamental level within the beads as well as on a larger scale in terms of the entire chromosome. It is tempting to speculate that perhaps evolutionarily there has been a fundamental organization which has been advantageous to preserve, even though the mechanism for preserving it has been altered in the evolutionary process.

Plan of This Thesis

The research to be presented in this thesis is largely an outgrowth of a fortuitous observation made during the course of preliminary experimentation directed towards the development of a rapid assay system for relaxation enzyme. It was reasoned that agarose gel electrophoresis could be used to monitor relaxation activity since this technique offered a means by which a large distinct separation of Form I and Form II SV40 DNA could be achieved. While investigating the efficacy of this technique as an assay system, it was noted that covalently closed relaxed SV40 DNA was resolved into a series of distinct species. At the time this research was initiated, the reports of Keller's findings (39) had not yet appeared in the literature and this independent observation was considered novel. Nevertheless, with the publication of Keller's report, it became apparent that the assay system was already well developed and characterized and had, in fact, served its purpose in facilitating the purification of relaxation enzyme. However, it was also equally apparent that the agarose gel system offered a unique approach to the analysis of supercoiled DNA and that the sensitivity of the technique was unrivaled by any other technique commonly employed for this purpose. In light of this, the plan of approach decided upon was first to characterize the agarose gel system in terms of a number of environmental variables, to investigate and interpret the multiple banding pattern observed with covalently closed SV40 DNA,

and extend the use of the technique to other covalently closed DNAs to determine if additional useful information could be obtained.

MATERIALS AND METHODS

The essential features of the methods which were employed and the materials used in these studies are presented in this section. Significant deviations in the methodology will be described in detail in connection with specific experiments in the section entitled RESULTS.

SV40 Growth and DNA Purification

SV40 strain 776 was used to infect confluent monolayers of CV-1 monkey cells grown in Dulbecco's Modified Eagle Medium (Gibco, Grand Island, New York) at a multiplicity of 0.01 pfu/cell. When approximately 30 percent of the cells showed a visible cytopathic effect, viral DNA was labeled by addition of either [^{3}H] -thymidine (20Ci/mmole, New England Nuclear, Boston, Massachusetts) to a concentration of 10 μCi/ml or sodium [^{14}C] -formate (61 mCi/mmole, Amersham-Searle, Arlington Heights, Illinois) to a concentration of 10 μCi/ml. Covalently closed DNA was isolated by Hirt lysis (98) when the infection had progressed to 95 to 100 percent visible cytopathic effect. The Hirt supernatant was deproteinized with chloroform:isoamyl alcohol (24:1) and banded twice in CsCl-ethidium bromide equilibrium gradients. CsCl was purchased from Kawecki-Berylco Industries, Reading, Pa. and was the high purity grade. The lower band in each gradient, consisting of covalently closed viral DNA, was collected and the ethidium bromide (Sigma,

St. Louis, Mo.) extracted with NaCl-saturated isopropanol. Following dialysis against 10 mM Tris, 10 mM NaCl, 1 mM EDTA, pH 8.1 (TEN), the DNA was treated with Ribonuclease A (Sigma) at a concentration of 40 µg/ml. The resulting ribonucleotides as well as nicked circular and oligomeric forms of SV40 DNA were removed by velocity sedimentation through 5 to 20 percent neutral sucrose gradients in an SW27 rotor in a Beckman L2-65B ultracentrifuge. The band of covalently closed monomeric SV40 DNA sedimenting at 21S was dialyzed, ethanol precipitated and redissolved in TEN for storage at 4°C. The specific radioactivity of the DNA was determined by spotting an aliquot of the solution on a Whatman 3MM filter paper disc. When dry, the disc was immersed in 10 ml of toluene containing 2 g/l of 2,5 diphenyl-oxazole (Isolabs, Akron, Ohio) and the amount of radioactivity determined in a Nuclear Chicago Mark II scintillation counter. DNA concentrations were determined by measuring absorbance at 260 nm in a Zeiss PMQII spectrophotometer. An absorbance of 1.0 was taken to represent a DNA concentration of 50 µg/ml. The specific radioactivity of tritium labeled DNA was generally 2-4 x 10^4 cpm/µg whereas that of ^{14}C labeled DNA was 1-3 x 10^3 cpm/µg.

Bacterial Strains and Plasmids

The closed circular DNAs of bacterial origin and the strains of Escherichia coli which harbor them are listed in Table 1. E.

coli C infected with ØX174am3, a lysis defective phage carrying an amber mutation in the E cistron, was the generous gift of Dr. Lawrence Grossman and was supplied as a frozen cell paste. All other strains were grown from frozen glycerol stocks stored at -90°C.

Prior to growth of the bacterial strains on a large scale, isolated colonies were tested for the presence of appropriate plasmid coded markers. Strains harboring ColE1 or ColE1 derivatives were tested for colicin resistance in L-broth essentially as described by Clewell (99). Colicin E1 was kindly supplied by Dr. Carolyn J. Collins. The plasmid pDC1 is temperature sensitive for DNA replication and was routinely tested for loss of colicin resistance at 42°C. Tetracycline and ampicillin resistance was determined by streaking the strains to be tested on tryptone plates containing 25 μg/ml tetracycline (Sigma) or 25 μg/ml ampicillin (Ayerst Laboratories, Inc., New York) respectively.

Bacterial Growth and DNA Purification

Colonies exhibiting the desired characteristics were picked for large scale growth. In general, plasmid-carrying strains were grown in L-broth without selective pressure. A 10 ml aliquot of L-broth was inoculated with the desired strain and incubated overnight at 28°C. An aliquot of this starter culture was then used as the inoculum for a larger culture ranging in volume from 200 ml to 1 liter. Bacterial growth was monitored by

TABLE 1

SUMMARY OF CLOSED CIRCULAR DNAs FROM *E. COLI*

DNA	MOLECULAR WEIGHT x 10^{-6}	STRAIN	MARKERS	SOURCE	REFERENCE
pDC1	1.8	HB101 (pDC1)	Colicin Resistance Ts DNA Replication	D. Charney	personal communication
pVH51	2.2	C600 (pVH51)	Colicin Resistance	D. Helinski	100
ØX174 RF	3.4	C (ØX174*am3*)	--	L. Grossman	101
pMB9	3.5	HB101 (pMB9)	Colicin Resistance Tetracycline Resistance	I. Dawid	102
ColE1	4.2	JC411 (ColE1) *Thy*$^-$	Colicin Resistance	D. Clewell	99
pSC101	5.8	C600 (pSC101)	Tetracycline Resistance	J. Morrow	103
ColE1*amp*	7.3	C600 (ColE1*amp*)	Colicin Resistance Ampicillin Resistance	R. Helling	104

measuring the optical density of the culture with a Klett-Summerson colorimeter equipped with a red filter (No. 66). The initial optical densities of the cultures were on the order of 20 Klett units. Because pDC1 is a temperature sensitive plasmid, cultures of strains harboring ColE1 or ColE1 derivatives were grown at 28°C, both as a matter of convenience when a number of cultures were grown simultaneously, and to insure accurate comparisons without having to be concerned about temperature effects. ColE1 and its derivatives may be amplified by addition of chloramphenicol to the medium (99). When these cultures reached an optical density of 100 to 120 Klett units, chloramphenicol (Parke Davis & Co., Detroit, Mich.) was added to a final concentration of 250 µg/ml. Immediately following the addition of the chloramphenicol, [^{3}H] -thymidine was added to 2 µCi/ml. After 16 to 18 hours of continuous aeration at 28°C, the cells were collected by centrifugation in a Beckman J-21 centrifuge at 4°C. Plasmids which could not be amplified with chloramphenicol were grown in a similar fashion and were labeled at the same optical density. Growth was monitored until the culture reached late log phase at which time the cells were collected by centrifugation.

The cell pellets obtained by centrifugation were washed twice with cold TEN and were resuspended in 0.05 M Tris, 25 percent sucrose, pH 8.1. DNA was extracted from the washed cells using the cleared lysis procedure. Lysozyme (Worthington Biochemical, Freehold, New Jersey) was added to a concentration of 5 mg/ml

and the cells were incubated on ice for 10 minutes. Following this incubation, EDTA was added to a concentration of 0.1 M and the incubation continued at 0°C for an additional 5 minutes. Cells were lysed by addition of 50 mM Tris, 62.5 mM EDTA, 0.4 percent sodium deoxycholate, 1 percent Brij 58 (polyoxyethylene 20 cetyl ether, Sigma). The cleared lysate was then centrifuged at 20 krpm to remove cellular debris. The resulting supernatant was collected and immediately extracted with chloroform:isoamyl alcohol to remove proteins. Occasionally, difficulty was encountered if the lysate was allowed to stand on ice for any length of time without further treatment. When protein extraction was delayed, there was frequently evidence of ω-protein activity (61) which resulted in the appearance of partially relaxed intermediates when the purified DNA was subjected to electrophoresis on agarose gels. This activity was never observed when the protein was removed immediately after centrifugation of the lysate. Following protein extraction, the DNA was banded to equilibrium as described for SV40 DNA. Most plasmid preparations were banded only once and were not treated with ribonuclease. For the purposes of the experiments performed, a single banding in CsCl-dye gradients followed by extraction of the ethidium, dialysis, and concentration of the DNA by ethanol precipitation gave acceptable levels of purity. Bacterial RNA species were rarely observed when plasmid DNA preparations were analyzed by gel electrophoresis although there was frequently a band of high molecular weight bacterial DNA visible in the gels.

Molecular Weight Determinations

Many of the experiments to be described required a reasonably accurate knowledge of the molecular weights of the closed circular DNAs used. Helling et al. (105) have reported that the electrophoretic mobility of linear DNA fragments on agarose gels is inversely proportional to the logarithm of the molecular weight. The linear forms of the bacterial DNAs listed in Table 1 were subjected to electrophoresis on 0.7 percent agarose slab gels and the mobilities calculated relative to SV40(L). The molecular weight of SV40 DNA was taken to be 3.25 million. Figure 2 shows a semi-logarithmic plot of molecular weight versus relative mobility. The curve was constructed using all of the monomeric plasmid DNAs used in this study as well as dimeric forms of several of the DNAs which were frequently present in small amounts in the DNA preparations. The plot is essentially linear to a molecular weight of about 5×10^6. Any significant error in the molecular weight values reported in the literature would introduce scatter into the plot. The lack of significant scatter is interpreted as indicating reasonably accurate molecular weight values. The departure from linearity when the molecular weight exceeds $5\text{-}6 \times 10^6$ is in agreement with results obtained by others using similar conditions (Robert Deans, personal communication).

Figure 2. Relative Electrophoretic Mobility of Linear DNA as a Function of Molecular Weight.

The linear forms of the DNAs used in these studies were subjected to electrophoresis on triplicate 0.7 percent agarose slab gels. Mobilities are calculated relative to that of SV40 linear DNA. In order of decreasing molecular weight, the points correspond to ColE1amp, ØX174 RF dimer, SV40 dimer, pSC101, ColE1, pMB9, ØX174, SV40, pVH51, and pDC1.

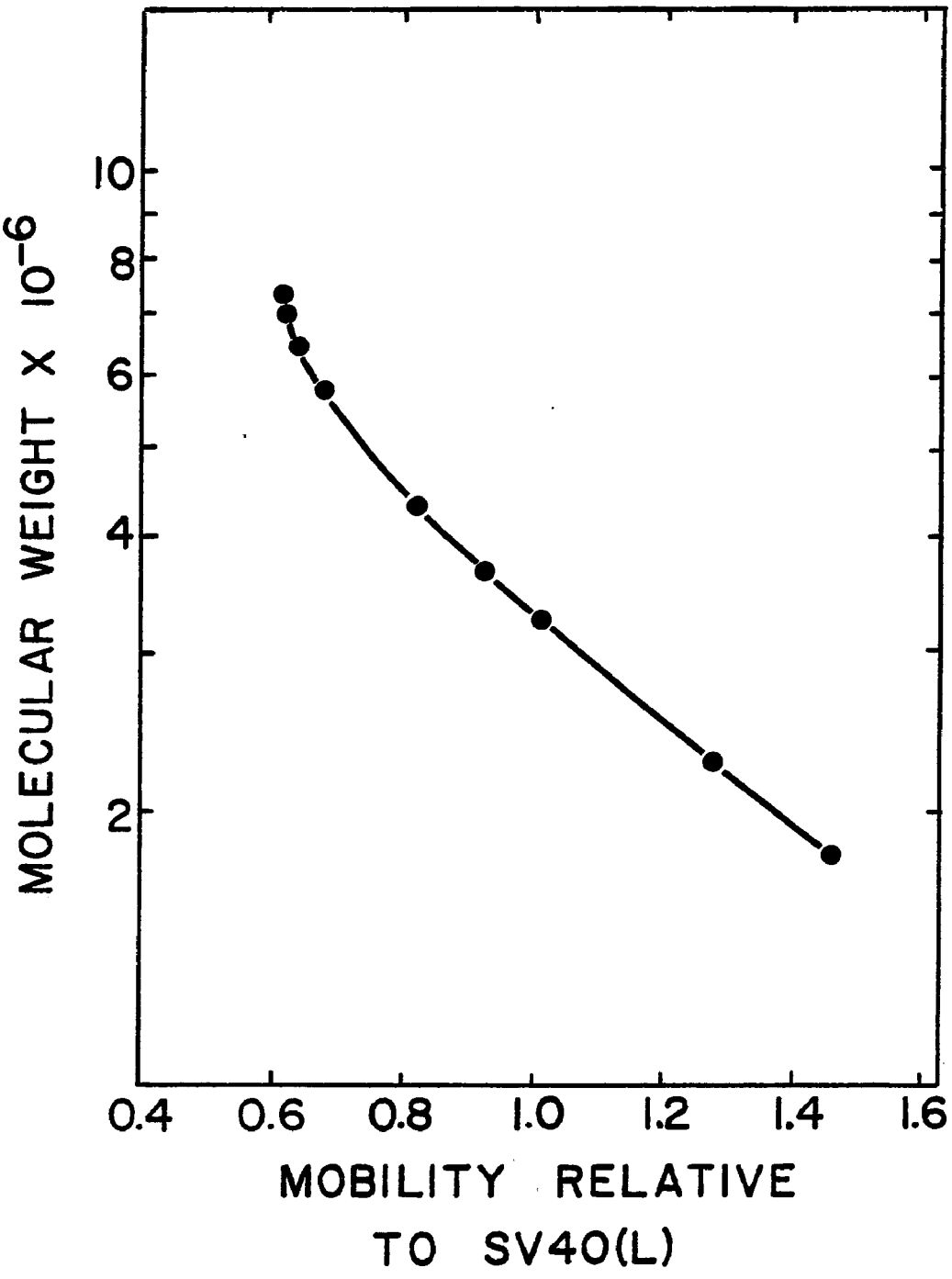

MOLECULAR WEIGHT X 10^{-6}
10
8
6
4
2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
MOBILITY RELATIVE
TO SV40(L)

Figure 2

Preparation of Covalently Closed Relaxed DNA

Two methods were used to generate covalently closed relaxed DNA from native supercoiled DNA. These two methods are shown in Figure 1.

Form II DNA was generated by limited digestion of native Form I DNA with DNase I in 10 mM Tris, 10 mM $MgCl_2$, pH 7.4, at a DNA concentration of 25 µg/ml. DNase I (Sigma, electrophoretically pure) was added to a concentration of 0.5 ng/ml and the reaction was carried out at 28°C. Nuclease activity was terminated by the addition of EDTA to a concentration of 20 mM after a 20 to 25 minute incubation period. Form II DNA and the unreacted supercoiled substrate were separated by velocity sedimentation on 5 to 20 percent neutral sucrose gradients. This procedure usually resulted in the conversion of approximately 60 percent of the Form I to nicked circular DNA. The nicked circular DNA isolated by velocity sedimentation was concentrated by ethanol precipitation and was used as the substrate for T4 ligase (Miles Laboratories, Kankakee, Ill.). The ligase reaction was performed as described by Modrich and Lehman (106) in a reaction mixture consisting of 66 mM Tris, 10 mM $MgCl_2$, 0.066 mM rATP (Sigma), 10 mM dithiothreitol, 1 mM EDTA, 50 µg/ml autoclaved gelatin (Difco, Detroit, Michigan) at pH 8.1. DNA concentrations ranged from 5 to 20 µg/ml. Reactions were terminated by addition of EDTA and sodium lauryl sarcosinate (Sigma) to final concentrations of 25 mM and 1 percent respectively. The reaction mixtures were centrifuged

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on 5 to 20 percent neutral sucrose gradients containing 40 μg/ml ethidium bromide to separate the Form I' DNA from the Form II substrate and linear DNA. The ethidium was extracted from the covalently closed relaxed DNA by adding NaCl to a concentration of 1.0 M and passing the DNA solution over a small (1-2 ml) column of Dowex AG 50W-X8 (BIO-RAD Laboratories, Richmond, Cal.). The DNA solution was then dialyzed against TEN, concentrated by ethanol precipitation, and redissolved in TEN for use.

Covalently closed relaxed Form I' DNA was also prepared by incubation of native supercoiled DNA with the relaxation enzyme from KB cells. The relaxation enzyme was the generous gift of Dr. Walter Keller and was supplied in a partially purified form in 0.6 M KPO_4, 2 mM 2-mercaptoethanol. The enzyme was stored at a concentration of approximately 0.3 mg/ml in 10 μl aliquots at -90°C. The reaction mixture for the relaxation reaction was 10 mM Tris, 0.2 M NaCl, 1 mM EDTA, pH 7.9. The relaxation enzyme was diluted tenfold in 0.02 M KPO_4, 1 mM dithiothreitol, 10 percent glycerol, 1 mg/ml BSA, pH 7 prior to addition to the reaction mixture. Reactions were terminated by addition of an equal volume of redistilled phenol and vortexing. The aqueous layer was removed after a brief centrifugation at 2 krpm in a Sorvall GLC-1 centrifuge and dialyzed against TEN for several hours. Although reaction mixtures could be applied directly to gels without dialysis, the dialysis step was found to be important for a number of reasons. If ethidium was added to a sample

containing phenol, the ethidium was preferentially extracted into the organic phase. Secondly, even in the absence of phenol, a DNA sample in a high salt buffer was poorly resolved on gels. Large amounts of salt in the sample typically resulted in broader more diffuse bands.

Electrophoresis

Electrophoresis was performed in both cylindrical gels and slab gels depending on the nature of the experiment. Agarose (SeaKem) was obtained from Marine Colloids Inc., Rockland, Maine and was usually prepared at a concentration of 0.7 percent in electrophoresis buffer consisting of 40 mM Tris, 5 mM sodium acetate, 1 mM EDTA, adjusted to pH 8.2 with acetic acid. Cylindrical gels were formed by pouring melted agarose in electrophoresis buffer into 0.6 x 16 cm gel tubes. After solidification of the agarose, the gel was partially extruded and cut to a length of 15 cm. For some experiments it was advantageous to use 27 cm gels which were prepared in a similar manner. Gels were retained in the glass tubes by covering the bottom of the gel tube with a nylon mesh and securing with a Tygon ring.

The apparatus used for cylindrical gels could accommodate a maximum of 12 gel tubes. When 27 cm gels were employed, a specially constructed lower buffer chamber was used which would accommodate the longer tubes.

Slab gels were prepared by pouring molten agarose between two Plexiglas plates separated by 0.4 cm spacers. Sample wells

were formed with a Lucite slot former which was removed after the agarose had solidified. The dimensions of the resulting gel were 24 x 13 x 0.4 cm. The apparatus used for the slab gels was similar to that described by Studier (107) except that the upper buffer chamber was modified with an overflow device so that buffer could be circulated between the upper and lower chambers by means of a peristaltic pump. Buffer cycling was found to be necessary to prevent large changes in the pH that occurred over the course of the run.

For many experiments, it was necessary to include ethidium bromide in the gel. Ethidium was added only when the agarose had cooled to 50°C and gels were poured immediately after addition of the dye. The dye concentrations of stock solutions were measured spectrophotometrically at a wavelength of 480 nm. Hinton and Bode (29) have reported that the extinction coefficient of ethidium bromide is somewhat concentration dependent. Therefore, absorbance measurements were always made on solutions of ethidium which were approximately 20 µg/ml. An abosrbance of one was taken to represent an ethidium concentration of 69.6 µg/ml. The measurement of ethidium concentrations was found to be necessary primarily because the ethidium as purchased from the supplier (Sigma) was not anhydrous. Absorbance readings were consistently 8 to 10 percent lower than that expected for anhydrous ethidium bromide which indicates that water of hydration could introduce a significant degree of error when not taken into account.

The power supplies used for electrophoresis were manufactured by Gelman (Model 38201-1, Ann Arbor, Mich.). Cylindrical 15 cm gels were routinely run at 6.7 V/cm while slab gels were run at 1.6 to 2.5 V/cm. Electrophoresis times were varied somewhat in accordance with the molecular weight of the DNA being examined but as a rule the times generally ranged from 3 to 4 hours for cylindrical gels and 18 to 22 hours for slab gels.

Samples for electrophoresis were made 5 percent in sucrose for cylindrical gels and 2.5 percent in Ficoll 400 (Pharmacia, Upsala, Sweden) for slab gels. Ficoll gave better resolution and less trailing with slab gels than did sucrose. The amount of DNA per sample was usually between 0.1 and 0.5 µg for cylindrical gels and between 0.4 and 1 µg for slab gels. Bromphenol blue (Sigma) was included at a concentration of 0.03 percent in all samples as a tracking dye. When ethidium bromide was present in the gel, it was also included in the sample mixture in a 0.5 to 2-fold weight excess over DNA.

DNA Quantification and Photography

Gels were stained for 45 minutes to an hour in freshly prepared electrophoresis buffer containing 1 µg/ml ethidium bromide. The DNA bands were visualized by placing the gel over a long-wave ultraviolet light source (C50 transilluminator, Ultraviolet Products, San Gabriel, Cal.). When DNA quantification was not required, photographs were taken with a Polaroid

MP-3 camera using Polaroid Type 55 P/N film, a UV filter (Ultraviolet Products) and a Kodak No. 23 Wratten gelatin filter. When quantification was required, photographs were taken on Kodak Pan-X film using a Crown Graphic camera equipped with a UV filter, and a Kodak No. 9 Wratten gelatin filter at an aperture of f4.7. Exposed film was developed in the dark in Kodak D11 developer for 13 minutes. A Kodak photographic step tablet (No. 1A, optical density range 0.05 to 3.05) was photographed periodically to assess the linearity of the film response. A 2 minute exposure time was determined to give the best response. Negatives were scanned on a Joyce-Loebl double beam recording microdensitometer (Model MKIII CS, Gateshead-on-Tyne, Great Britain). Background levels were established for each trace by scanning the film between sample channels. Reconstruction experiments showed that under the conditions used, the heights of the peaks in the densitometer trace were proportional to the amount of DNA in each band. Amounts of SV40(I) DNA from 0.35 to 1.05 μg were electrophoresed in the presence of 15 ng/ml ethidium bromide for 18 hours. Following staining and photography, the gel photographs were traced by densitometry. For each band in the Form I series, the peak height in the densitometer trace was linearly related to the amount of DNA in the original sample. For most of the work presented, the assumption was made that all of the bands in a given sample channel have the same shape. Examination of densitometric

profiles generally indicated that this assumption was valid. This method of DNA quantification was also used successfully by Wang (41). When this mode of analysis was employed, care was taken to minimize exposure of the gel to ultraviolet light prior to photography. Prolonged exposure of the gel to ultraviolet light resulted in a significant degree of photochemical bleaching which decreased the intensity of the fluorescent DNA bands. Prolonged exposure also tended to increase the amount of background fluorescence in the gel. Gels exposed to ultraviolet light for extensive periods of time could be restored to a usable state by soaking in the staining solution.

DNA was also quantitated by scintillation counting. Fluorescent bands were excised from the gels and placed in 10 ml of ACS scintillation fluid (Amersham-Searle) for aqueous samples. The agarose slices containing DNA were agitated at 37°C on a reciprocating shaker for 24 hours prior to counting. Preliminary experiments indicated that this incubation period was required to achieve maximum counting efficiency. There was good agreement between this method and the densitometric data. However, because of the difficulty in excising closely spaced bands, this method was not routinely employed.

Data Analysis

All calculations and least-squares curve fitting was done with the aid of an HP-25 programmable calculator (Hewlett-Packard, Cupertino, Cal.). The least squares procedure used

was that outlined in the manual of program applications supplied by Hewlett-Packard (108). For data approximating a linear relationship of the form $y = mx + b$, the slope of the least squares line was calculated according to the equation

$$m = \frac{\Sigma xy - \frac{\Sigma x \Sigma y}{n}}{\Sigma x^2 - \frac{(\Sigma x)^2}{n}} \qquad (12)$$

where n is the total number of paired data points (x,y) analyzed. The y-intercept of the plot was determined from the relationship

$$b = \frac{\Sigma y}{n} - m\frac{\Sigma x}{n} \qquad (13)$$

The coefficient of correlation was calculated for each plot and is a measure of how closely the least squares equation fits the experimental data. The relationship used to calculate this quantity (r^2) is

$$r^2 = \frac{\left[\Sigma xy - \frac{\Sigma x \Sigma y}{n}\right]^2}{\left[\Sigma x^2 - \frac{(\Sigma x)^2}{n}\right]\left[\Sigma y^2 - \frac{(\Sigma y)^2}{n}\right]} \qquad (14)$$

The value of r^2 is always between zero and one. The closer r^2 is to unity, the better the least squares curve fits the experimental data.

The method by which experimental data was analyzed will be presented with the results. The quantities which were substituted for x and y in the equations listed above are described fully in

relation to the experimental data. The degree of dispersion about mean values obtained from the data is given as the standard deviation which was calculated using the equation

$$\sigma = \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x^2)}{n}}{n - 1}} \qquad (15)$$

RESULTS

Behavior of Various Conformers of SV40 DNA on Agarose Gels

During the early stages of this work, agarose gel electrophoresis of DNA had been confined primarily to linear DNA fragments generated by restriction enzyme cleavage (105). Since the object of this study was to investigate the properties of covalently closed DNA, a number of experiments were performed to assess the effects of a number of parameters related to the gel system itself. It was hoped that by investigating the effects of such variables as agarose concentration, electric field strength, and ionic strength, the system could be optimized to give maximum resolution and perhaps allow a number of qualitative statements to be made regarding the state of the DNA within the gel and its mode of migration.

The experiment which led to the linear relationship between the logarithm of the molecular weight of a linear DNA and the relative mobility shown in Figure 2 simultaneously led to similar relationships for native supercoiled and nicked circular DNAs over the range of molecular weights examined. Electrophoresis was performed in triplicate in 0.7 percent agarose slab gels using DNAs ranging in molecular weight from 1.8×10^6 (pDC1) to 7.3×10^6 (ColE1amp). The fortuitous presence of dimeric and trimeric forms in several of the DNA preparations allowed placement of additional data points. The mobilities

Figure 3. Relative Electrophoretic Mobility as a Function of Molecular Weight for Circular DNA Molecules

The solid circles (●——●) refer to native supercoiled DNA molecules. Open circles (O——O) represent points obtained for nicked circular DNAs. The mobilities of each of the DNAs in each conformation are calculated relative to the mobilities of the equivalent conformational types of SV40 DNA. The DNAs used in the construction of the plot are the same as those used for Figure 2. In addition, SV40 trimers (molecular weight 9.75×10^6) are included in the Form I plot and pVH51 dimers (molecular weight 4.4×10^6) are included in both plots.

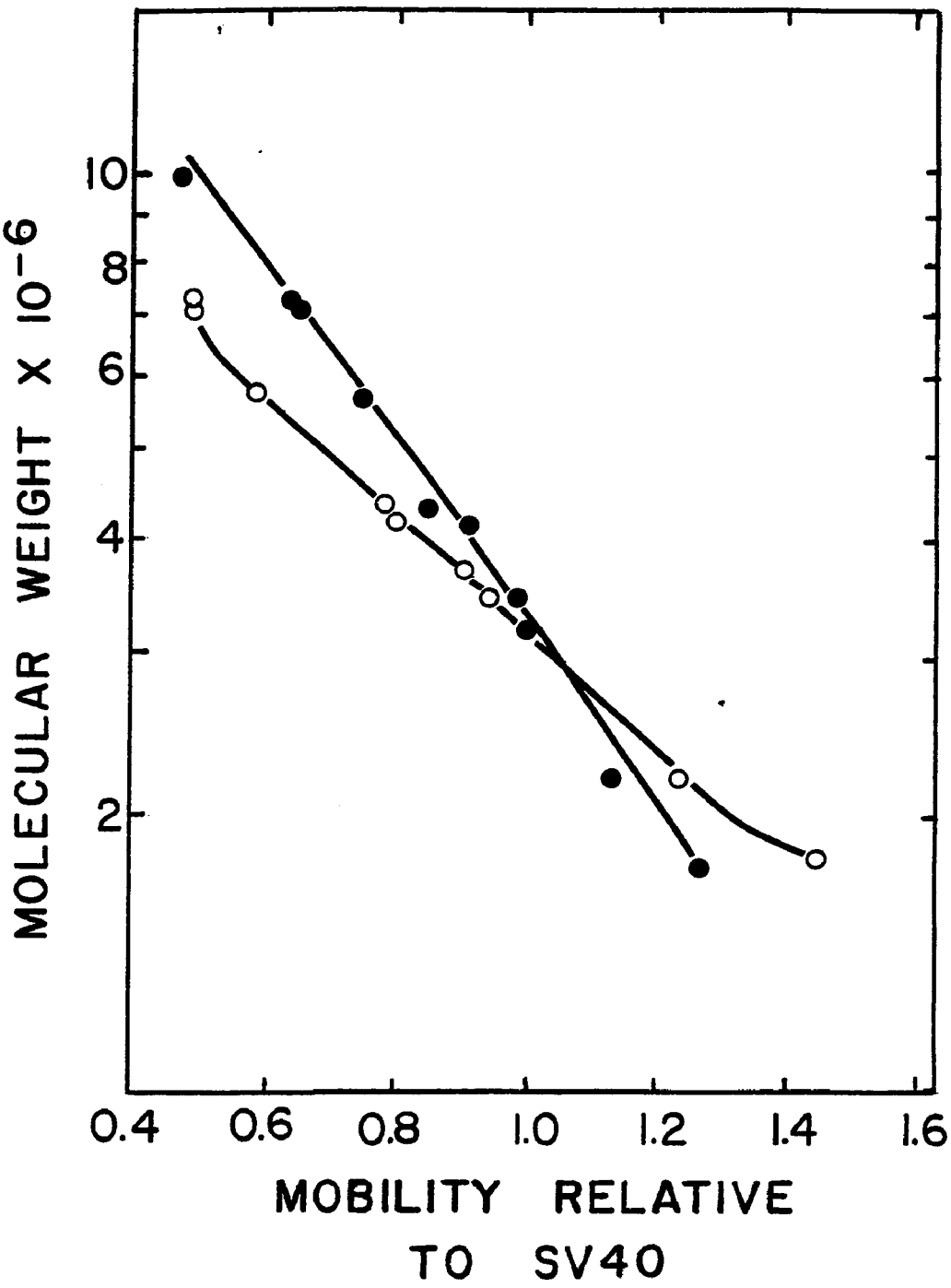

MOLECULAR WEIGHT X 10⁻⁶
10
8
6
4
2
8
0.4
0.6
0.8
1.0
1.2
1.4
1.6
MOBILITY RELATIVE
TO SV40

Figure 3

of nicked circular DNAs were calculated relative to SV40(II) while those of native supercoiled DNAs were calculated relative to SV40(I). The data for each of the two conformational types are shown in Figure 3. Both curves are essentially linear over the molecular weight range examined. For nicked circular DNA, the curve in Figure 3 appears to depart from linearity at molecular weights in excess of about 7×10^6. A similar departure from linearity may occur when the molecular weight of the DNA is below about 2×10^6. However, with only a single data point this conclusion is subject to some uncertainty. The plot for Form I DNAs does not deviate significantly from linearity within the range of molecular weights investigated. It is apparent from these curves that the ratio of the mobility of Form I DNA relative to Form II is dependent on the molecular weight of the DNA. Under the conditions in which these gels were run, SV40(I) migrates approximately 1.38 times as fast as SV40(II). Given this ratio, the ratio of mobilities of Form I and Form II for any DNA within the molecular weight range encompassed by this study may be calculated. This same calculation may be extended to linear DNAs by employing the curve shown in Figure 2. Under these conditions of electrophoresis, SV40(L) migrates approximately 1.12 times as rapidly as SV40(II).

The effects of varying agarose concentration were explored using the various forms of SV40 DNA. Cylindrical gels were

prepared containing different agarose concentrations but were otherwise identical. A mixture of the three forms of SV40 DNA was loaded onto each gel and electrophoresis carried out at 6.7 V/cm for lengths of time ranging from 3 hours for the 0.4 percent agarose gel to 7 hours for the 2.0 percent gel. The mobilities of each of the three DNA species was calculated as the migration rate in cm/second divided by the field strength in V/cm. The retardation coefficient is a useful quantity which can be obtained by plotting the logarithm of the mobility of a DNA species versus the concentration of agarose in the gel. Ferguson (109) demonstrated a linear relationship between the logarithm of the mobility (M) of a protein and the gel concentration. Fisher and Dingman (34) and Dingman et al. (35), using the composite agarose-polyacrylamide system, also showed that this relationship was true for DNA. The relationship between log μ and gel concentration (C) is

$$\log \mu = \log \mu_o - K_R C \qquad (16)$$

The quantity K_R, the retardation coefficient, reflects the properties of the gel as well as the size and shape of the migrating molecule. Figure 4 shows a plot of log (μ) versus gel concentration. Extrapolation to zero agarose indicates that in free solution, SV40(I) and (II) should have the same or nearly the same mobilities while linears should migrate more slowly. The retardation coefficients for SV40(I), (II), and (L) are calculated to be 0.60, 0.92, and 0.46 respectively. The low

retardation coefficient for linear DNA reveals that the mobility of this species of SV40 DNA is least affected by increasing agarose concentration. The points at which the plots intersect in Figure 4 are the isomobility points, above and below which a reversal in migration order of the species occurs. When the agarose concentration is less than 1.4 percent, Form I SV40 DNA migrates more rapidly than either Form II or linears. However, when the agarose concentration exceeds 1.4 percent, a reversal in the migration order occurs such that linear DNA has the greatest mobility, followed by Form I and Form II SV40 DNA. Below 0.4 percent agarose, a reversal in the migration order of SV40(II) and linears should occur but this could not be tested because gels containing less than 0.4 percent agarose were extremely fragile.

The magnitude of the gradient of potential applied across the gel was also investigated. A mixture of SV40 Form I, Form II, and linear DNA was applied to 0.7 percent agarose gels and electrophoresis performed at a constant product of voltage and time (300 V·hrs). Figure 5 shows a plot of mobility relative to that of the lowest voltage gradient used (0.83 V/cm) *versus* the magnitude of the voltage gradient. Form II and linear molecules seem to be affected in a similar manner while Form I DNA is significantly less sensitive to changes in the field strength. In this case, a comparison of the mobilities of SV40 Form I and linear DNA relative to SV40(II) will lead to the erroneous

Figure 4. Logarithmic Relationship Between Mobility and Agarose Concentration.

The mobilities (M) of SV40 (I) (●——●), (II) (○——○) and linears (□——□) were calculated as the migration rate in cm/second divided by the field strength in v/cm. Electrophoresis was performed for 3 hours for 0.4 percent agarose, 4 hours for 0.7 percent agarose, 5 hours for 1.0 percent, and 7 hours for 1.4, 1.7, and 2.0 percent agarose gels. The extrapolations to zero percent agarose are shown as broken lines.

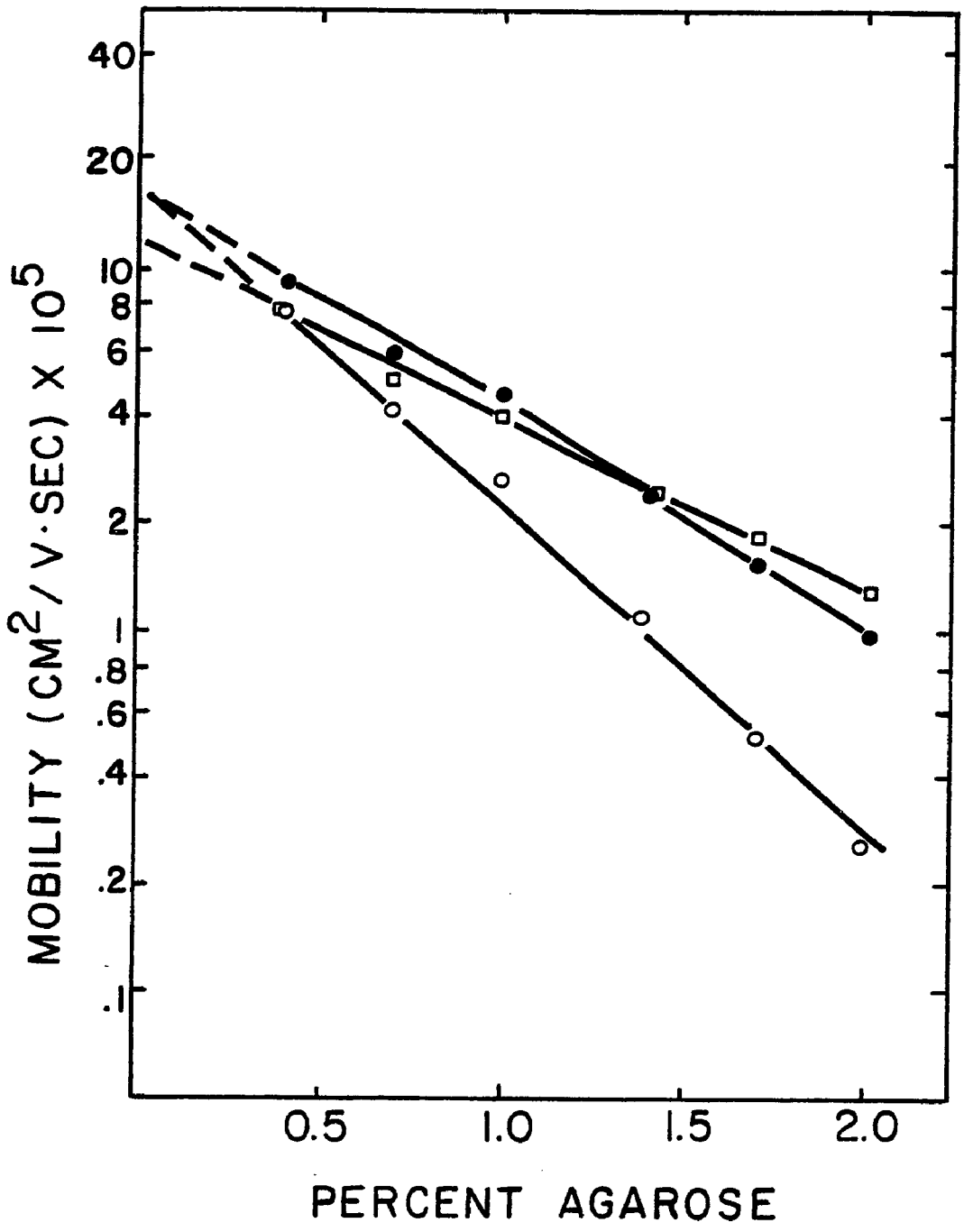

40
20
10
8
6
4
2
.8
.6
.4
.2
.1
MOBILITY (CM²/V·SEC) X 10⁵
0.5
1.0
1.5
2.0
PERCENT AGAROSE

Figure 4

Figure 5. Relative Electrophoretic Mobility as a Function of Electric Field Strength.

Mobilities of SV40 Form I (□——□), Form II (○——○) and linears (●——●) are calculated relative to the mobility observed at the lowest voltage examined across a 15 cm gel (0.83 V/cm).

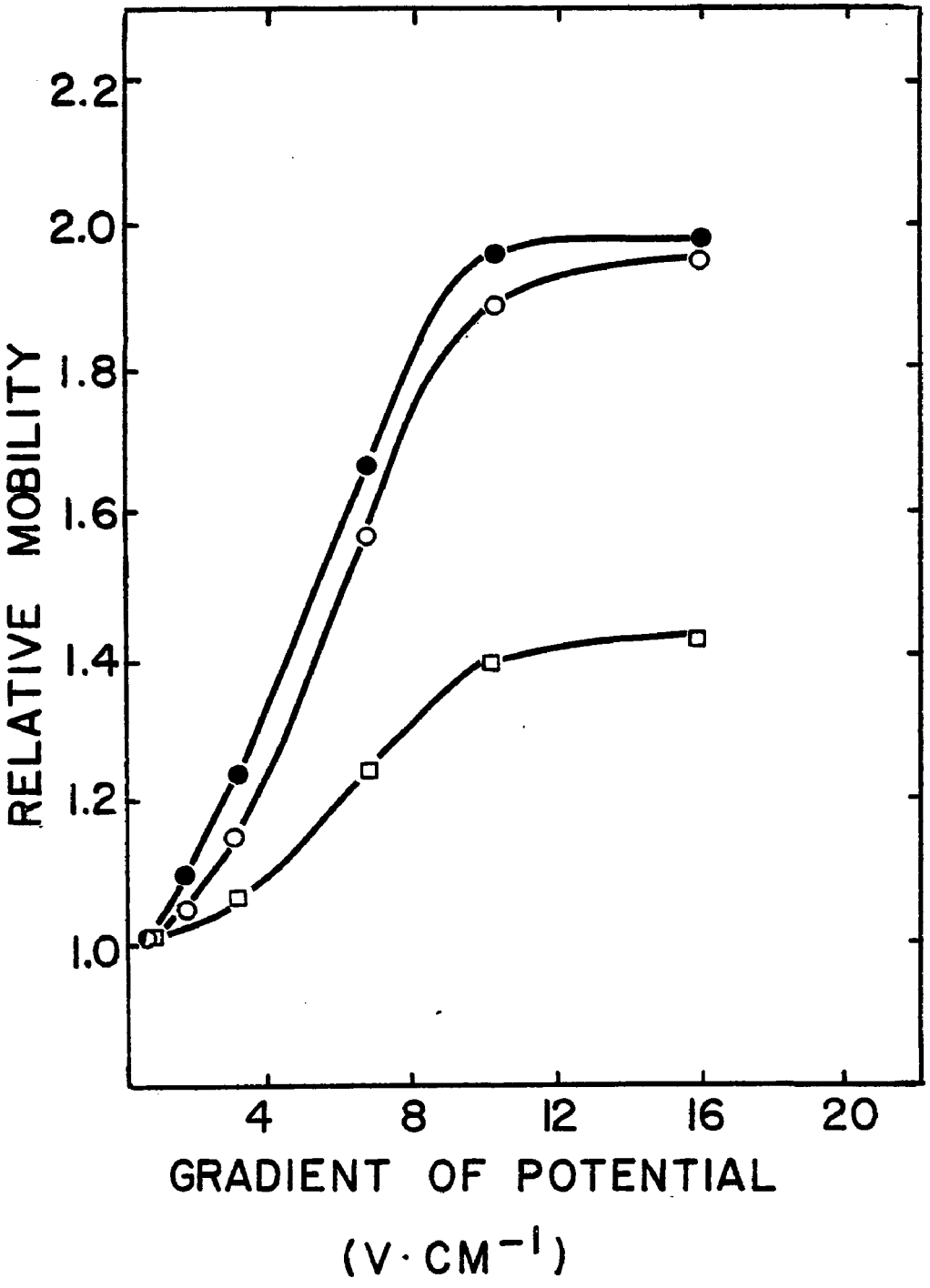

2.2
2.0
1.8
1.6
1.4
1.2
1.0
RELATIVE MOBILITY
4
8
12
16
20
GRADIENT OF POTENTIAL
(V·CM⁻¹)

Figure 5

conclusion that Form I DNA is dramatically affected by the magnitude of the voltage gradient while linear DNA is only slightly affected. While the relative separation of Form I and Form II decreases with increasing field strength, the decrease is attributable to a more pronounced change in the mobility of Form II as the field strength is increased.

The effects observed when the field strength is varied cannot be attributed to temperature. In preliminary experiments, a small thermocouple was inserted into a 0.7 percent agarose gel and the temperature monitored over a 2 hour period at a field strength of 6.7 V/cm. The internal gel temperature rose only 2 to 3 degrees and stabilized as long as the lower buffer chamber in the apparatus was filled to capacity. In experiments requiring field strengths greater than 10 V/cm, a water jacketed cylindrical gel apparatus was used which maintained a uniform temperature.

A third parameter which was investigated was the effect of ionic strength. This was examined by simply varying the buffer concentration in both the gels and the buffer chambers of the gel apparatus. The ionic strength was determined by measuring the conductance of the electrophoresis buffer with a Radiometer Copenhagen conductivity meter (type CDM2c, Copenhagen, Denmark) and comparing it to a standard curve prepared from a series of NaCl solutions. Figure 6 shows the effect of increasing ionic strength on the mobilities

of SV40(I) and SV40(L) relative to SV40(II). Each point represents the average of duplicate gels. Increasing the ionic strength has a slight effect on the relative mobility of Form I DNA over the range of ionic strengths investigated. However, when the data are expressed in these terms, the relative mobility of linear DNA appears to be quite sensitive to the ionic environment. Upon closer examination of the gels, however, it was concluded that the absolute migration rates of both Form I and Form II DNA actually increased with increasing ionic strength. Moreover, the differential increase in the rate of migration in Forms I and II was significantly greater than that of the linear DNA. The curves in Figure 6 reflect therefore, a slightly greater increase in the mobility of Form II relative to Form I as the ionic strength is increased, and a much greater increase in the mobilities of both Form I and Form II relative to linears. The change in the position of the linear band between Forms I and II was quite pronounced. At very low ionic strengths, the linear band migrated just behind Form I but quickly assumed a position slightly ahead of Form II as the ionic strength was increased.

Possible explanations for the observed behavior of these three conformers of SV40 DNA under the conditions investigated will be presented in the DISCUSSION. In terms of optimizing and characterizing the system, these studies proved useful. The objective was to increase the separation of Form I and

Figure 6. The Effect of Increasing Ionic Strength on the Relative Mobilities of SV40(I) and SV40(L) DNA.

Ionic strength is expressed in terms of NaCl concentrations having the same conductivity as the electrophoresis buffers used in the experiment. The mobilities of SV40(I) (●——●) and SV40(L) (○——○) are calculated relative to that of SV40(II). Each point represents the average of duplicate gels.

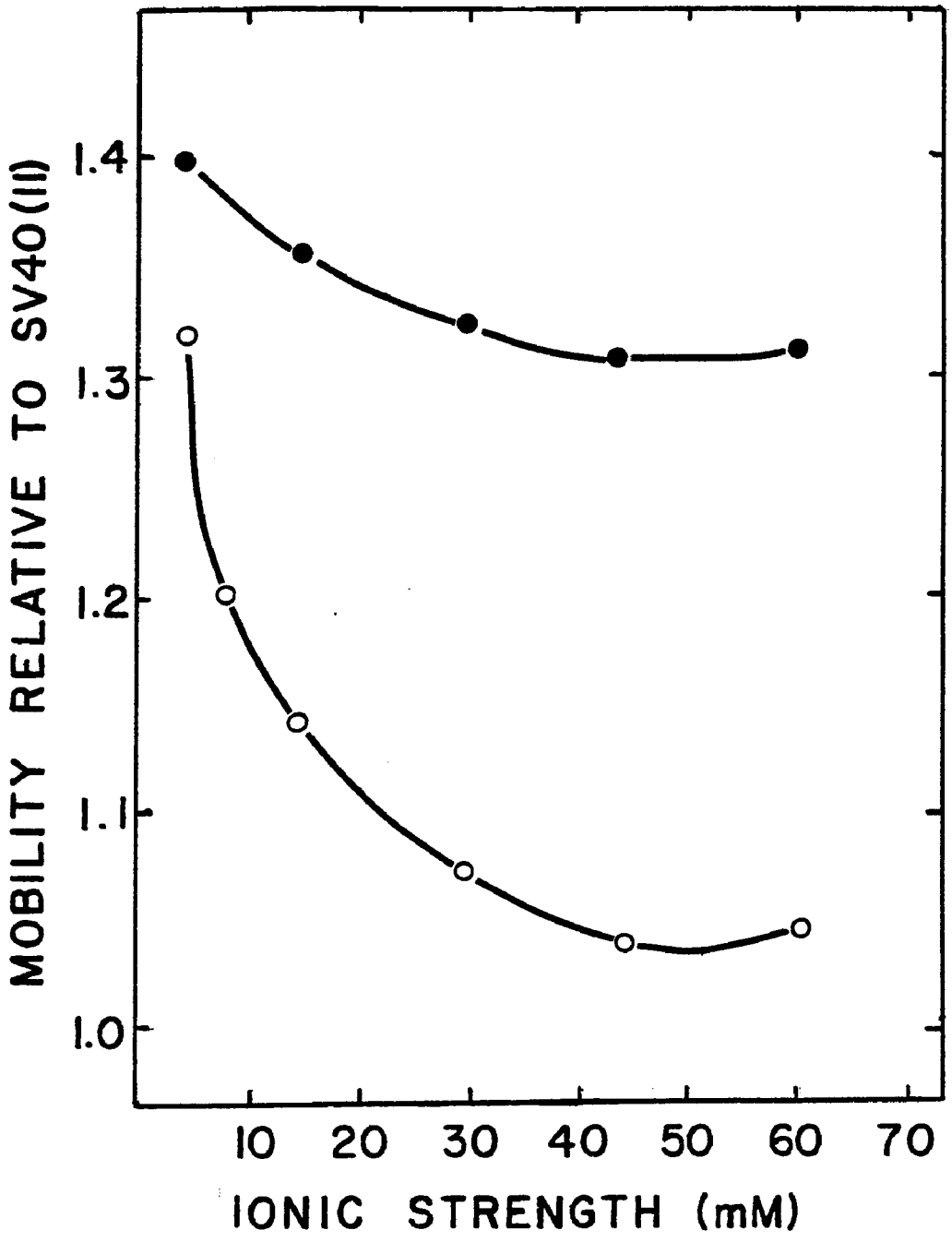

MOBILITY RELATIVE TO SV40(II)
1.4
1.3
1.2
1.1
1.0
10
20
30
40
50
60
70
IONIC STRENGTH (mM)

Figure 6

Form II species while attempting to maintain linears in a position in which they would not interfere with quantitative analysis of bands migrating between Form I and Form II. This was necessary if the effects of variations in superhelix density were to be studied since the most important region of the gel for these purposes lies between the Form I and Form II positions. Although linears could be eliminated from this region by using an agarose concentration of 0.4 percent, some degree of resolution was sacrificed at this concentration. In addition, mechanical difficulties were encountered with 0.4 percent gels. On the other hand, linears could be eliminated from the region of interest by using an agarose concentration of 1.4 percent or greater. With these gels, no mechanical difficulties were encountered and the resolving power was improved. However, these gels seemed to have a high degree of background fluorescence and became inconvenient to use because of the long electrophoresis times required. Although the time could be reduced in many cases by using higher voltage gradients, this was generally accompanied by a loss of resolution. A loss of resolution was also apparent when the ionic strength was less than that of standard electrophoresis buffer. The system which was used most extensively in the experiments that follow was 0.7 percent agarose in the standard electrophoresis buffer. Linear DNA was removed from the samples prior to electrophoresis by sedimentation on neutral

sucrose gradients. Voltage gradients were maintained below 3 V/cm. This system seemed to be most acceptable in terms of its resolution, convenience of operation, and the quality of the resulting photographic record.

Resolution of Covalently Closed Relaxed Forms of SV40 DNA by Agarose Gel Electrophoresis

Early experiments aimed at developing a rapid assay system for relaxation enzyme led to the rather surprising observation that covalently closed relaxed Form I' SV40 DNA formed by treatment of SV40(II) with ligase did not migrate as a homogeneous species on agarose gels. The insert in Figure 7 shows a typical pattern obtained when nicked circular SV40 DNA is sealed at 37°C and subjected to electrophoresis as described. Instead of migrating as a single species, the Form I' DNA is resolved into a series of bands. The intensity of the bands is not uniform across the pattern. The densitometric profile shown in Figure 7 reveals that the DNA is distributed in an approximately symmetrical manner about a central most intense band. In light of Keller's work in which he demonstrated that agarose gels were capable of clearly resolving intermediates in the relaxation enzyme reaction differing by a single superhelical turn (26), the finding that Form I' SV40 DNA was resolved into a series of bands was interpreted as being the result of thermally induced fluctuations in the conformation of the DNA at the time of covalent closure. Prior to covalent closure, both the tertiary

Figure 7. Densitometric Profile of SV40(I') DNA.

The densitometer trace is the profile of the pattern shown in the insert. Electrophoresis and densitometry were performed as described in the text. Migration is from left to right. This tracing demonstrates the loss of resolution at very low superhelix densities. The band at the extreme left represents SV40(II) and two or three SV40(I') components. The baseline was established by tracing each side of the sample channel (lower two traces) and averaging.

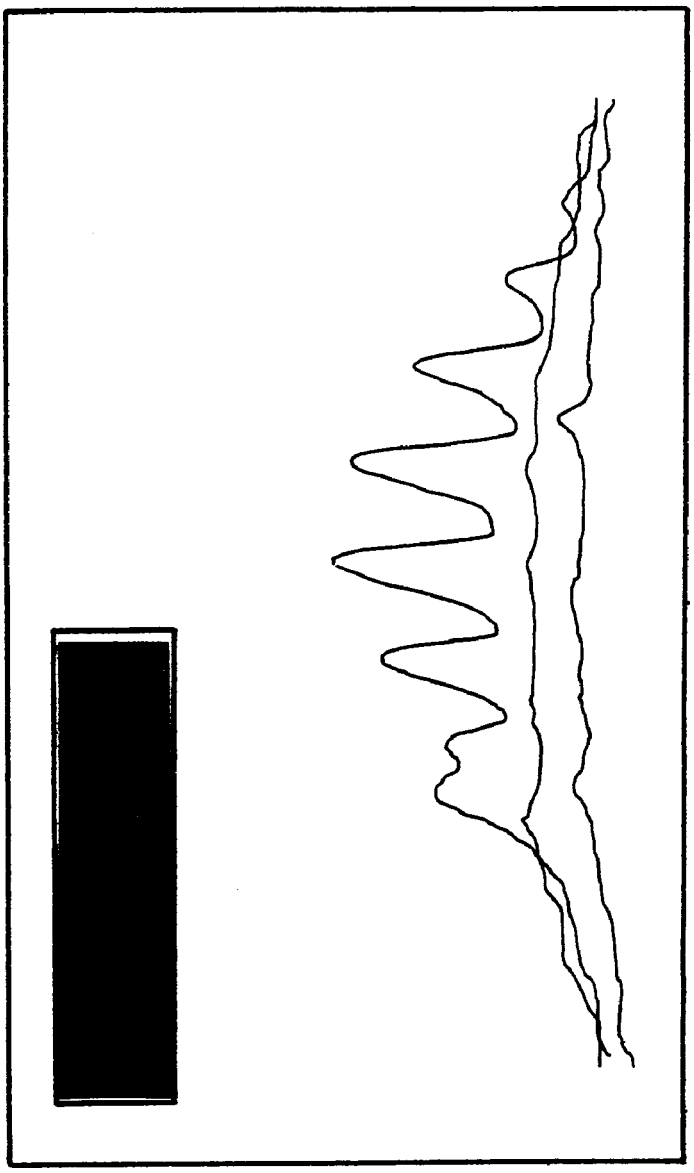


Figure 7

structure and the value of β for a Form II molecule in solution will fluctuate. When closure occurs, a value of α is established in the molecule which represents the sum of these two terms at the instant closure occurs. Following closure, τ and β will continue to fluctuate but the value of α in any given molecule will be preserved. The action of ligase on Form II is therefore expected to generate a natural population of molecules which vary with respect to α. The relative intensity of a given band in the resulting gel pattern should reflect the probability that a molecule will be in the state represented by that particular band. The methods for analyzing the patterns will be discussed in greater detail in the following section.

Although this interpretation may explain the existence of multiple species, it does not explain the actual physical appearance of the pattern in the gel. Under the conditions of the ligase reaction, the most probable state of the molecule is expected to be that with zero superhelical turns. This probability should be approximated by the relative intensity of the most intense band in the distribution. Yet, the most intense band in the pattern shown in Figure 7 does not co-migrate in the gel with the Form II which represents this average state. Since the environmental conditions within the gel are significantly different from those of the ligase reaction mixture, the effect of this change was investigated. Agarose gels were prepared in buffer consisting of 60 mM Tris, 1 mM EDTA, pH 7.6 instead

of the usual electrophoresis buffer. One gel contained, in addition, $MgCl_2$ at a concentration of 10 mM. This buffer system is essentially the same as that used for the ligase reaction. The gels were placed in a water jacketed electrophoresis apparatus through which water was circulated from a thermostatically controlled bath maintained at 37°C, the temperature at which the ligation reaction was performed. SV40(I') was applied to each gel and electrophoresis performed as usual. Following staining in ethidium, a normal series of bands was observed in the gel prepared in buffer without $MgCl_2$ whereas in the gel prepared with $MgCl_2$ all of the DNA migrated with Form II as a single band. Although a change in temperature between that of the ligase reaction and that at which electrophoresis is performed is expected to influence the position of the bands in the gel, the primary factor influencing the position of the bands must be the change in ionic conditions since a full complement of species was observed in the absence of $MgCl_2$ even though electrophoresis was performed at the same temperature as the ligase reaction. A reduction in the ionic strength is known from Wang's work (13) to cause the primary helix to unwind slightly. This would introduce supercoiling of the positive sense into a covalently closed relaxed molecule. The appearance of a series of bands on agarose gels can be attributed to a uniform

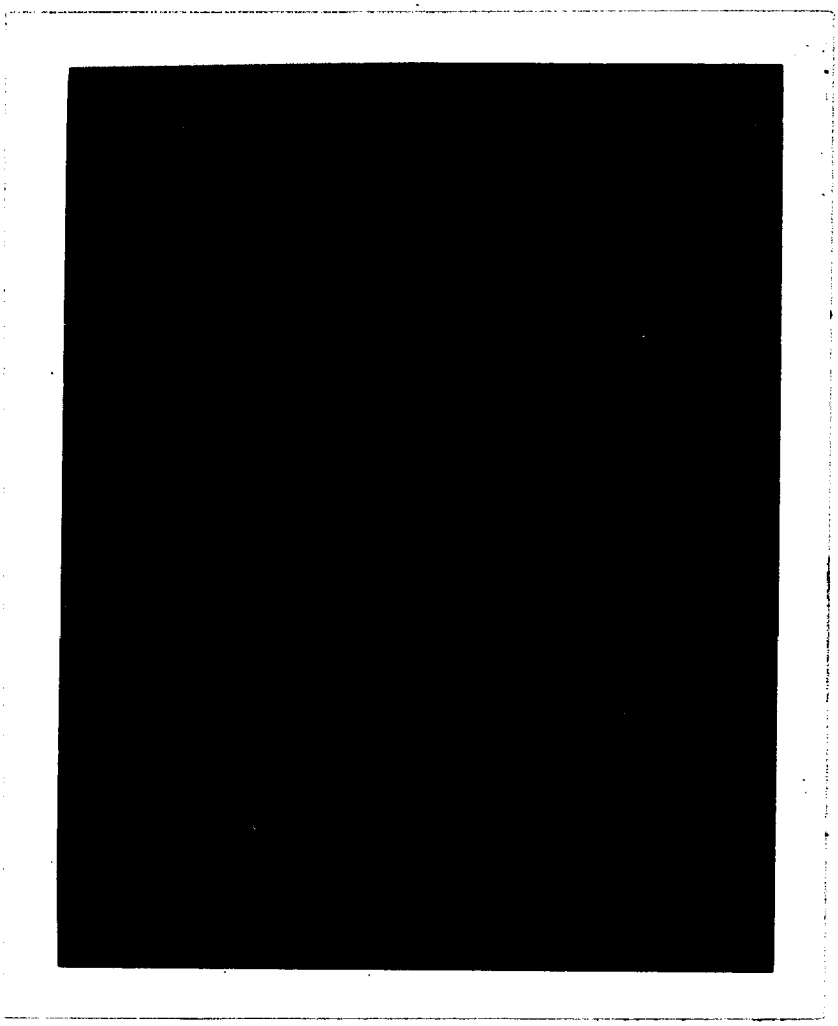


Figure 8

Dye Titration of SV40(I') DNA on Agarose Gels.

The SV40(I') DNA used was formed from SV40(II) by treatment with T4 ligase at 37°C. Gels are 0.7 percent agarose containing ethidium bromide in concentrations increasing from left to right in 10 ng/ml increments from zero to 110 ng/ml. Migration is from top to bottom. The uppermost band in each gel is SV40(II).

introduction of superhelical turns to all components of the Form I' population to an extent related to the magnitude of the difference in the ionic environment. Based on Wang's results, the sense of the superhelical turns introduced as a result of transferring the DNA from the ligation environment to the electrophoresis environment is expected to be positive.

To confirm this, a dye titration experiment was performed. This technique will be discussed only briefly at this point and will be considered in greater detail in a later section. A series of cylindrical 0.7 percent gels was prepared containing increasing concentrations of ethidium bromide in 10 ng/ml increments from 0 to 110 ng/ml. A sample mixture containing SV40(I') was applied to the gels and electrophoresis was performed for 105 minutes at 6.7 V/cm on 15 cm gels. The results of the titration are shown in Figure 8. The initial gel which contains no ethidium shows well resolved components of SV40(I') DNA. As the ethidium concentration increases, the band set migrates more rapidly with respect to Form II until it is no longer resolved and migrates as rapidly as Form I DNA. The monotonic increase in mobility indicates that in the absence of dye, the band set is positively coiled. If, in the initial condition, the turns were negative, the mobility of the band set would first decrease and then increase. This will be demonstrated in the section dealing with dye titrations.

The assumption has been made to this point that each band differs from its neighbor by a single superhelical turn. Topological differences between DNA molecules in adjacent bands must be integral but need not necessarily be unit differences. However, any topological increment other than unity between adjacent bands must be regarded as improbable. The functional groups in the Form II moleucle at the site of the nick are in proper alignment for joining once per 360° rotation about the phosphodiester bond opposite the break.

Experimentally, evidence was obtained which strongly suggests that the bands are space in unit turn increments. Ligase reactions were performed at 0°, 14.5°, 23.5°, and 38.5°C using nicked circular SV40(II) DNA as the substrate. Reaction mixtures were temperature equilibrated before addition of the enzyme and the volume of enzyme added was sufficiently small (1-2 percent of the total volume) as to cause negligible temperature perturbations. Care was taken to terminate the reactions at the same temperature as they were performed. The products of the four reactions are shown in Figure 9 and the densitometric profiles are shown in Figure 10. There is clearly a relationship between the position of the band set in the gel and the temperature of ligation. To determine what this relationship was in more quantitative terms, the centers ($\bar{n}$) of each of the densitometer traces for each of the DNA samples

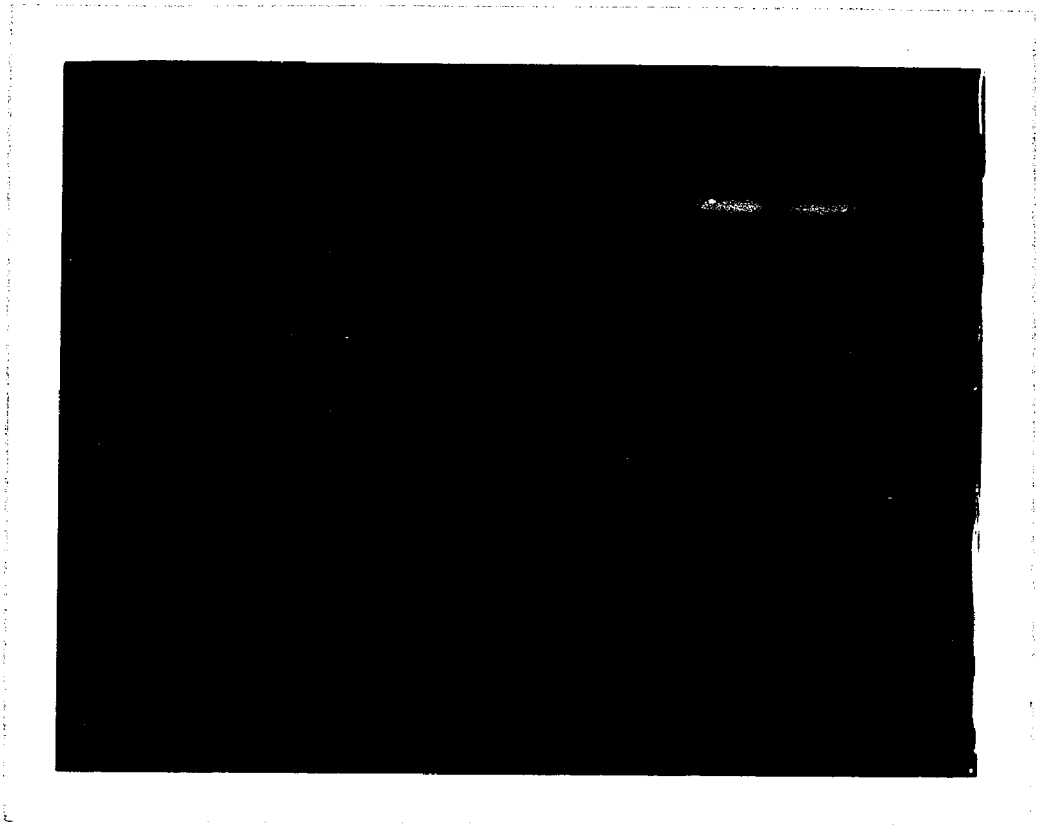


Figure 9

The Effect of Ligation Temperature on the Position of SV40(I') Components in the Gel.

The direction of migration in the fluorescence photograph is from top to bottom. The uppermost band in each sample channel corresponds to SV40(II). Ligation reactions and electrophoresis were performed as described in MATERIAL AND METHODS. Samples are, from left to right, Channels 1 and 2, 0°C; Channels 3 and 4, 14.5°C; Channels 5 and 6, 23.5°C, and Channels 7 and 8, 38.5°C.

Figure 10. Densitometric Profiles of SV40 DNA Ligated at Four Different Temperatures.

Ligase reactions were performed as described in the text. The direction of migration in this figure is left to right. The band at the extreme left in each trace is SV40(II). The arrows denote the calculated centers of each of the distributions.

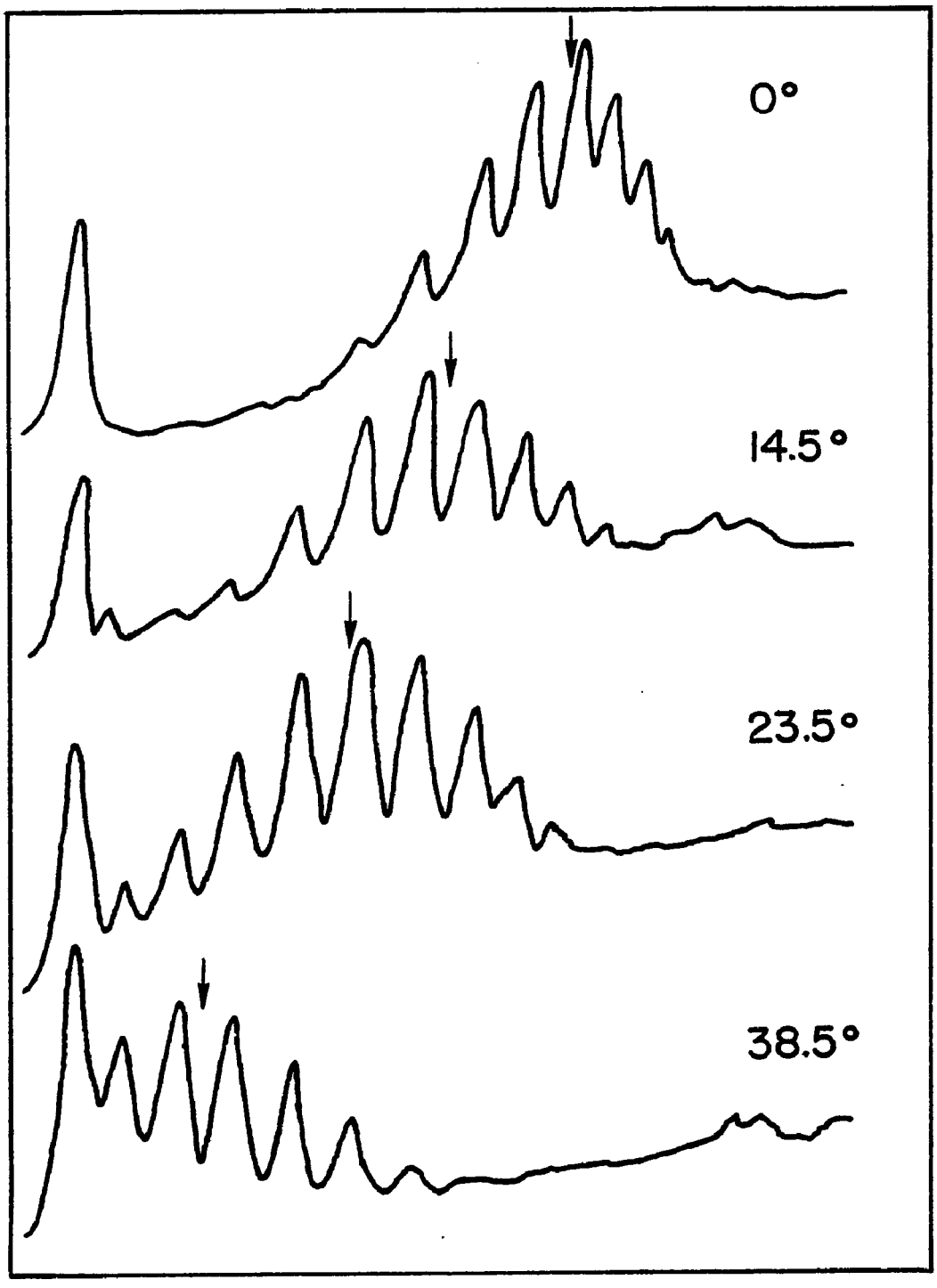


Figure 10

were calculated using the equation

$$\bar{n} = \frac{\Sigma mn}{\Sigma m} \qquad (17)$$

where n refers to the number assigned to each band in a distribution when the bands are numbered consecutively from one end to the other and m is the measure of DNA mass which in this case was the height of each peak in the densitometric profile. The use of the above equation necessitates the consideration of an equal number of bands on each side of the center of a distribution which is approximated visually from the densitometer trace. The centers of each of the distributions shown in Figure 10 are identified by arrows. It is this point which corresponds to zero superhelical turns under the conditions in which the DNA was sealed. It should be noted that the center of a distribution need not correspond precisely to the most intense band. The center of the distribution will correspond to the most intense band if, in the Form II molecule, the time-averaged positions of the free 3'-hydroxyl and 5'-phosphoryl groups at the nick occupy the same positions as they would if the phosphodiester bond were present. Since this situation is unusual, a small amount of twist is required to bring the two functional groups into proper alignment for joining. This small amount of twist displaces the center of the distribution away from the central band. Pulleyblank et al. (40) have termed this quantity ε which, in terms of superhelical turns, may be

defined as $-0.5 < \varepsilon \leq 0.5$. The central most intense band in the distribution represents the smallest angular displacement from zero which is required to join the functional groups at the nick.

Examination of the densitometric profiles shown in Figure 10 indicates that as the ligation temperature is increased, the average value of τ for the band set decreases upon transfer of the DNA to the environment in which electrophoresis is performed. Since, in the gel, all of the DNA is in the same environment, the mobility differences can be attributed to differences in the initial state in which the DNA was ligated. There is sufficient overlap of the patterns to allow estimation of temperature effects by direct band counting. Figure 11 shows a plot of $\Delta \alpha$ versus temperature for SV40 DNA. Based on the assumption that adjacent bands differ by a single superhelical turn, the number of turns from the calculated center of the 38.5°C reaction sample to the centers of the remaining patterns was counted. The plot is linearly related to the ligation temperature and has a negative slope. The slope of this plot divided by the number of base pairs in SV40 DNA (4900) yields the temperature coefficient for the change in average base rotation angle of the DNA. The magnitude of this quantity was calculated to be $-1.2 \pm 0.1 \times 10^{-2}$ °/base pair/°C. The negative sign indicates that as the temperature increases, the Watson-Crick duplex structure unwinds. In a previously published report

Figure 11. Change in the Topological Winding Number as a Function of Temperature for SV40 DNA.

The number of turns from the center of the 38.5°C reaction product to the centers of the remaining patterns were counted and plotted against temperature. Each point represents the average of two measurements.

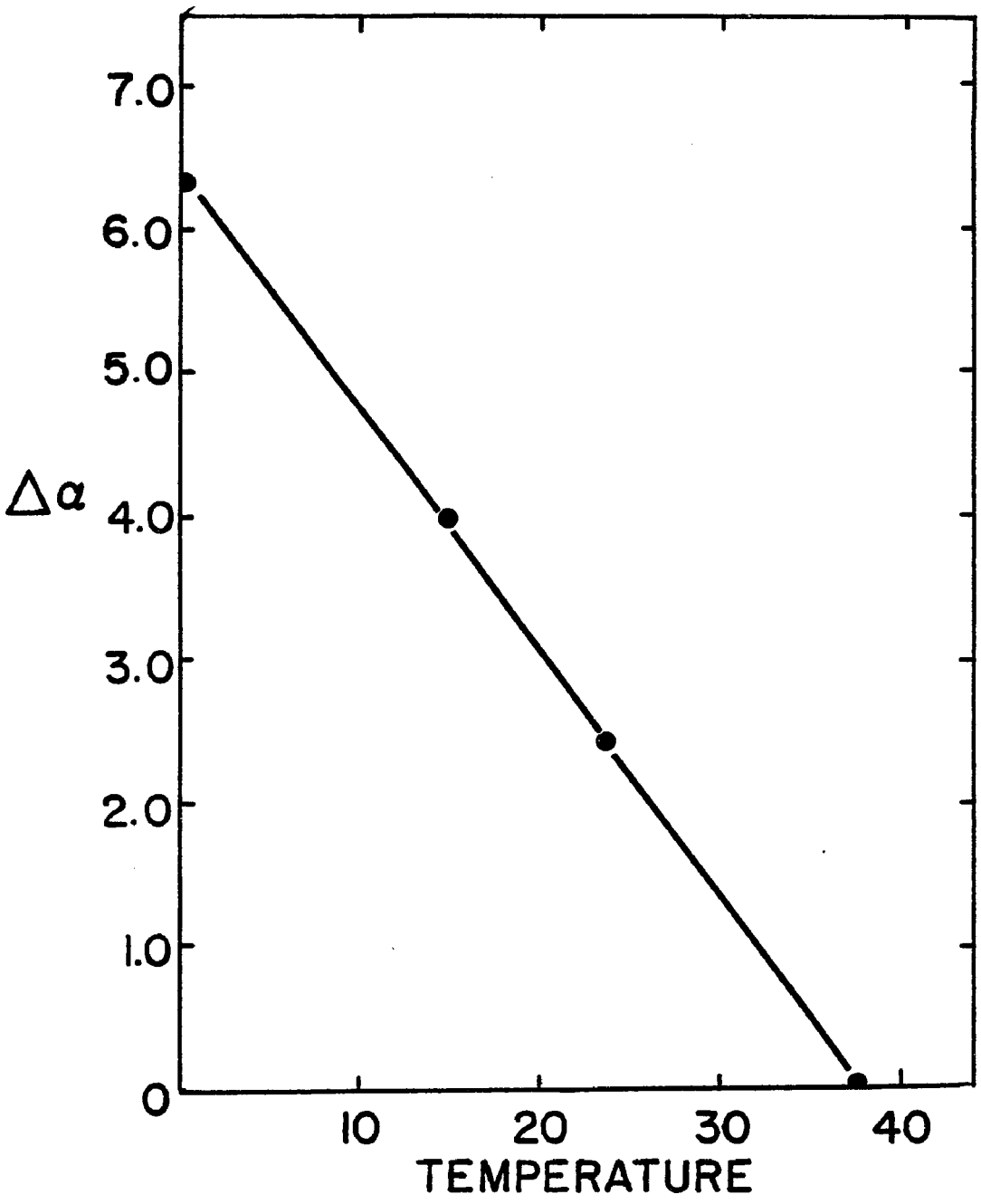


Figure 11

by Wang (13), this same quantity was calculated to be 4-5 x 10^{-2} °/base pair/°C. The calculation leading to this figure incorporated a 12° duplex unwinding angle for ethidium bromide. When recalculated using the 26° unwinding angle for ethidium, a value of about 1.1 x 10^{-2} °/base pair/°C was obtained, which is in very close agreement with the value obtained by electrophoretic methods. This close correspondence serves to indicate that adjacent bands in the distributions differ by a single superhelical turn since such a close correspondence would not be possible if the winding increment was some value other than one. This result also serves to confirm the accuracy of the more recently reported value for the duplex unwinding angle of ethidium bromide of 26° (25).

The Boltzmann Distribution and the Free Energy of Superhelix Formation

The distribution of DNA species in Form I' patterns clearly represents an equilibrium distribution of conformations. The distribution of topological isomers consistently obtained when SV40(I') was subjected to electrophoresis on agarose gels suggested that an analysis of the relative proportion of DNA in each band might lead to useful information concerning the free energy of superhelix formation.

The most probable configuration of the DNA at equilibrium is that at which $\tau = 0$. Departures from this state will occur

at a frequency which reflects the magnitude of the free energy change involved. DNA molecules in thermal equilibrium with the surroundings may be considered to be distributed over a number of states. The number of molecules in the ith state (N_i) relative to the number in the state with the lowest energy (N_o) is given by the Boltzmann equation

$$N_i/N_o = g_i \exp -G_\tau/RT \qquad (18)$$

where g_i is a degeneracy factor which in the case of this analysis is equal to one. $G_{\tau,\tau=i}$ is the free energy of the ith state and R and T have their usual meaning. Since $\tau = 0$ is the most probable state at equilibrium, the free energy of the state $\tau = i$ may be expressed as

$$G_{\tau=i} - G_{\tau=0} = -RT \ln N_i/N_o \qquad (19)$$

Since at equilibrium $G_{\tau=0}$ is taken to be the ground state, this term may be dropped from the expression.

Bauer and Vinograd demonstrated empirically that the free energy of superhelix formation is approximately proportional to the square of the superhelical winding number (28). The form of their expression relating the free energy to the superhelical winding number takes the usual form of an expression describing torsional potential. Therefore, the free energy difference between the ground state ($\tau_i=0$) and the ith state ($\tau=i$) will be proportional to a constant (A) multiplied by the square of the displacement (τ^2) (110). A similar line of reasoning was

followed by Depew and Wang (41) and Pulleyblank, et al. (40). The proportionality constant relating τ^2 to G_τ is expected to be unique in a given medium at a given temperature. The form of the equation which was applied to the analysis of the distributions is Gaussian and is given by

$$-\ln N_i/N_o = G_\tau/RT = A\tau^2 \qquad (20)$$

To determine N_i and N_o, SV40(I') species resolved on gels were photographed. The fluorescent intensities of the bands were evaluated by densitometry and the centers of the distributions determined by application of equation 17. Beginning from the center, appropriate values of τ were assigned to each band. The natural logarithm of the DNA mass (peak height) was calculated for each of the species in the distribution and plotted versus τ^2. The best least-squares fit of the plot was obtained by substituting τ^2 and the natural logarithm of the DNA mass for x and y respectively in equations 12, 13, and 14. The y-intercept of this plot is the natural logarithm of N_o, the DNA mass at the center of the distribution where $\tau = 0$. This value is used to calculate the relative DNA mass in each band (N_i/N_o). The least-squares procedure was then applied to the paired data points (τ^2, $-\ln N_i/N_o$) yielding a line passing through the origin with a slope of A.

SV40 DNA nicked and ligated as described in MATERIALS AND METHODS was analyzed in this manner. The levels of background

fluorescence were evaluated by tracing between sample channels on the fluorescence photograph and the heights of the peaks were measured from this baseline. A plot of $-\ln N_i/N_o$ versus τ^2 for SV40(I') DNA is shown in Figure 17 (open circles). The slope of the plot (A) was determined to be 0.16 with a standard deviation of 0.015 for 14 determinations. To provide a measure of confirmation, bands were exicsed from three SV40(I') patterns and counted in ACS scintillator. The level of radioactivity in each band was used as an indication of DNA mass. The average value of A obtained by this method of determination was 0.16 with a standard deviation of 0.006. Substitution of this value of A into equation 20 permits the calculation of G_τ for SV40 DNA. A plot of G_τ as a function of τ is shown in Figure 12. Other experiments to be described in detail indicate that native SV40 DNA isolated from infected cells has approximately -25 superhelical turns under electrophoresis conditions at 23°C and a buffer ionic strength equivalent to 35 mM NaCl. This leads to a value of 59 kcal/mole for the molar free energy of superhelix formation at 25°C.

The traces shown in Figure 10 were evaluated in the manner described in order to investigate the temperature dependence of the coefficient A. The values of the coefficient A for SV40 DNA ligated at 0, 14.5, 23.5, and 38.5°C were found to be 0.17, 0.15, 0.15, and 0.17 respectively. These values represent the

Figure 12. The Molar Free Energy of Superhelix Formation as a Function of the Superhelical Winding Number for SV40 DNA.

The derivation of the equation leading to this plot is described in the text. The value of τ for native SV40 DNA was taken to be -25 and was obtained from dye titration data (see following section).

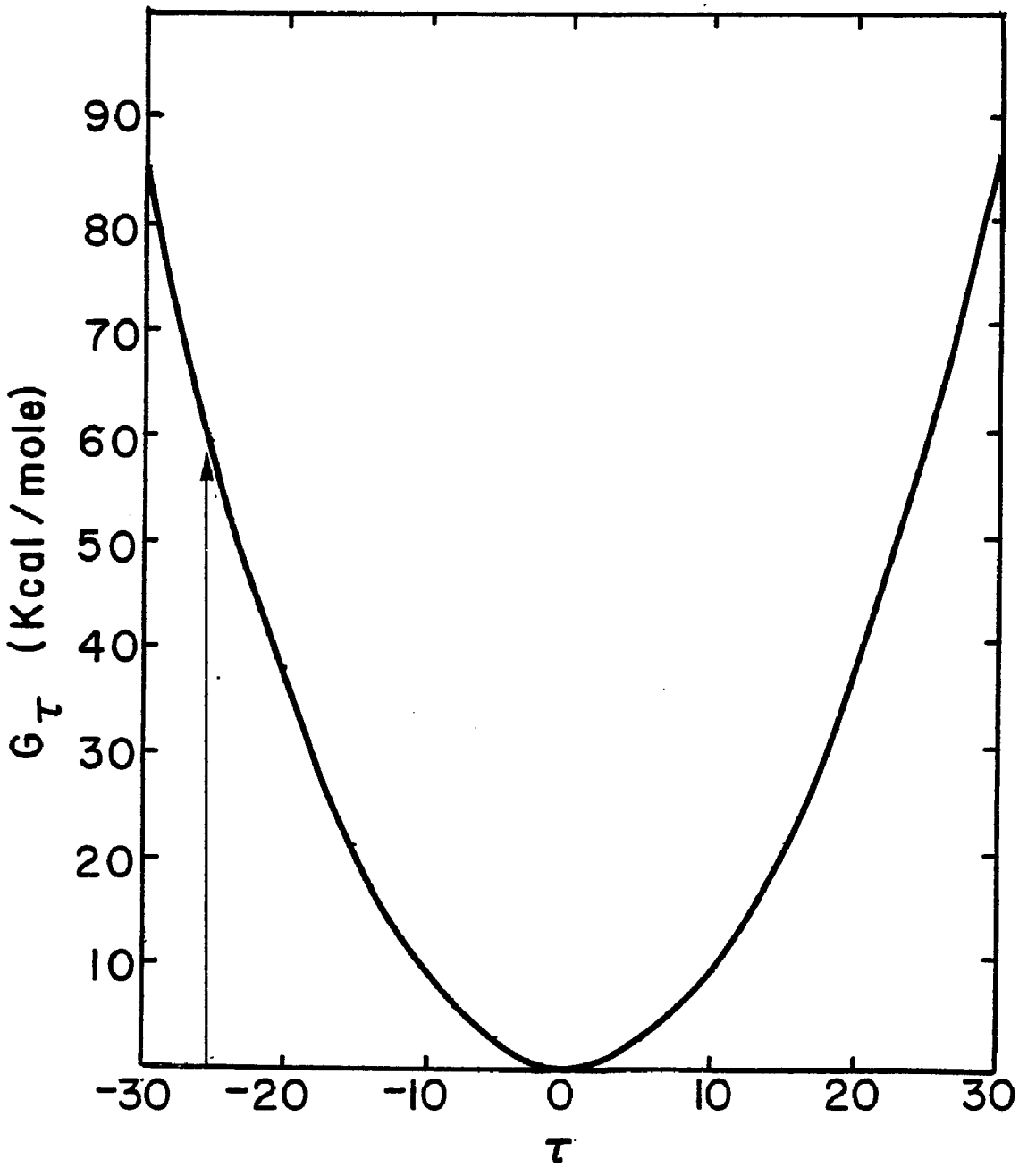


Figure 12

average of two determinations for each temperature. The apparent insensitivity of A to temperature changes indicates that the major factor contributing to the free energy of superhelix formation is loss of entropy.

The influence of molecular weight on the magnitude of A was investigated using a series of plasmid DNAs isolated from E. coli. In general, these DNAs were relaxed by using the relaxation enzyme from KB cells which was kindly supplied by Dr. Walter Keller. Although the reaction mixture used for the relaxation enzyme differs in ionic strength and composition from the ligation reaction mixture, preliminary experiments with SV40 DNA indicated that the magnitude of the coefficient A was not influenced by the change in conditions. The change in ionic conditions did, however, influence the position of the band sets in the gel. Covalently closed Form I' patterns were frequently poorly resolved because many of the species in the band set co-migrated with Form II. Under the conditions of electrophoresis, species possessing only a few turns have mobilities which are indistinguishable from that of Form II (see Figures 7 and 9,38.5°C). The relative position of the limit product of the relaxation enzyme reaction in the gel compared to the ligase product indicates that the duplex is underwound in 0.2 M NaCl (relaxation reaction) compared to its state in 10 mM $MgCl_2$ (Ligase reaction). To adequately resolve

Figure 13. Densitometric Profiles of Covalently Closed Relaxed DNAs.

The traces shown correspond to: A. pDC1, B. pVH51, C. SV40, D. ØX174 RF, E. pMB9, and F. ColE1. With the exception of SV40, the profiles shown are from DNAs relaxed by relaxation enzyme. SV40(I') DNA was relaxed through the sequential use of DNase I and T4 ligase. The limit products of the relaxation enzyme reaction were electrophoresed in the presence of 4-8 ng/ml EtBr to increase mobilities. The inclusion of EtBr in the gel was not necessary for SV40(I'). Migration in each trace is from left to right. The peak at the extreme left in each trace corresponds to the nicked circular form of each DNA.

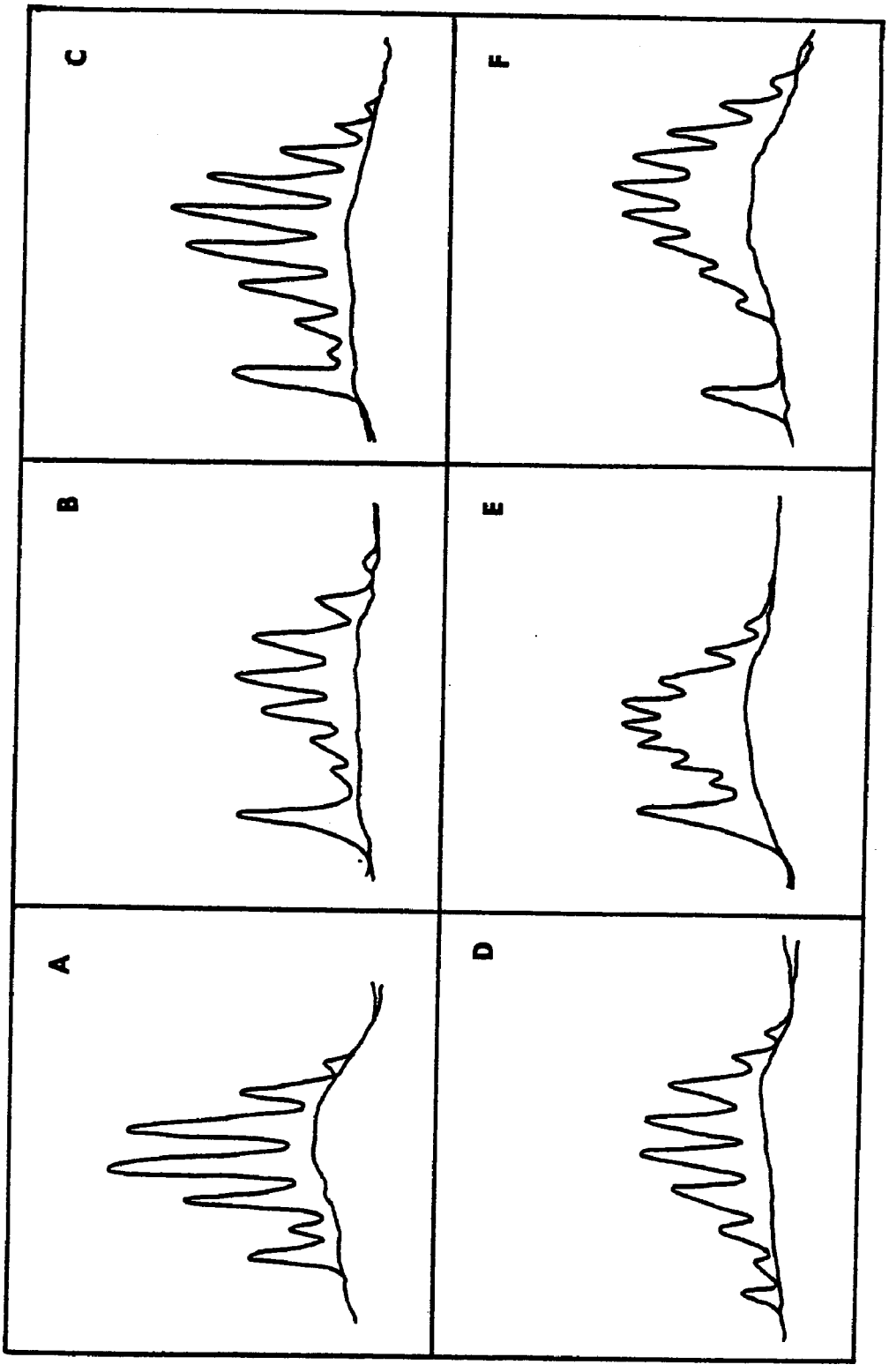


Figure 13

Figure 14. Densitometric Profiles Covalently Closed Native DNAs.

The traces shown correspond to: A. pDC1, B. pVH51, C. SV40, D. ØX174 RF, E. pMB9, F. ColE1. All DNAs were partially relaxed for the analysis by including EtBr in the gels at a concentration of approximately 15 ng/ml. Migration in each trace is from left to right. The peak at the extreme left side of each trace corresponds to the nicked circular form of each DNA.

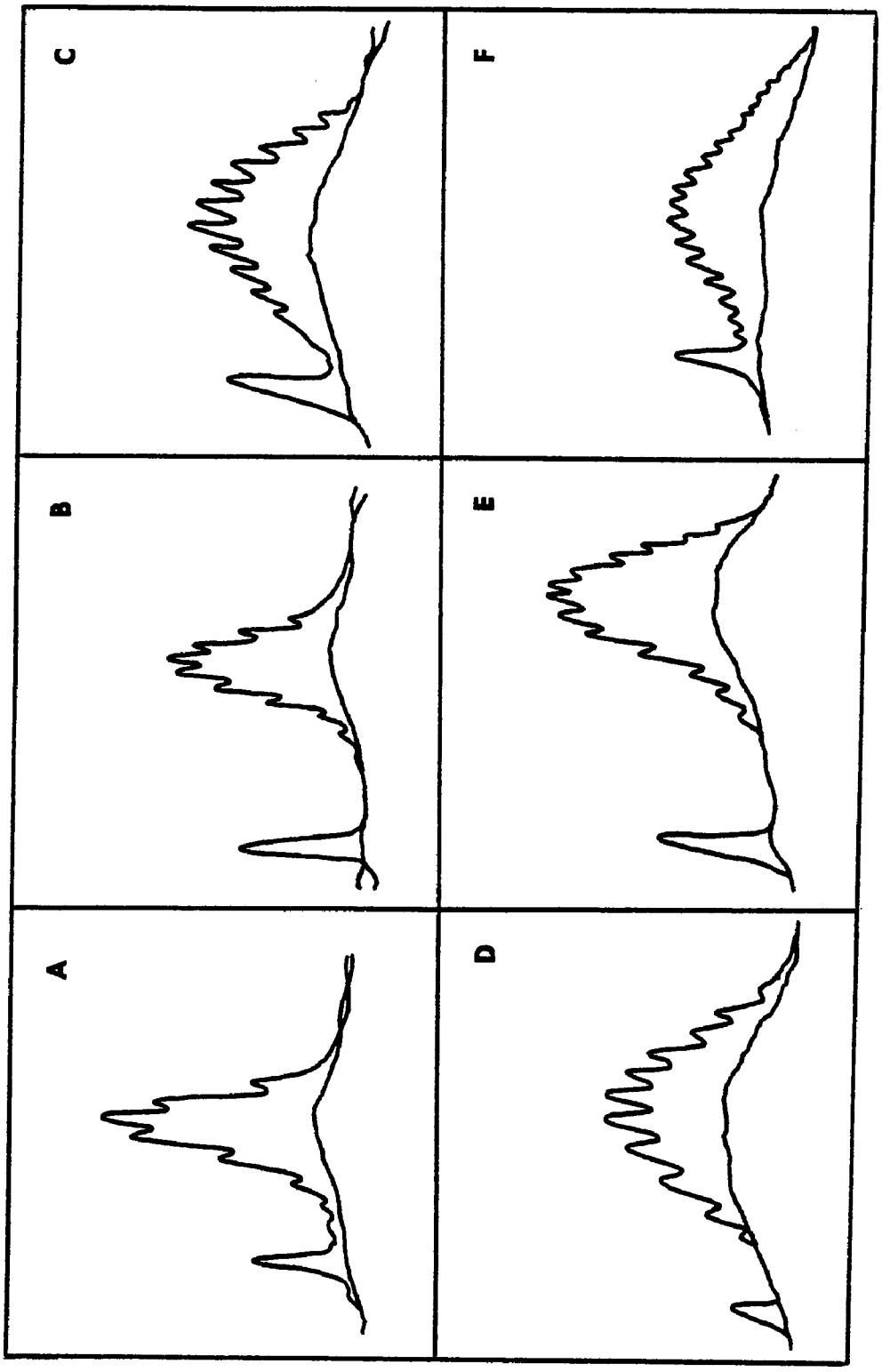


Figure 14

Form I' components, a small amount of ethidium bromide (4 - 8 ng/ml) was included in the gels. This increased the mobility of all covalently closed species relative to Form II and permitted visualization of all components.

Five Form I' DNAs from bacterial sources were analyzed in the manner described for SV40(I') DNA. These DNAs (pDC1, pVH51, ØX174 RF, pMB9, and ColE1) cover a molecular weight range of 1.8 million to 4.2 million. The densitometric profiles of these DNAs are shown in Figure 13. A profile of SV40(I') is included for comparison. It should be noted that there is a positive relationship between the molecular weight of the DNA and the number of components in the Form I' pattern. Plots of $-\ln N_i/N_o$ *versus* τ^2 for each DNA are shown in Figures 15 through 20 (open circles). The figures are arranged in order of increasing molecular weight. As the molecular weight of the DNA increases, the magnitude of the slope (A) decreases. The data are summarized in Table 2. Assuming that G_τ is proportional to molecular weight when the superhelix density is constant, the magnitude of the coefficient A should be inversely proportional to the number of nucleotides in the DNA molecule. This relationship is illustrated in Figure 21 (open circles). The plot is linear with a slope of 1.6×10^3 and extrapolates through the origin. The extrapolation through the origin indicates that at infinite molecular weight, the energy required to introduce a superhelical turn into the molecule approaches zero as a limit.

Figure 15. The Negative Logarithm of Relative Concentration versus τ^2 for Covalently Closed Relaxed and Native pDC1 DNA.

Densitometric profiles of native and covalently closed relaxed pDC1 DNA were analyzed as described in the text. Open circles refer to pDC1(I') while solid circles refer to pDC1(I). The Form I' plot represents the average of 10 determinations and has a slope of 0.32. The Form I plot is the average of 9 determinations and has a slope of 0.22.

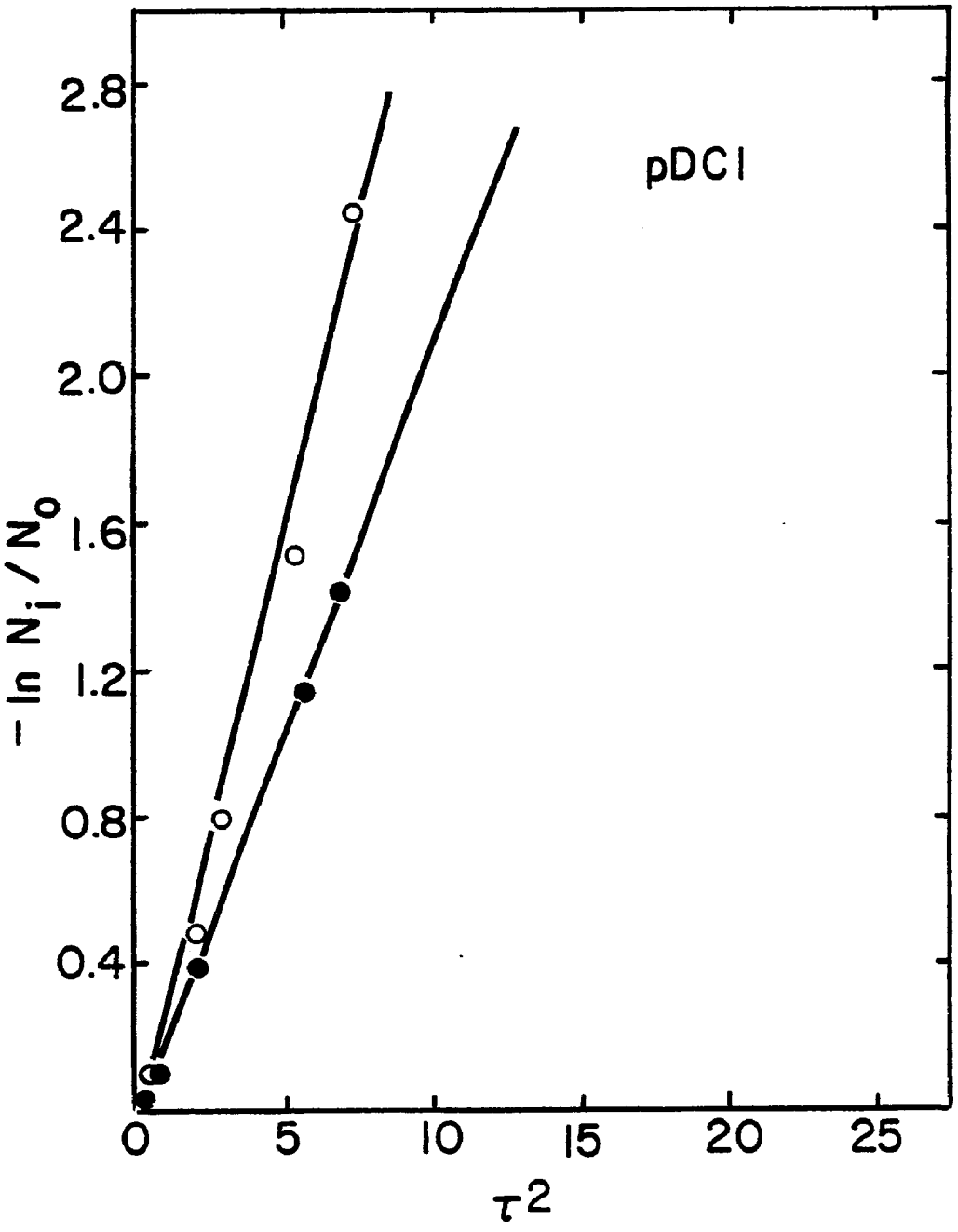


Figure 15

Figure 16. The Negative Logarithm of Relative Concentration versus τ^2 for Covalently Closed Relaxed and Native pVH51 DNA.

Densitometric profiles of native and covalently closed relaxed pVH51 DNA were analyzed as described in the text. Open circles refer to pVH51(I') while solid circles refer to pVH51(I). The Form I' plot represents the average of 7 determinations and has a slope of 0.25. The Form I plot is the average of 14 determinations and has a slope of 0.11.

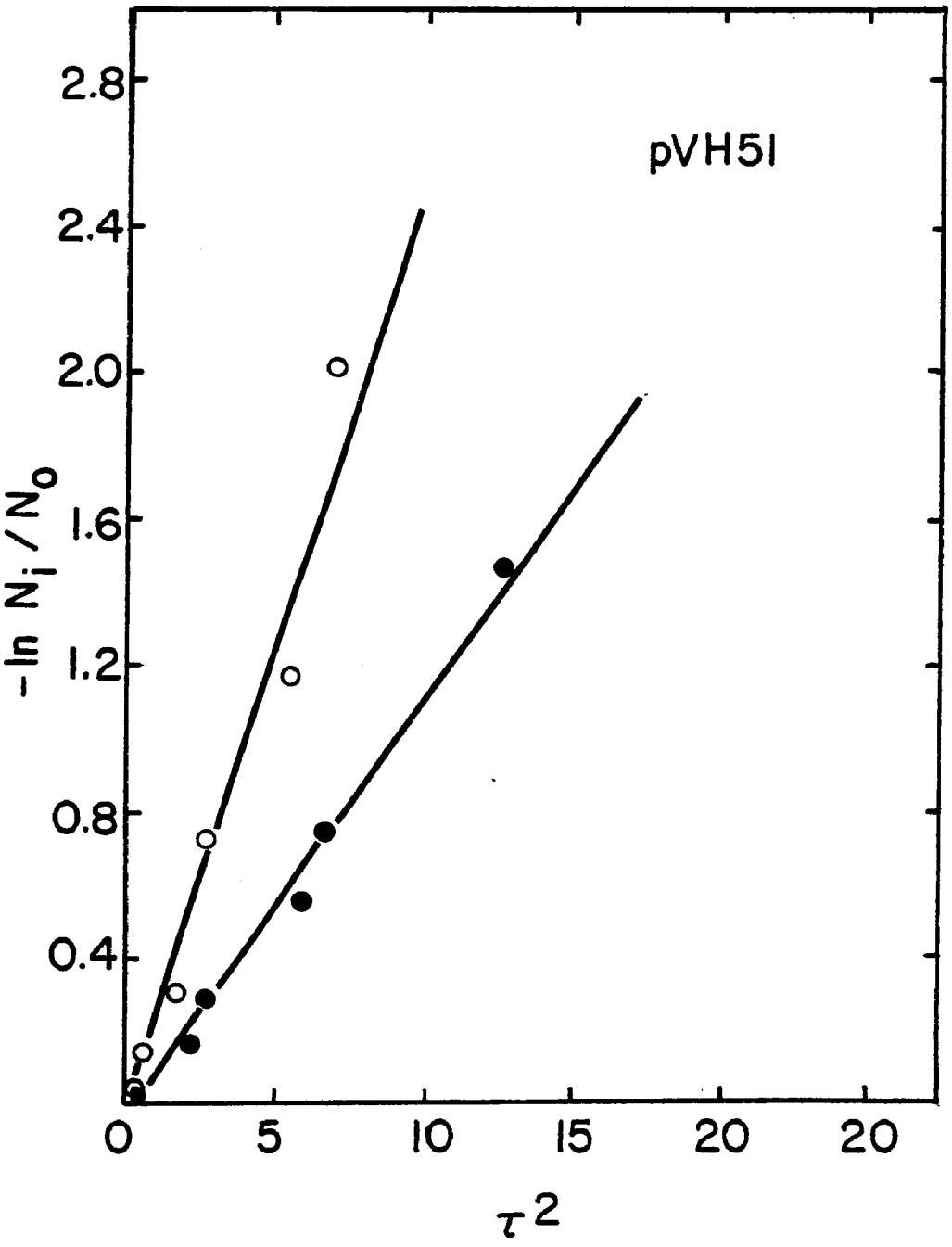


Figure 16

Figure 17. The Negative Logarithm of Relative Concentration *versus* τ^2 for Covalently Closed Relaxed and Native SV40 DNA.

Densitometric profiles of native and covalently closed relaxed SV40 DNA were analyzed as described in the text. Open circles refer to SV40(I') while solid circles refer to SV40(I). The Form I' plot represents the average of 14 determinations and has a slope of 0.16. The Form I plot is the average of 14 determinations and has a slope of 0.04.

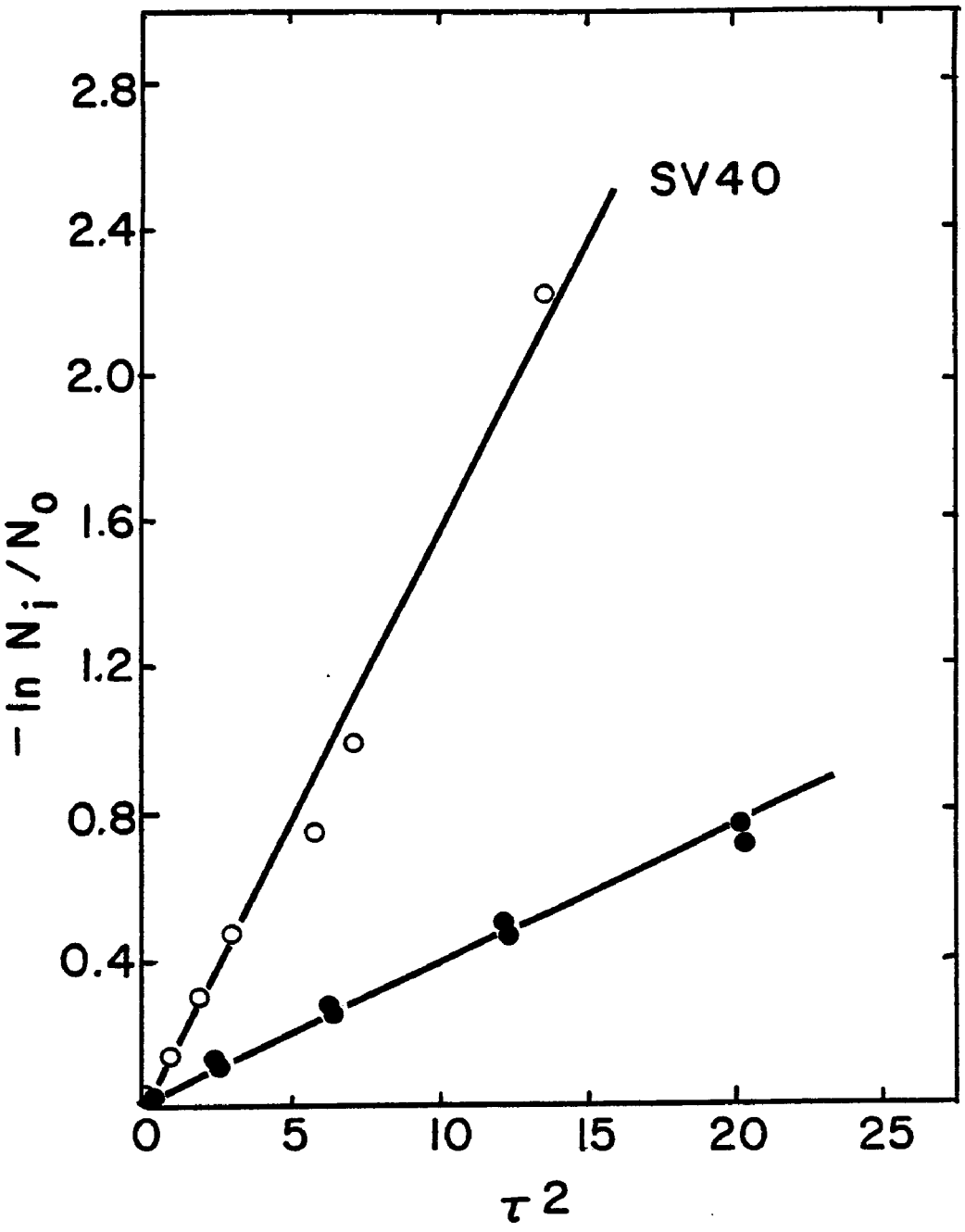


Figure 17

Figure 18. The Negative Logarithm of Relative Concentration *versus* τ^2 for Covalently Closed Relaxed and Native ØX174 RF DNA.

Densitometric profiles of native and covalently closed relaxed ØX174 RF DNA were analyzed as described in the text. Open circles refer to ØX174 RF(I') while solid circles refer to ØX174 RF(I). The Form I' plot represents the average of 3 determinations and has a slope of 0.16. The Form I plot is the average of 6 determinations and has a slope of 0.07.

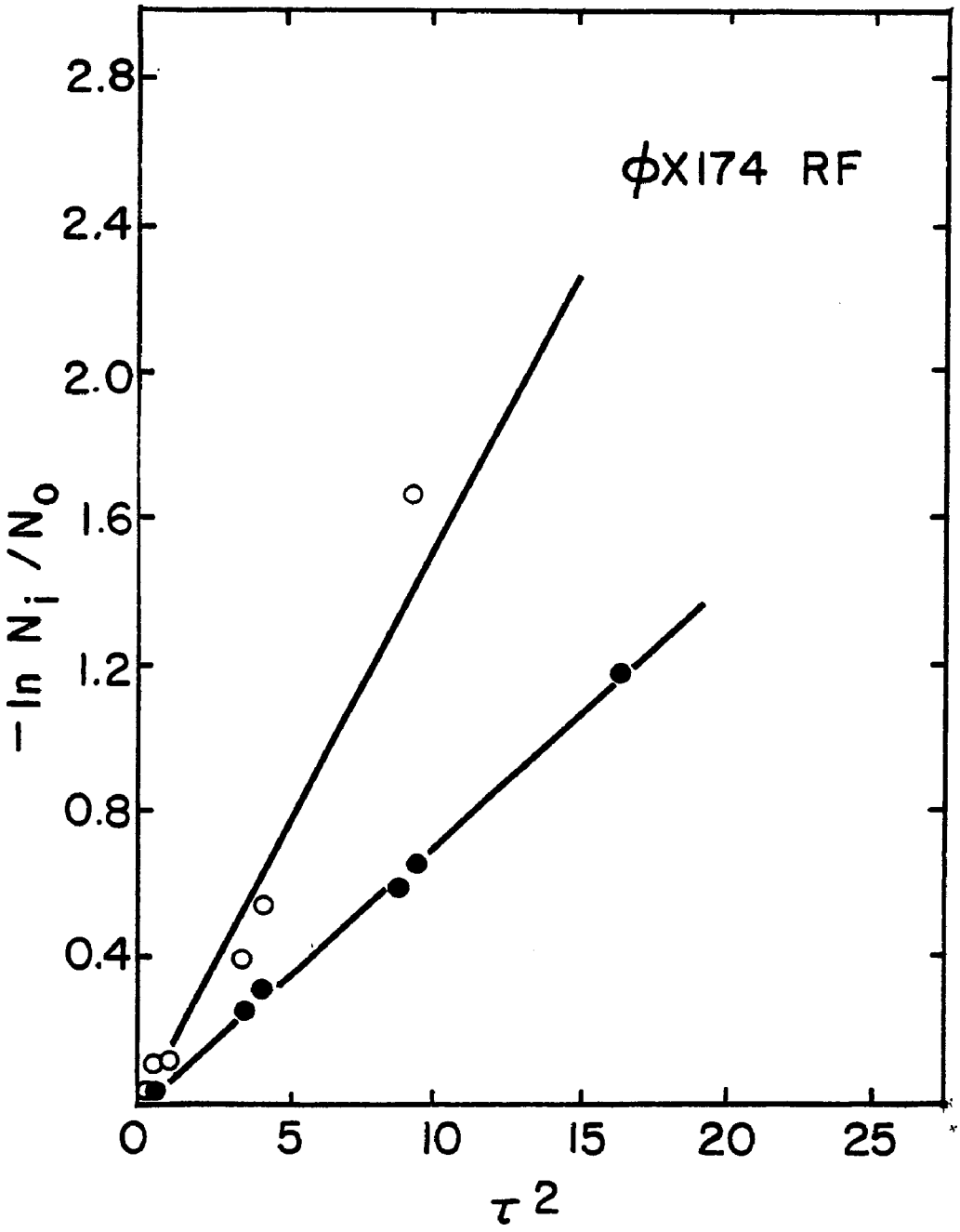


Figure 18

Figure 19. The Negative Logarithm of Relative Concentration *versus* τ^2 for Covalently Closed Relaxed and Native pMB9 DNA.

Densitometric profiles of native and covalently closed relaxed pMB9 DNA were analyzed as described in the text. Open circles refer to pMB9(I') while solid circles refer to pMB9(I). The Form I' plot represents the average of 11 determinations and has a slope of 0.15. The Form I plot is the average of 12 determinations and has a slope of 0.05.

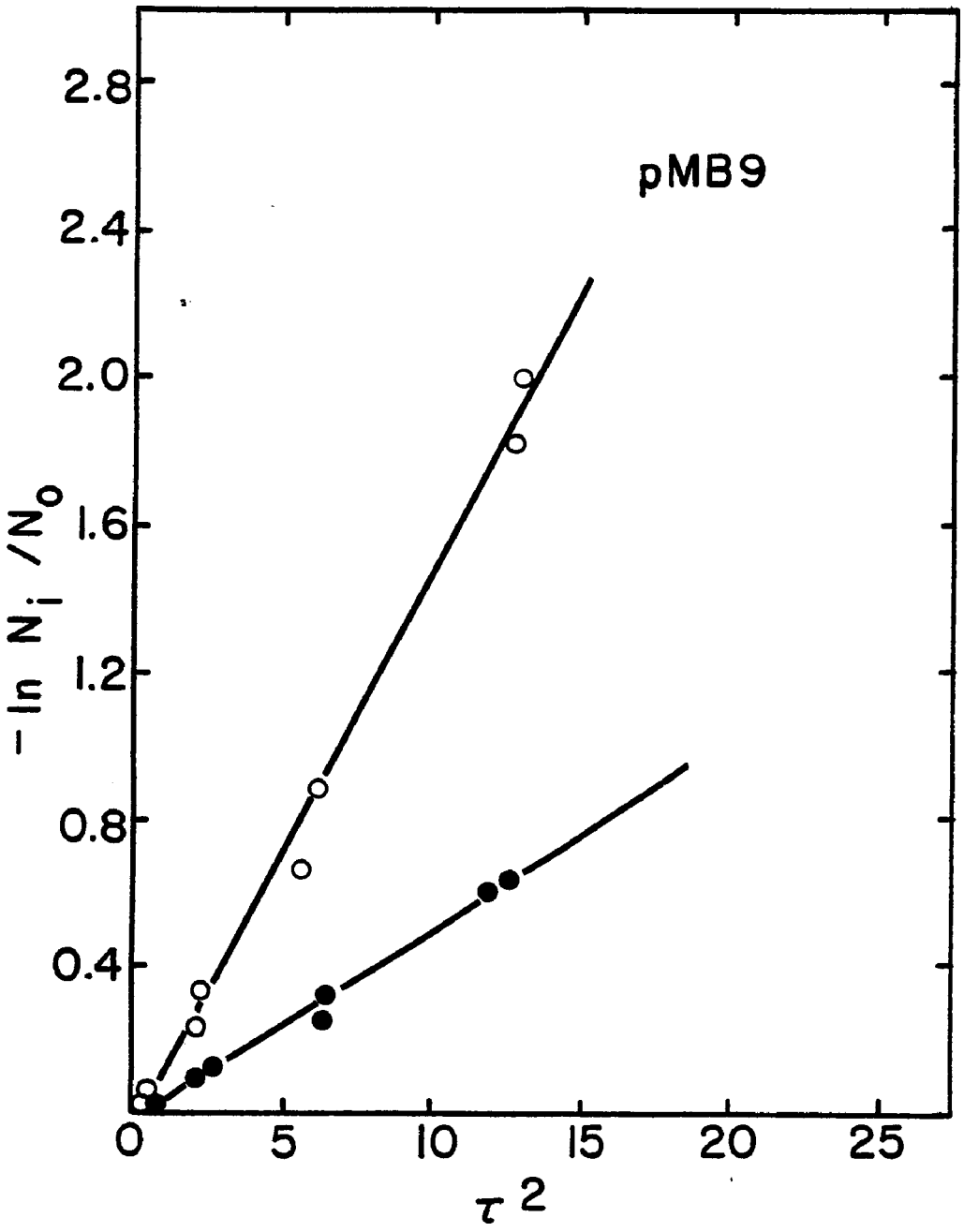


Figure 19

Figure 20. The Negative Logarithm of Relative Concentration versus τ^2 for Covalently Closed Relaxed and Native ColE1 DNA.

Densitometric profiles of native and covalently closed relaxed ColE1 DNA were analyzed as described in the text. Open circles refer to ColE1(I') while solid circles refer to ColE1(I). The Form I' plot represents the average of 8 determinations and has a slope of 0.12. The Form I plot is the average of 5 determinations and has a slope of 0.02.

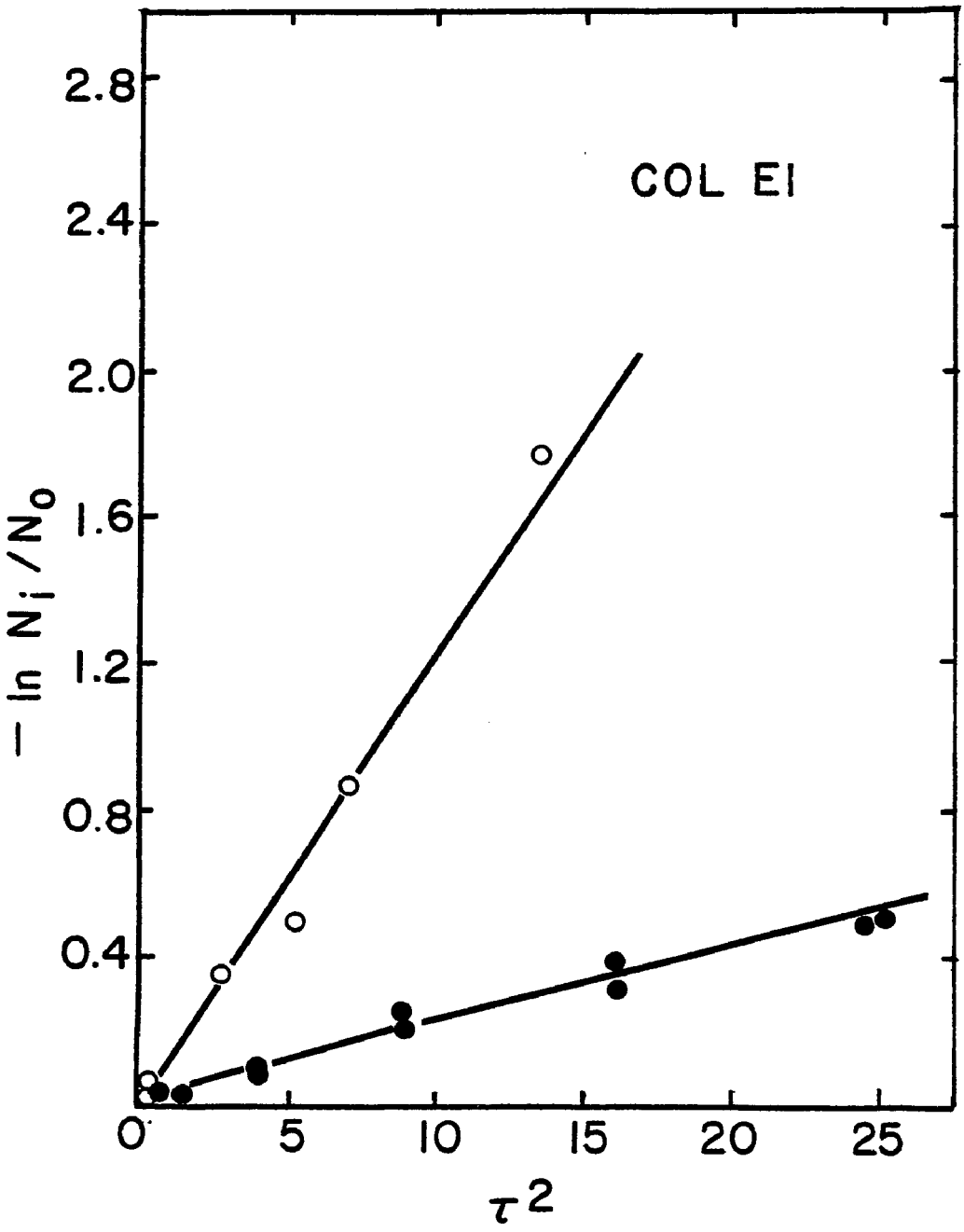


Figure 20

Figure 21. The Relationship Between the Coefficient A and DNA Length for Covalently Closed Relaxed and Native DNAs.

Coefficients for each DNA were obtained by densitometric analysis as described in the text. DNA length is expressed in terms of the number of nucleotides in each DNA which was calculated from the molecular weight (N), using a value of 331 as the average molecular weight of a nucleotide in sodium DNA. Open circles refer to Form I' DNAs while solid circles refer to native DNAs. The data point for SV40(I), denoted by the arrow, was not included when the least-squares curve fitting procedure was applied to Form I DNAs. The Form I plot below the X-axis is shown as a broken line because the validity of such an extrapolation to infinite molecular weight is questionable.

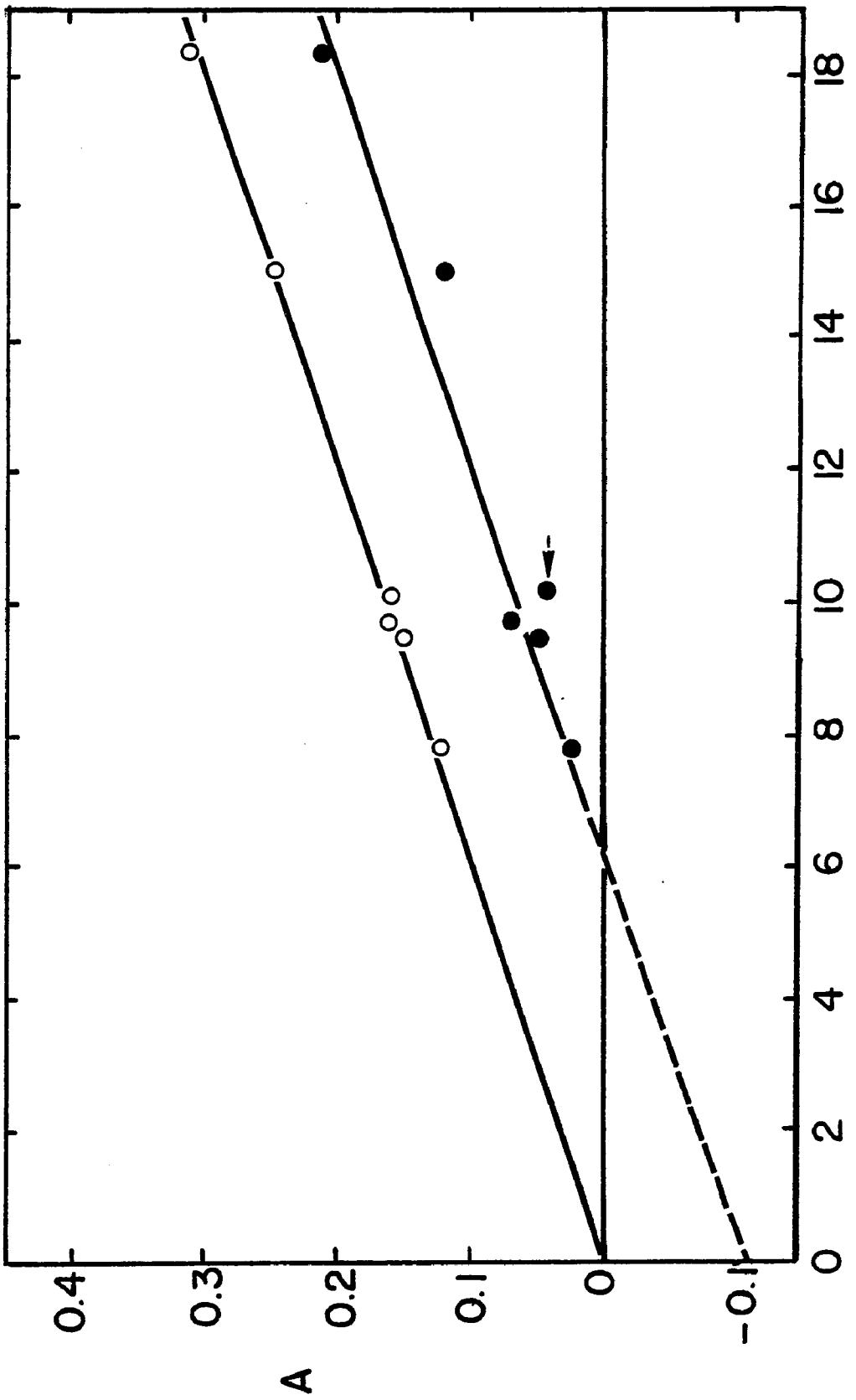


Figure 21

TABLE 2

DETERMINATION OF A FOR A VARIETY OF COVALENTLY CLOSED RELAXED FORM I' AND NATIVE SUPERCOILED FORM I DNAs

DNA	$A_{(I')}$	NO. ANALYZED	STD. DEV.	$A_{(I)}$	NO. ANALYZED	STD. DEV.	MW x 10^{-6}
pDC1	0.32	10	0.030	0.22	9	0.015	1.8
pVH51	0.25	7	0.022	0.11	14	0.011	2.2
SV40	0.16	14	0.015	0.04	14	0.005	3.25
ØX174 RF	0.16	3	0.012	0.07	6	0.008	3.4
pMB9	0.15	11	0.015	0.05	12	0.006	3.5
ColE1	0.12	8	0.012	0.02	5	0.001	4.2

TABLE 3

SUMMARY OF COEFFICIENTS AND CONSTANTS OBTAINED FOR COVALENTLY CLOSED DNAs RELAXED IN VITRO

DNA	N	A	k ($x10^{-3}$)
pDC1	5400	0.32	1.7
pVH51	6600	0.25	1.6
SV40	9800	0.16	1.6
ØX174 RF	10300	0.16	1.6
pMB9	10600	0.15	1.6
ColE1	12700	0.12	1.5

The slope of the plot in Figure 21 defines a constant k which relates A to molecular weight. This relationship is defined by the equation $AN = k = 1.6 \times 10^3$ and is summarized for the DNA investigated in Table 3. The constant k may be used to derive an equation for the molar free energy of superhelix formation for any DNA when the number of nucleotides (N) is known. The general relationship which follows from this is

$$\frac{G_\tau}{RT} = \frac{k}{N} (\tau)^2 \qquad (21)$$

and is applicable to any closed circular DNA.

The Distributions of Species in Covalently Closed Native DNAs

If multiple species are present as the limit product of enzyme reactions leading to the formation of covalently closed DNA, it is reasonable to assume that the native supercoiled form of each DNA also consists of a collection of species differing in superhelix density. It is normally impossible to observe the components of native supercoiled DNA because at the relatively high superhelix densities of these DNAs, each DNA migrates as a single species on 0.7 percent agarose gels. However, if ethidium bromide is included in the gel, a native DNA can be relaxed to a degree which permits resolution of its components. This type of relaxation will not alter the topological winding number and will therefore have no effect on the distribution of DNA among the various species. Experimentally, the amount of ethidium which resulted in a satisfactory degree

of relaxation was approximately 15 ng/ml in standard electrophoresis buffer. Small adjustments in the amount of EtBr concentration needed to achieve the desired result were occasionally necessary but rarely exceeded a difference of 1 to 2 ng/ml. SV40(I) DNA was subjected to electrophoresis in the presence of EtBr and was clearly resolvable into a series of bands. Like the SV40(I') distribution, the distribution of DNA among the various species appeared to be Gaussian. However, the native distribution was different from the covalently closed relaxed distribution in two respects. First, there were considerably more species observable in the Form I distribution than in the Form I' distribution when equivalent amounts of DNA were analyzed. There appeared, on the average, to be 14 to 15 observable species comprising the Form I distribution as compared to about 10 for SV40(I'). Secondly, the relative spacing between adjacent bands on the gel was diminished to about half of that routinely obtained with SV40(I').

To investigate the characteristics of Form I distributions SV40 as well as the five bacterial DNAs were analyzed in their native forms. The analysis was confined to a relatively narrow molecular weight range because the components of the DNA populations could not be resolved in the presence of ethidium bromide when the molecular weight of the DNA was large (greater than about 5×10^6). Densitometric profiles of each DNA were obtained from fluorescence photographs and are shown in Figure 14.

The patterns were analyzed in the same manner as those for the Form I' DNAs. The plots of $-\ln N_i/N_o$ versus τ^2 for each of the six DNAs are shown in Figures 15 through 21 (solid circles). The results have been plotted with the Form I' data for purposes of comparison. In each case, the slope of the line obtained for the native DNA is significantly less than that obtained when the DNA had been subjected to enzymatic relaxation. The values obtained for the coefficient A for each of the native DNAs are summarized with the Form I' data in Table 2. In Figure 21, the values of the coefficient A for each native DNA are plotted versus the reciprocal of the number of nucleotides. The resulting plot represents the best least-squares fit of all data points except SV40(I) and is a linear relationship. The plot intercepts the x-axis at $N = 1.6 \times 10^4$ nucleotides. The portion of the plot below $A = 0$ is depicted as a broken line because it is not clear that such an extrapolation to infinite molecular weight is valid. Nevertheless, two important observations may be made from this plot. First, the slope of the line is parallel or very nearly parallel to the line obtained for the covalently closed relaxed DNAs. However, the line for the native DNAs is displaced downward by a uniform amount which is independent of molecular weight. Secondly, the data point obtained for SV40(I) lies very close to this line. The value of A for SV40(I) was not included when the least-squares procedure was applied to the native DNAs because the distribution

of components which constitute SV40(I) DNA are presumed to reflect the intracellular state of the DNA in the eukaryotic cell. Since the other five DNAs were isolated from E. coli, the distributions of their components are presumed to reflect the state of the DNA in the prokaryotic intracellular environment. In view of the fact that SV40(I) was isolated from eukaryotic cells, the close proximity of the SV40 data point to the line established for bacterial DNAs is rather surprising. This will be dealt with in greater detail in the DISCUSSION.

Rearrangement of equation 21 leads to the prediction that the number of observable species in a given Form I' distribution should be proportional to the square root of the number of nucleotides comprising the DNA. Fluorescence photographs of the patterns for each DNA were inspected and the number of bands present in each were counted. A plot of the number of observable species versus the square root of N, the number of nucleotides, is presented in Figure 22. The data for the covalently closed relaxed DNAs are represented by the open circles while the solid circles represent data points for native DNAs. The relationship is reasonably linear for Form I' DNAs as expected. The same plot for the native forms of the DNAs is not linear and represents a higher order relationship.

The decrease in the relative spacing between Form I bands relative to Form I' bands was investigated to determine whether

Figure 22. The Number of Observable Species in a Population as a Function of the Square Root of the Number of Nucleotides.

Fluorescence photographs of the band patterns for both the covalently closed relaxed and native forms of each DNA were examined and the number of species counted. Each point represents the average from the available data for each DNA.

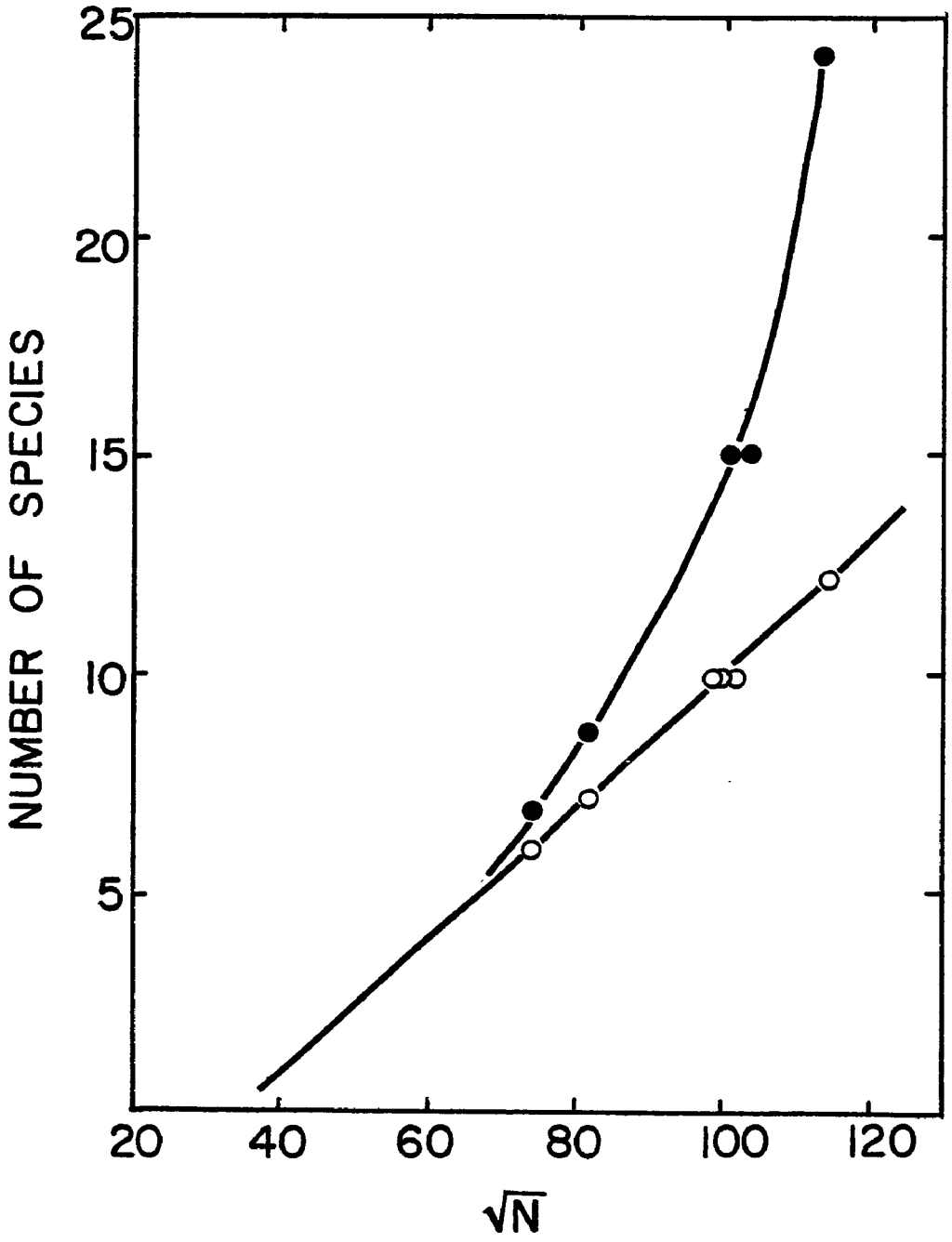


Figure 22

it reflected a fundamental difference in the DNA or whether it was a phenomenon peculiar to the analytical method. To ascertain the cause of the decreased spacing, two experiments were performed. In the first experiment, the time course of the enzymatic conversion of SV40(I) to SV40(I') using the KB relaxation enzyme was monitored on gels containing 15 ng/ml ethidium bromide. This ethidium concentration was found to be ideal in that the components of native DNA are fully displayed yet the limit product of the relaxation reaction is not so coiled that resolution is lost. A relaxation enzyme reaction was run at 23°C using SV40(I) as a substrate. Aliquots of the reaction mixture were removed periodically and the reaction quenched by addition to 50 µl of phenol and mixing. Following centrifugation to separate the phases, the aqueous phase containing the DNA was removed and prepared for electrophoresis. Samples were not dialyzed after phenol extraction since the volumes were small and uniform quantitative recovery of the DNA was unlikely. The samples obtained at each time point were loaded onto 0.7 percent agarose slab gel containing 15 ng/ml ethidium bromide and electrophoresis performed as described. The resulting gel pattern showing the progressive relaxation of SV40(I) is presented in Figure 23. Several observations may be made from this photograph. First, the breadth of the Form I distribution is significantly greater (Channel 1) than that of the limit product. Secondly, the

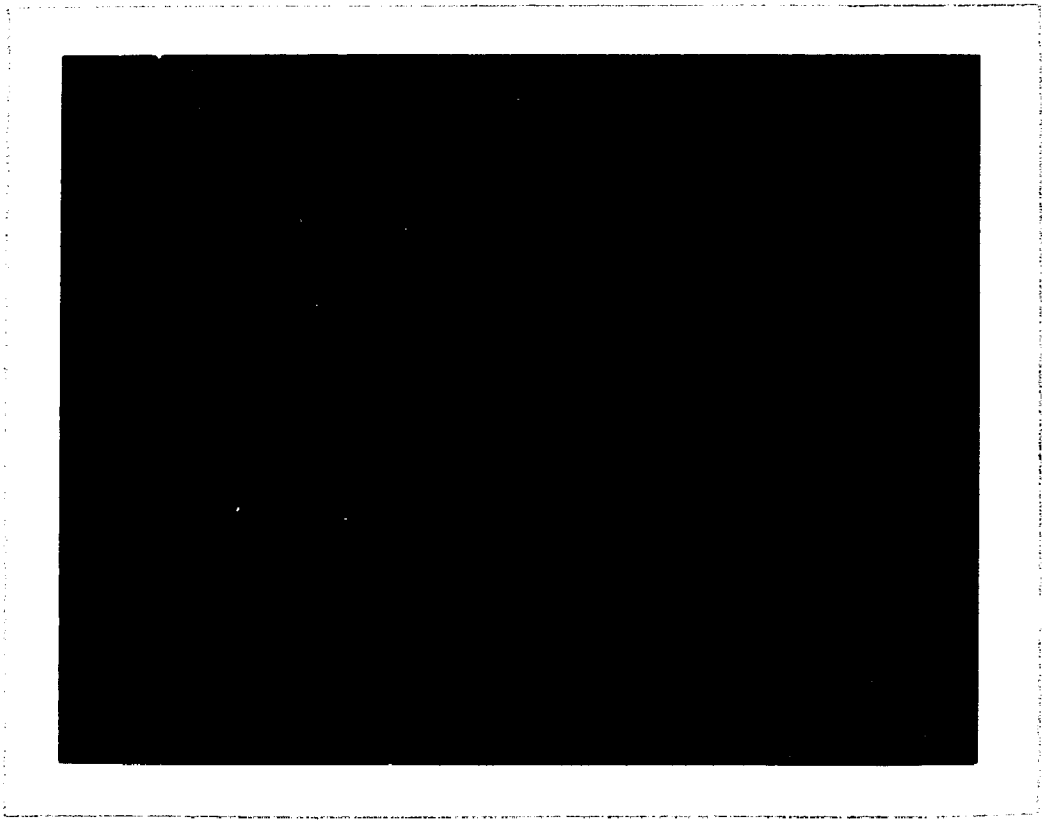


Figure 23

Progressive Relaxation of SV40(I) by Relaxation Enzyme

The relaxation reaction was performed on approximately 10 μg of SV40(I) DNA at 23°C. Aliquots were removed at time intervals during the reaction, quenched with phenol, and prepared for electrophoresis. The gel was 0.7 percent agarose and contained 15 ng/ml ethidium bromide. Under these conditions, the Form I substrate is negatively supercoiled while the limit product (Form I') is positively supercoiled. The direction of migration is from top to bottom. Time intervals are as follows: Channel 1, 0 min.; channel 2, 1 min.; channel 3, 2 min.; channel 4, 4 min.; channel 5, 5 min.; channel 6, 7 min.; channel 7, 12 min.; and channel 8, 30 min.

presence of ethidium bromide must be responsible for the reduction in relative spacing between adjacent species since in the presence of ethidium, the spacing between adjacent species in both the Form I and Form I' patterns are identical. Co-migration of reaction intermediates with Form II DNA was not achieved in this experiment because of the presence of residual phenol in the sample. The phenol extracts the ethidium added to the sample mixtures and causes the DNA to scavenge ethidium as it passes through the gel. Eventually an equilibrium is established between the DNA and the ethidium within the gel. However, before equilibrium is reached the mobilities of the covalently closed species are not uniform and co-migration is not observed. Densitometric analysis of the Form I substrate and the Form I' limit product resulted in values of A of 0.04 and 0.15 respectively. This result verifies the nature of the product and indicates that although ethidium reduces the relative spacing, it does not alter the outcome of the analysis.

A second experiment was performed to follow the reduction in relative spacing as a function of the ethidium bromide concentration. Samples of SV40(I') DNA were prepared both by the use of relaxation enzyme and the sequential use of DNase I and ligase. Aliquots of these samples were applied in duplicate to a series of 0.7 percent agarose slab gels containing ethidium bromide in concentrations ranging from 0 to 18 ng/ml. Ethidium

concentrations were carefully checked spectrophotometrically so that correction factors could be applied. Above an ethidium concentration of 8 ng/ml, SV40(I) DNA was also included as a sample for comparison. Measurements of band spacings were made from photographs of the gels in which a metric scale had been included as an internal standard. The results are illustrated in Figure 24. The curve was constructed using the average of the spacings measured for the relaxation enzyme and ligase-generated Form I' DNAs. The rationale behind averaging was that it would minimize the influence of differences in spacing which were solely attributable to position in the gel. Furthermore, care was taken to measure the distances between the same bands in each distribution for each gel. Measurements were made over a two band span and divided by two to obtain the average band spacing. The distances between bands are reported relative to the spacing in the absence of ethidium. The reduction in spacing occurs fairly rapidly at very low ethidium concentrations and reaches what appears to be a minimum value at or near 14 to 16 ng/ml. Form I included in these gels had exactly the same relative spacing as the Form I' DNAs. This experiment demonstrates that the winding increment between adjacent bands in the SV40(I) distribution is one turn. Additional observations reported in the following section will demonstrate that this reduced spacing is a minimum which in the concentration range in which the phenomenon is observable, appears to be insensitive to the amount of ethidium bound.

Figure 24. Decrease in Relative Band Spacing With Increasing EtBr Concentration in the Gel.

Each point represents the average of measurements made on duplicate samples of relaxation enzyme-generated and ligase-generated SV40(I') DNA. The ethidium concentrations have been corrected by a factor obtained by spectrophotometric determination of the EtBr in the stock solution.

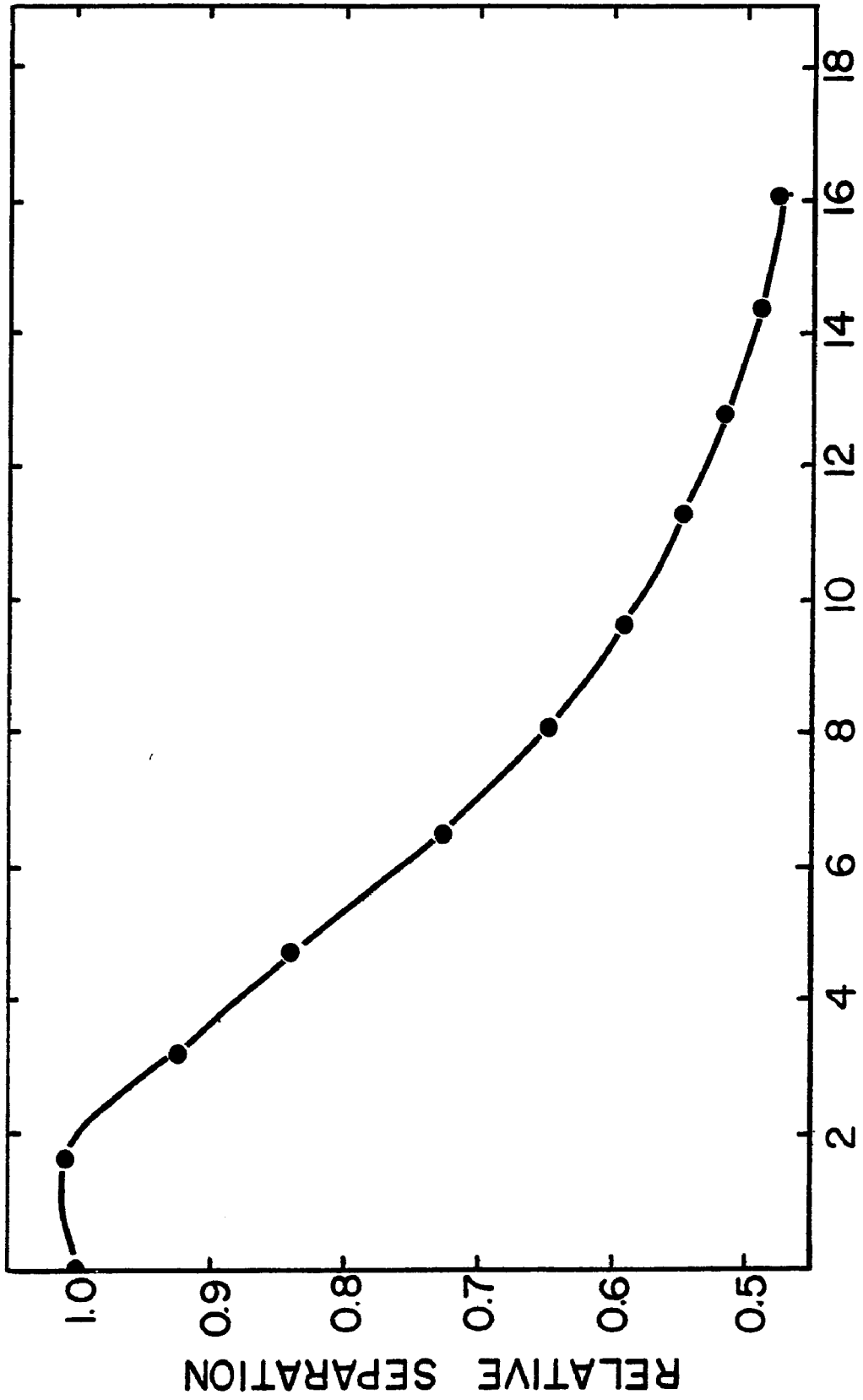

RELATIVE SEPARATION
1.0
0.9
0.8
0.7
0.6
0.5
2
4
6
8
10
12
14
16
18
ETHIDIUM CONCENTRATION (ng·ml⁻¹)

Figure 24

Dye Titrations of Covalently Closed DNA

From the time this work was initiated, it seemed reasonable to apply the technique of agarose gel electrophoresis to dye titrations of supercoiled DNA. Agarose gels are highly sensitive to the tertiary structure of DNA and will separate covalently closed supercoiled molecules from nicked circular molecules of the same molecular weight. It was demonstrated in the previous section that ethidium could be used in the gel to partially relax native supercoiled DNA so that the populations of topological isomers could be examined. However, when a slab gel apparatus is used, the effect of only one ethidium concentration can be examined at a time. Cylindrical gels offer a means for circumventing this apparent limitation.

The electrophoretic titration technique takes advantage of the fact that ethidium is positively charged. During electrophoresis, the ethidium migrates towards the cathode while the DNA migrates towards the anode at a rate determined principally by the shape of the molecule at the dye concentration within the gel. In order for meaningful data to be obtained, this migration rate must be uniform and this requires that an equilibrium condition exist within the gel between the DNA and the dye. To insure a state of equilibrium, measures must be taken to preclude scavenging of ethidium by the DNA when electrophoresis is initiated and to terminate electrophoresis before the ascending ethidium boundary reaches the leading DNA

band. As long as these requirements are met, the number of ethidium concentrations which can be examined simultaneously is limited only by the capacity of the apparatus. In point of fact, even this need not be a problem since another apparatus may be connected to the same power supply, thereby doubling the capacity.

Termination of electrophoresis at an appropriate time is a simple matter to control and requires only a knowledge of where the ethidium boundary is with respect to the DNA. The ascending dye boundary is clearly visible in gels containing large amounts of ethidium (10 μg/ml) and was found to have a mobility about equal to that of the tracking dye, brom phenol blue. Electrophoresis was therefore terminated before the tracking dye had migrated a distance half as great as the length of the gel.

The rapid equilibration of the DNA with the ethidium in the gel can be accomplished by adding a large excess of ethidium to the samples. Rapid equilibration requires that the large amount of bound ethidium in the sample dissociate quickly and be removed from the DNA so that the DNA is exposed to the steady-state dye concentration in the gel. A control experiment indicated that dissociation is a rapid process. Samples were prepared containing 0.35 μg of a mixture of SV40(I) and SV40(II) DNA. Ethidium bromide was added to each sample to give final concentrations ranging from zero to 200 μg/ml and

the samples were applied to 15 cm 0.7 percent agarose gels containing no ethidium. Following electrophoresis, it was found that the migration of Form I relative to Form II was independent of the amount of ethidium in the sample. This demonstrates that the bound ethidium dissociates before any detectable alteration in migration can occur, even when the ethidium concentration is 10^4 times greater than that required to relax SV40(I) completely. The only requirement for ethidium in the sample is that its concentration be high enough that the DNA will have bound to it more ethidium than will be bound to it in the gel. Lower concentrations in the sample will cause the DNA to scavenge ethidium until equilibrium is reached. For this reason, samples for titration were brought to a convenient volume in electropohresis buffer and ethidium was added in a 5 to 7-fold weight excess over DNA.

Dye titrations were routinely performed on 27 cm agarose gels at 1.1 V/cm for 15 hours at room temperature. Initially, 15 cm gels were used at 6.7 V/cm but the resolution was comparatively poor and there was difficulty in determining the critical free dye concentration (c'). In addition, the electropohresis buffer was modified to contain 20 mM NaCl. In the presence of the salt, the affinity of the dye for the DNA is reduced so that small errors in the determination of the ethidium concentration and dilution errors have a smaller effect. Normally, a series of gels was prepared for a titration

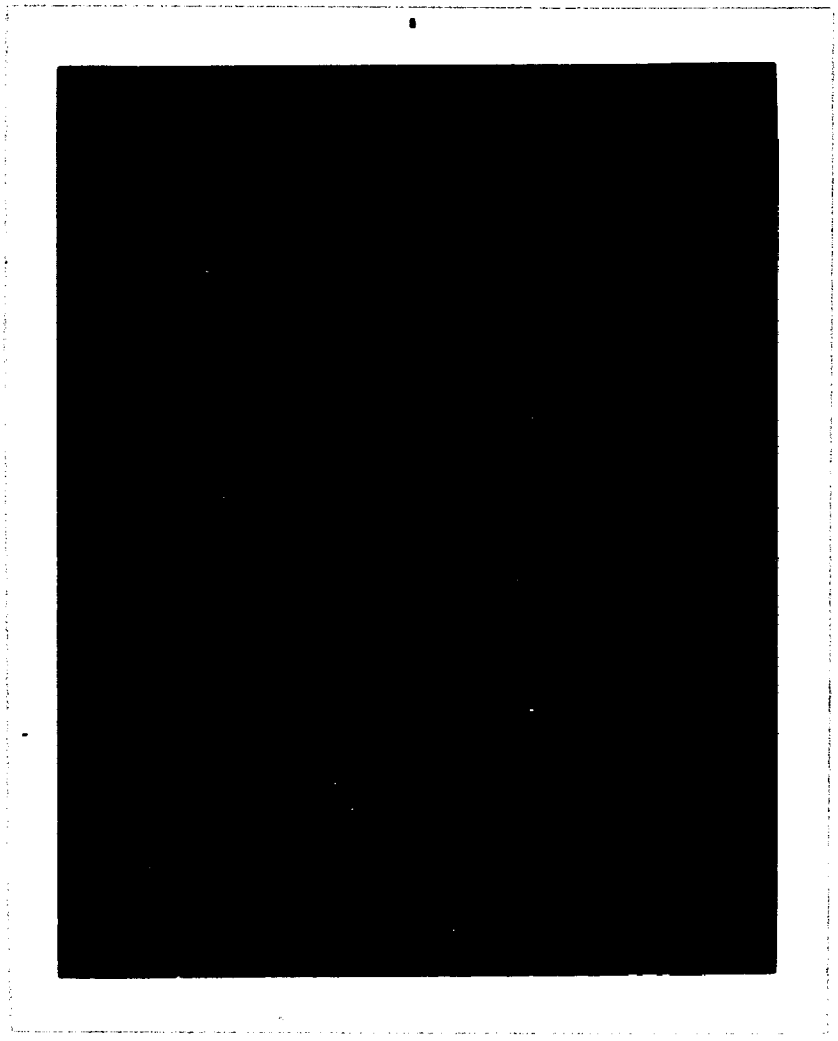


Figure 25

Dye Titration of Native pDC1 DNA

The dye titration was performed according to the electrophoretic technique described in the text. The ethidium concentration in this series of gels increases in increments of approximately 10 ng/ml from left to right. The gel at the extreme left contained no ethidium. The direction of migration is from top to bottom. The uppermost band in each gel is pDC1(II).

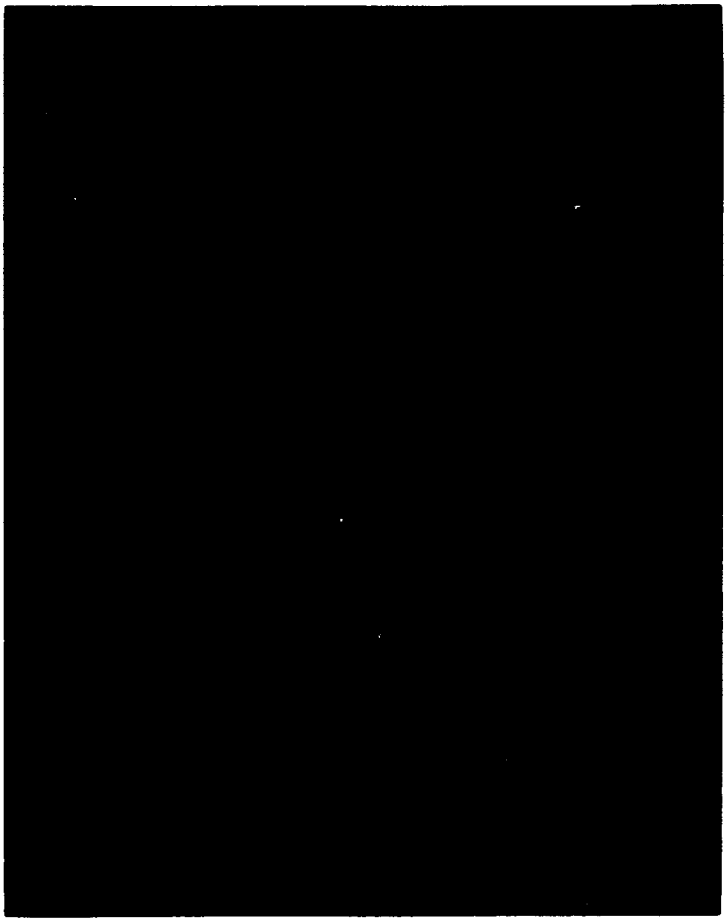


Figure 26

Dye Titration of Native pVH51 DNA.

The dye titration was performed as described in the text. The ethidium concentration in this series of gels increases in increments of approximately 10 ng/ml from left to right. The gel at the extreme left contained no ethidium. Migration is from top to bottom. This series of gels demonstrates the capacity to simultaneously titrate two DNAs in the same series of gels. The more rapidly migrating set of DNA species is pVH51 monomer while the more slowly migrating DNA is the dimeric form of pVH51.

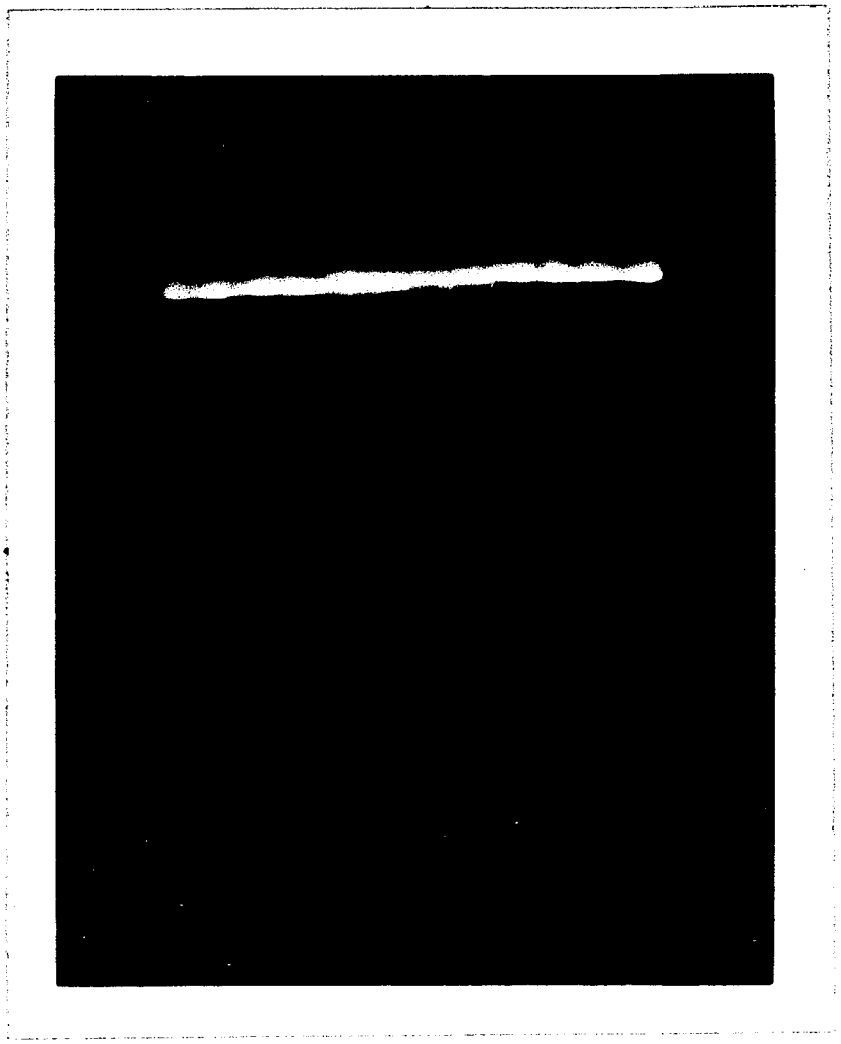


Figure 27

Dye Titration of Native SV40 DNA

The dye titration was performed as described in the text. The ethidium concentration in this series of gels increases in increments of approximately 10 ng/ml from left to right. The gel at the extreme left contained no ethidium. Migration is from top to bottom.

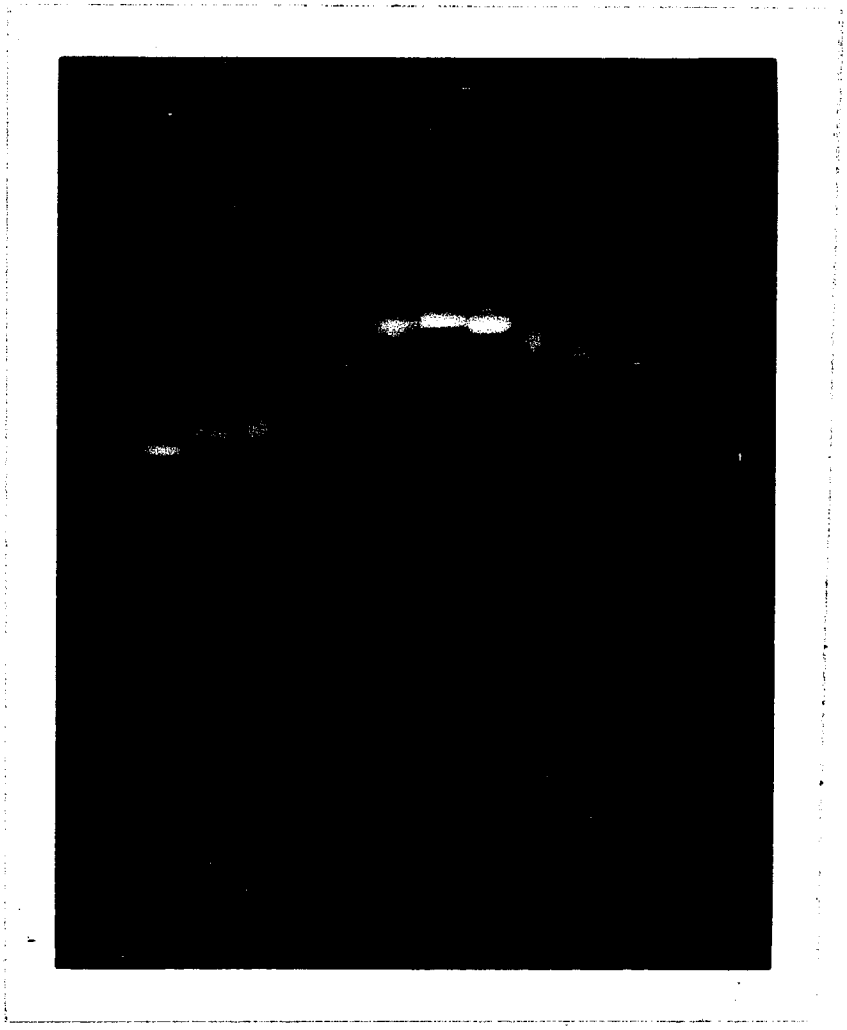


Figure 28

Dye Titration of Native ØX174 RF DNA

The dye titration was performed as described in the text. The ethidium concentration in this series of gels increases in increments of approximately 10 ng/ml from left to right. The gel at the extreme left contained no ethidium. Migration is from top to bottom. The titration curve of dimeric ØX174 RF DNA can be seen faintly just above the monomeric DNA in the photograph.

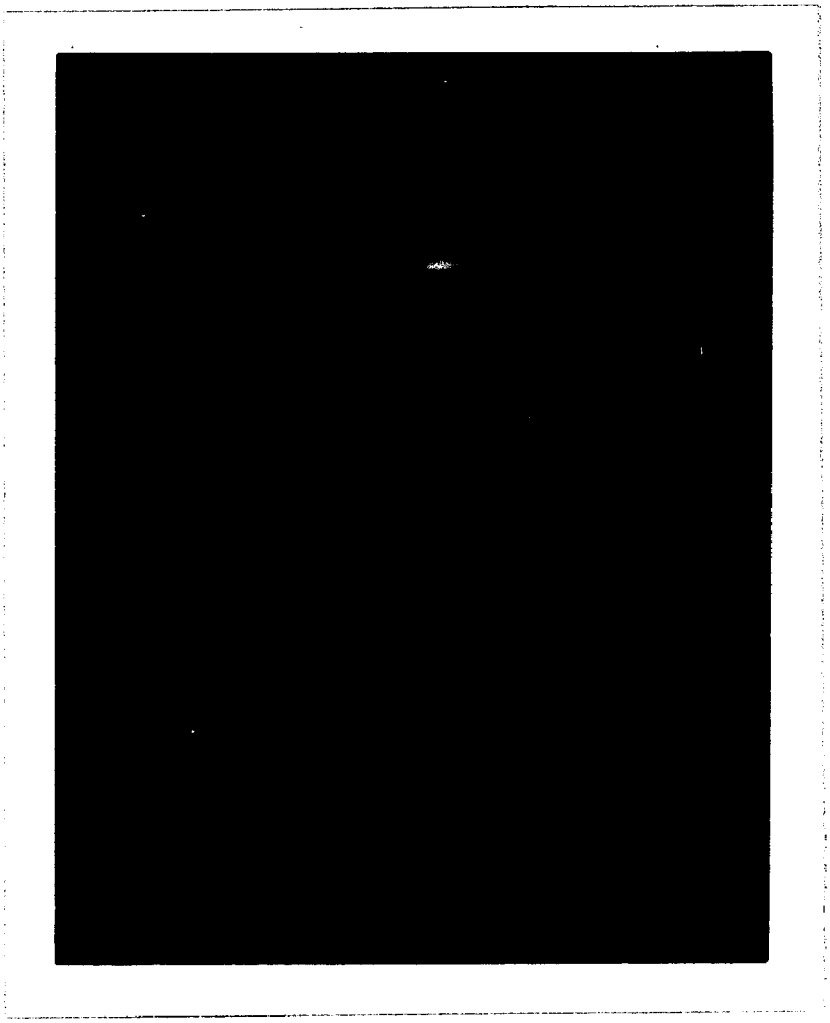


Figure 29

Dye Titration of Native pMB9 DNA

The dye titration was performed as described in the text. The ethidium concentration in this series of gels increases in increments of approximately 10 ng/ml from left to right. The gel at the extreme left contained no ethidium. Migration is from top to bottom. Dimeric pMB9 DNA can be seen migrating behind pMB9(II).

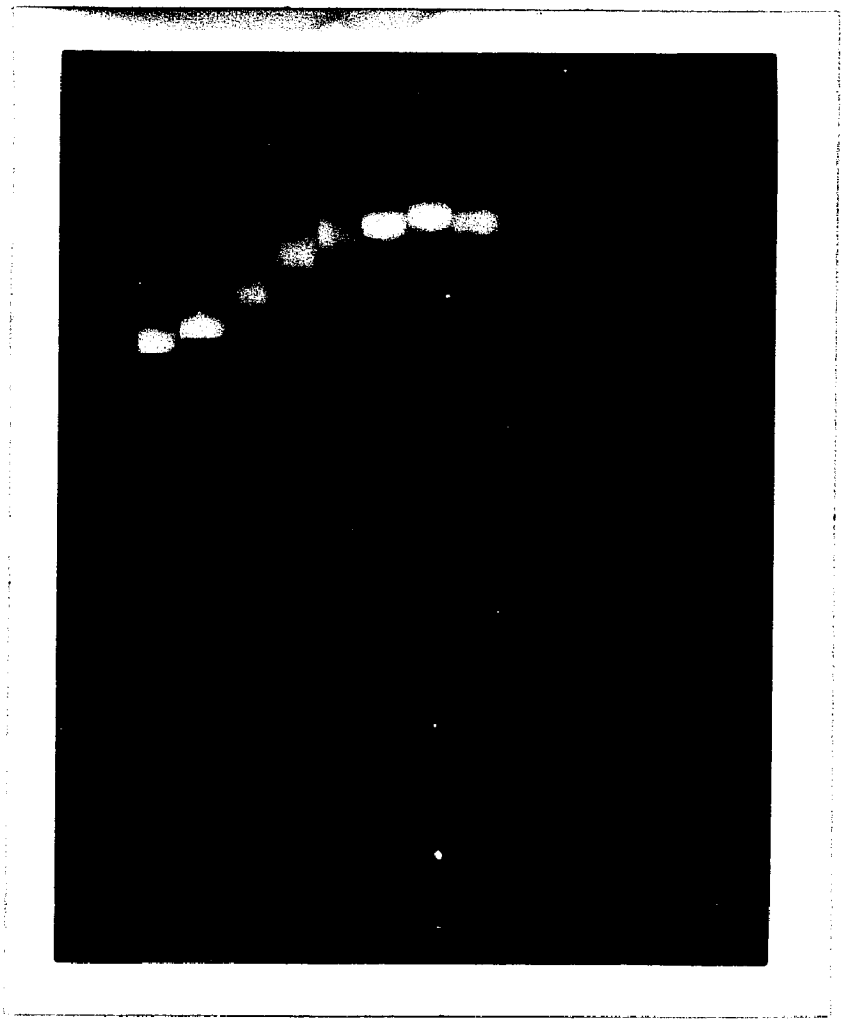


Figure 30

Dye Titration of Native ColE1 DNA

The dye titration was performed as described in the text. The ethidium concentration in this series of gels increases in increments of approximately 10 ng/ml from left to right. The gel at the extreme left contained no ethidium. Migration is from top to bottom.

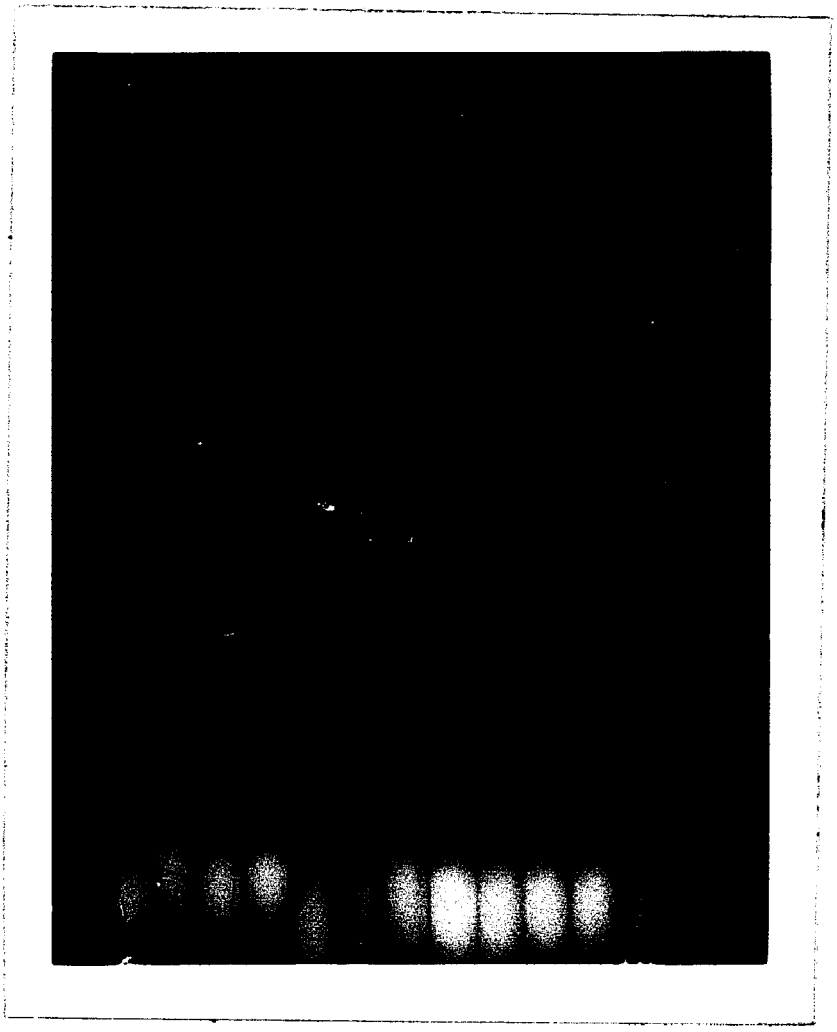


Figure 31

Dye Titration of Native pSC101 DNA

The dye titration was performed as described in the text. The ethidium concentration in this series of gels increases in increments of approximately 10 ng/ml from left to right. The gel at the extreme left contained no ethidium. Migration is from top to bottom. Component species of DNA populations are not resolvable at this molecular weight in the presence of the dye.

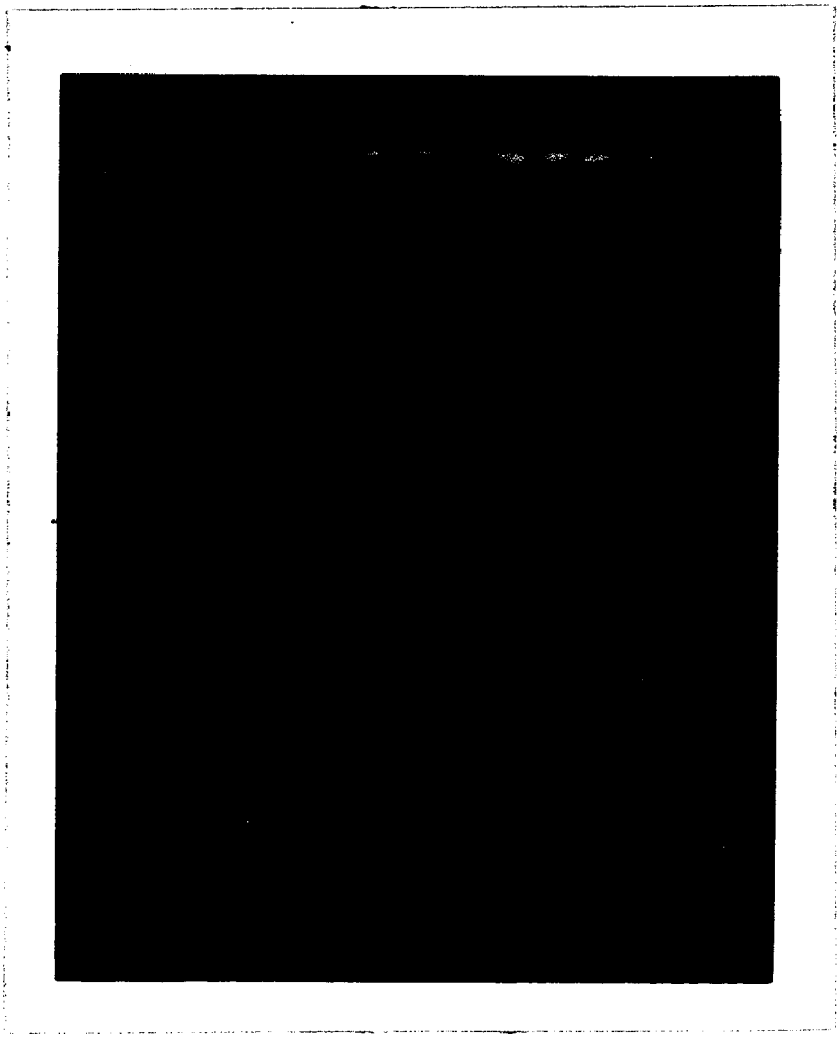


Figure 32

Dye Titration of Native ColEl*amp* DNA

The dye titration was performed as described in the text. The ethidium concentration in this series of gels increases in increments of approximately 10 ng/ml from left to right. The gel at the extreme left contained no ethidium. Migration is from top to bottom. Component species are not resolvable at this molecular weight in the presence of dye.

containing ethidium in concentration increments of 10 ng/ml. This figure was later corrected to reflect the true concentration based on a spectrophotometric determination of the ethidium concentration in the stock solution. The increment in ethidium concentration was always somewhat less than 10 ng/ml but was generally within 90 percent of this value.

Dye titrations of native pDC1, pVH51, SV40, ØX174 RF, pMB9, ColE1, pSC101, and ColE1amp DNA are shown in Figures 25 through 32. The entire titration of each DNA was performed in a standard 12-place cylindrical gel apparatus and covered a range of ethidium concentrations from zero to approximately 110 ng/ml. The critical free dye concentration is that dye concentration at which the relaxed Form I DNA co-migrates with Form II. This can be estimated within an error of less than 10 percent under these conditions. When the critical free dye concentration did not correspond exactly to one of the ethidium concentrations used in the titration, the dye concentration in the gel which most closely approximated c' was used. It was occasionally noted that co-migration of Form I DNA with Form II did not occur even though there was a clear minimum in the titration. The reason for this behavior is unclear but it never seemed to affect the apparent c' calculated from the titration. From the figures it is apparent that in spite of the wide range of molecular weights, the DNAs titrated are all relaxed within a fairly narrow range of dye concentrations. The data for these DNAs are presented in Table 4.

TABLE 4

SUMMARY OF CRITICAL FREE DYE CONCENTRATION FOR DNAs TITRATED

DNA	$c'(ng \cdot ml^{-1})$	$c'(M)$	NO. TITRATIONS	TEMP. AT WHICH DNA WAS SYNTHESIZED (^{o}C)
pDC1	61	1.5×10^{-7}	2	28
pVH51	60	1.5×10^{-7}	2	28
SV40	47	1.2×10^{-7}	3	37
ØX174 RF	53	1.3×10^{-7}	2	37
pMB9	57	1.4×10^{-7}	2	28
ColE1	56	1.4×10^{-7}	2	28
pSC101	65	1.6×10^{-7}	1	37
ColE1amp	65	1.6×10^{-7}	1	37

In some of the preparations titrated, the dimeric form of the DNA was present. In all cases, the titration curve for the dimeric form of the DNA was identical to that for the monomeric form. This capacity for dual titration serves to emphasize the value of this technique since, not only can the titration be performed quickly but the simultaneous titration of two DNAs within the same series of gels provides a direct means by which the superhelical densities of two DNAs may be compared under identical conditions. Two DNAs of the same molecular weight may also be examined on the same set of gels if the application of the second DNA sample to the gels is delayed for a suitable period of time after electrophoresis of the first sample has begun.

In relation to an observation made in the preceding section concerning the relative spacing between bands in the presence of ethidium bromide, the dye titration serves to illustrate three points. First, the components of the distributions are visible on either side of the minimum in the titration curve. Secondly, the relative spacing between bands is independent of the dye concentration since the distance between adjacent bands is the same at dye concentrations greater than c' as it is at dye concentrations less than c'. This phenomenon was observed in standard electrophoresis buffer as well. Thirdly, it was not possible to resolve components of a DNA population in the presence of ethidium bromide above a molecular weight

limit which lies between 4.4 million (pVH51 dimer) and 5.8 million (pSC101).

The data presented in Table 4 can be used to obtain the superhelix density of each DNA. By employing the Scatchard equation (equation 7), the molar ratio of bound dye per nucleotide at equilibrium (ν_c) can be calculated. At this point, a knowledge of the association constant for the ethidium:DNA binding reaction is required. The ionic strength of electrophoresis buffer containing 20 mM NaCl was determined from conductance measurements and expressed in terms of equivalent NaCl concentrations. This permitted the association constant for the binding reaction to be estimated from the data of Hinton and Bode (29). By interpolation, the association constant was found to be $2.0 \pm 0.2 \times 10^6$ 1/M.

Experimental evidence was obtained which indicates that this value of the association constant is reasonable. Relaxation enzyme was used to relax two samples of SV40 DNA. One sample was relaxed at 32°C while the other reaction was performed at 43°C. From the known change in base rotation angle as a function of temperature, the difference in superhelical densities between these two DNA samples is calculated as

$$\Delta\sigma = \frac{-10\phi}{180}\Delta\nu_c = \frac{0.12}{360}\Delta T \qquad (22)$$

The duplex unwinding angle (ϕ) of ethidium is taken to be 26°. The quantity ΔT refers to the difference in the temperatures

at which the two DNAs were relaxed. Solving for $\Delta\nu_c$ in terms of ΔT leads to the expression

$$\Delta\nu_c = 2.3 \times 10^{-4} \Delta T \qquad (23)$$

This relationship is substituted into the Scatchard equation and K_a is found in terms of $\Delta c'$ and ΔT.

$$K_a = \frac{2.3 \times 10^{-4} \Delta T}{\Delta c' (\nu_m - 2.3 \times 10^{-4} \Delta T)} \qquad (24)$$

Since, for the reaction conditions employed in which the temperatures differed by only 11°C, the term $2.3 \times 10^{-4} \Delta T$ is very much smaller than ν_m which is taken to be 0.18 and the denominator in equation (24) may be simplified to $\Delta c'(\nu_m)$.

Titrations were performed on each of the two samples on identical sets of gels in which the increment on ethidium concentration was nominally 1 ng/ml. The concentrations were corrected by a factor obtained from a spectrophotomatric determination of the ethidium concentration of the stock solution. Samples were subjected to electrophoresis as described for the previous titrations and were stained and photographed for examination. Although it was difficult to determine precisely the critical free dye concentration for each of the two samples, an estimate of the difference was made to the nearest 0.5 ng/ml. Taking the correction factor into account, the difference in c' for the two samples at room temperature in electrophoresis buffer containing 20 mM NaCl was determined to be approximately 2.2 ng/ml. Conversion of this value to molar concentration and

substitution into equation 24 yields a value for the association constant of approximately 2.5 x 10^6 1/M. The value of K_a as determined from the data of Hinton and Bode is within the limits of experimental error of this method which is estimated to be on the order of 25 percent.

The use of the Scatchard equation and the data presented in Table 4 yields values of ν_c which, in turn, can be used to calculate the superhelix density of each DNA. Because a value for ϕ of 12° was used extensively in much of the previous literature, superhelical densities were calculated using values of 12° as well as 26° for purposes of comparison. When the value of ϕ is taken to be 12°, the relationship between σ_o and ν_c is

$$\sigma_o = -0.67\ \nu_c \qquad (25)$$

while a 26° unwinding angle yields the relationship

$$\sigma_o = -1.44\ \nu_c \qquad (26)$$

The results of these calculations are presented in Table 5. Although the superhelical winding number is dependent on the molecular weight of the DNA, the superhelix densities of the various DNAs are reasonably constant regardless of the source. The values for the superhelix density of the DNAs obtained from these titrations are somewhat lower than those reported by Wang (21) and Vinograd (9,31). However, these two investigators obtained their values by performing their titrations in very high ionic strength media which causes an increase in the superhelix density of the DNA (21).

TABLE 5

SUPERHELIX DENSITIES AND SUPERHELIX WINDING NUMBERS FOR NATIVE CLOSED CIRCULAR DNAs AS DETERMINED BY DYE TITRATION ON AGAROSE GELS

DNA	c' (M)	ν_c	σ_{o12}	τ_{o12}	σ_{o26}	τ_{o26}
pDC1	1.5×10^{-7}	0.042	-0.028	-8	-0.060	-16
pVH51	1.5×10^{-7}	0.042	-0.028	-9	-0.060	-20
SV40	1.2×10^{-7}	0.035	-0.023	-11	-0.050	-25
ØX174 RF	1.3×10^{-7}	0.037	-0.025	-13	-0.053	-27
pMB9	1.4×10^{-7}	0.039	-0.026	-13	-0.056	-30
ColE1	1.4×10^{-7}	0.039	-0.026	-16	-0.056	-36
$(pVH51)_2$	1.5×10^{-7}	0.042	-0.028	-19	-0.060	-40
pSC101	1.6×10^{-7}	0.044	-0.029	-25	-0.063	-55
$(ØX174\ RF)_2$	1.3×10^{-7}	0.037	-0.025	-26	-0.053	-54
$(pMB9)_2$	1.4×10^{-7}	0.039	-0.026	-27	-0.056	-59
ColE1amp	1.6×10^{-7}	0.044	-0.029	-32	-0.063	-69

MOLAR FREE ENERGY OF SUPERHELIX FORMATION AND FREE ENERGY PER BASE PAIR FOR NATIVE SUPERCOILED DNAs TITRATED

TABLE 6

DNA	A*	G_τ (kcal/mole)	g_τ (cal/mole base pair)
pDC1	0.32	49	18
pVH51	0.25	59	18
SV40	0.16	59	12
ØX174 RF	0.16	69	13
pMB9	0.15	80	15
ColE1	0.12	92	15
$(pVH51)_2$	0.12	114	17
pSC101	0.09	160	18
$(ØX174\ RF)_2$	0.08	140	13
$(pMB9)_2$	0.08	160	16
ColE1amp	0.07	200	18

*Values of A for DNAs larger than ColE1 were calculated from the relationship A = k/N. All other values of A were determined experimentally.

The information presented in Table 5 can be used in conjunction with the data presented in the preceding section and equation 21 to determine the molar free energy of superhelix formation for each DNA at 25°C. At constant superhelix density, the free energy per 10 base pairs should also be constant and is an intensive quantity. The results of these calculations are shown in Table 6. Values for the coefficient A for DNAs larger than ColE1 were calculated from the relationship $A = k/N$ where N is the number of nucleotides in the DNA. The calculations presented in Table 6 demonstrate that although G_τ increases four to five-fold over the range of molecular weights investigated, g_τ, the free energy per base pair remains relatively constant. Variability in these values reflects variability in the superhelix densities of the DNAs.

DISCUSSION

Characteristics of the Electrophoresis System

As demonstrated in the previous section, agarose gel electrophoresis is a technique with high resolution capabilities which has proven useful for analyzing DNA in a variety of conformations. The results obtained indicate that the absolute mobility as well as the relative mobility of a DNA molecule is strongly dependent upon the conditions under which electrophoresis is performed. This dependence serves to increase the utility of the technique since conditions may be varied in such a manner as to enhance the separation of specific forms of a particular DNA.

Based on the results presented, some hypotheses of a qualitative nature may be offered to explain the observed electrophoretic behavior of DNA on agarose gels. However, because the nature of the agarose matrix is poorly understood, and the nature of the interaction between the DNA and the matrix is unknown, definitive statements concerning the shape of a DNA molecule and the influence of electrophoresis conditions are impossible. Arnott et al. (111) have studied the structure of agarose by x-ray diffraction, optical rotation, and computerized model building and concluded that, upon solidification, agarose forms parallel double helices which coalesce to form a porous network. They suggest that the network of fibers is an unusual

system of internal channels and comparatively large voids through which water molecules and particles can diffuse.

Fisher and Dingman (34) and Dingman et al. (35) have demonstrated a linear relationship between the logarithm of electrophoretic mobility for a given DNA species and the concentration of polyacrylamide in the composite gel system. This relationship has a negative slope which represents the retardation coefficient (109). The retardation coefficient reflects the properties of the gel as well as the size and shape of the migrating molecule. The data presented in Figure 4 shows that a linear relationship also exists between the logarithm of the electrophoretic mobility, and the gel concentration in the agarose gel electrophoresis system. Extrapolation of the plots in Figure 4 to zero agarose indicates that in free solution, SV40(I) and (II) should have the same or nearly the same mobilities while linear molecules should migrate more slowly. Similar data has recently been obtained in a more detailed comprehensive study by Johnson and Grossman (112) for the agarose gel system. The slope of the log mobility versus percent agarose plot was least for linear DNAs which indicates that linear DNA is least affected by increasing agarose concentration. This result is consistent with the suggestion offered by Dingman et al. (35) to explain the behavior of linear DNA in the composite system. They proposed that because retardation coefficients for linear

DNAs were small and minimally influenced by molecular weight, the linear duplex molecules were migrating through the gel matrix end-on. Though it may explain the observed behavior, it is intuitively a difficult concept to accept.

The isomobility points determined for PM2 DNA by Johnson and Grossman (112) are somewhat different than those observed in this study with SV40 DNA. SV40(II) and (L) co-migrated in 0.4 percent agarose which is similar to the concentration determined by extrapolation of the data of Johnson and Grossman (112). However, they determined that the isomobility point for PM2(I) and (L) was 0.92 percent agarose while in this study using SV40 DNA, the isomobility point was found to be 1.4 percent agarose. The differences are attributable to two factors. The molecular weight of PM2 DNA is about twice that of SV40 and their data was obtained using a voltage gradient of about half that used for the experiment with SV40 DNA. From Figures 2 and 3, it is clear that the relative mobility of Form I and linears with respect to Form II is not independent of molecular weight. Furthermore, Johnson and Grossman have noted that the isomobility points are influenced by the magnitude of the voltage gradient.

Dingman et al. (35) noted that in the composite agarose-polyacrylamide gel system, a large increase in the mobility of circular DNA species occurred as the voltage gradient was

increased but noted only a modest increase in the mobility of duplex linear DNA. In contrast, the results of Johnson and Grossman (112) as well as the result of this study indicate a large increase in the mobilities of both linear and nicked circular DNA and only a moderate increase in the mobility of Form I DNA with increasing field strength. The results of Dingman et al. (35) would seem to be more consistent with the interpretation that linear molecules migrate end-on. Nevertheless, the results obtained in this study may indicate that as the field strength increases, the DNA may be forced through some of the smaller pores in the matrix or that a favorable orientation of the molecule becomes less important. This leads to speculation that the decreased response of Form I DNA to increasing field strength reflects the greater rigidity of the molecule as compared to Form II and linears which are expected to be somewhat more flexible.

The increased mobility observed as the ionic strength was increased was unexpected since a greater degree of charge masking at higher ionic strengths should cause a decrease in the electrophoretic mobility. Johnson and Grossman (112) have noted a similar effect and suggest that increased gel temperature may be responsible. An increase in the rate of heat production is expected to accompany increases in ionic strength. No attempt was made to control temperature in the experiment

performed in this study to test the effect of varying ionic strength.

The experiments designed to investigate the effects of ionic strength and field strength on the mobility of SV40 DNA demonstrate that it is not sufficient to consider relative mobility alone. If only relative mobilities are considered, the result of both of these experiments lead to erroneous interpretations.

Perhaps the most surprising feature of the agarose gel system is its ability to discriminate between covalently closed DNA species differing by only a single superhelical turn. Espejo and Lebowitz (42) note that there is a correlation between the intrinsic viscosity of a circular DNA and the ability of the gel matrix to resolve the species. Waring has shown that very little change in the intrinsic viscosity of a native covalently closed molecule occurs until about halfway through the titration to ν_c (113). The range of superhelix densities which can be resolved for a given DNA appears to correspond well to the greatest changes in the intrinsic viscosity. This observation may also explain why species are not resolved when the number of superhelical turns is very close to zero. The spacing between adjacent bands extending from Form II to Form I is also approximately sigmoidal which is consistent with this interpretation.

The decrease in relative spacing between adjacent species in the presence of EtBr was troublesome in that it limited the range of molecular weights which could be investigated. The reasons for the decrease in spacing are not obvious. It cannot be due entirely to charge neutralization by the ethidium or to an ethidium-induced lengthening or stiffening of the DNA since there does not appear to be any significant dependence on ethidium concentration above 15 to 16 ng/ml in electrophoresis buffer. From the titration photographs, it can be seen that bands are equally spaced at ethidium concentrations both greater and less than ν_c, representing differences in ethidium concentrations as great as 80 to 90 ng/ml.

Analysis of Distributions of Covalently Closed DNA

The agarose gel technique presented an opportunity to perform a type of analysis which is rarely possible in most physical chemical systems. Unlike most systems in which the behavior of whole populations is observed, the electrophoretic technique employed in this study allowed the analysis to be extended to components of DNA populations and resulted in a reasonably unambiguous evaluation of the effects of some environmental factors as well as thermodynamic parameters.

Perhaps the principal advantage to the electrophoretic technique is that for most of the analyses, it was not necessary

to know the binding constant for the EtBr:DNA binding reaction or the duplex unwinding angle for ethidium. Because each of these factors represents a source of error when the analysis used requires a knowledge of these quantities, the information obtained by the electrophoretic technique is considered more reliable.

The Temperature Coefficient of the Average Base Rotation Angle

The experiment to determine the change in average base rotation angle as a function of temperature permitted an unambiguous assessment of this quantity. The difference between the calculated experimental value of -1.2×10^{-2} °/base pair/°C and Wang's originally reported value of -4 to -5×10^{-2} °/base pair/°C (13) reflects primarily the inaccurate value of ϕ used in Wang's calculation. A comparison of the two values, however, results in a calculated value of ϕ of 29 to 36 degrees which is somewhat higher than the accepted value of 26° but is still consistent with the results of Pulleyblank and Morgan (24). Pulleyblank *et al.* (40) and Depew and Wang (41) also used the agarose gel electropohresis technique to evaluate the temperature coefficient of the average base rotation angle and reported values of $-1.4 \pm 0.1 \times 10^{-2}$ and -1.2×10^{-2}°/base pair/°C respectively. Both Pulleyblank *et al.* (40) and Depew and Wang (41) used a number of different DNAs varying in molecular weight and base composition in their analyses and obtained essentially

identical results for each. Although the results of this study and the results reported by Depew and Wang (41) are the same, the value obtained by Pulleyblank et al. (40) is slightly higher. The cause of this slight discrepancy is unknown.

The value obtained in the temperature experiment represents the average change in base rotation angle as a function of temperature. As such, it is not possible to evaluate differential effects which may occur in A-T or G-C rich regions of the DNA. However, based on the comparative studies of Depew and Wang (41) and Pulleyblank et al. (40), these differneces are expected to be small. The change in average base rotation angle is likely to be rather uniform over all base pairs in the molecule and not localized predominantly in, for example, one or a few A-T rich regions. If this were the case, raising the temperature from 0° to 38.5°C would result in the unpairing of 60 to 65 base pairs in a DNA the size of SV40. Since these temperatures are well below the melting point for SV40 DNA, such extensive disruption of the primary helix is energetically unfavorable and must be considered highly improbable.

Thermal Fluctuations in Chain Conformation

The presence of multiple species in Form I' preparations is attributed to thermal fluctuations in chain conformation. The generation of topological isomers may be considered to reflect the sum of two contributing factors; fluctuations along the primary helix and fluctuations in the overall tertiary

configuration of the molecule. It should be noted that insofar as the topological winding number is concerned, the origin of the distortion is unimportant. The distributions themselves represent equilibrium distributions of distortions which reflect the free energy changes which take place during the departure from the ground state with zero superhelical turns. Treatment of Form II DNA with ligase results in a population of species because the distortions present in the molecules are essentially trapped by ring closure. Although the fluctuations will continue after covalent closure, it is not possible to alter the topological winding number established in the molecule at the time of closure without introducing a scission.

The treatment of Form I DNA with relaxation enzyme is perhaps a more interesting situation. In this case, the negatively supercoiled substrate has associated with it a large positive free energy of superhelix formation. In the presence of relaxation enzyme, the DNA loses this free energy through the progressive loss of supercoils until the equilibrium state is reached. As with Form II and ligase, this state is characterized by the absence of supercoils. The limit product of the relaxation reaction is not a uniform species but rather an equilibrium distribution of species like that observed in the limit product of the ligase reaction. In spite of the fact that there is no longer a force driving a change in the average topological winding number of the population, the relaxation enzyme continues

to function even after equilibrium is reached. Thus, although the instantaneous value of α for an individual molecule may change at equilibrium, its time-averaged value is equal to β and the net distribution of species remains constant. Pulleyblank et al. (40) have shown that relaxation enzyme from LA9 mouse cells will convert a relaxed substrate which is homogeneous with respect to its topological winding number into the same equilibrium distribution of species that is observed when Form I is used as the substrate. They separated PM2(I') components on agarose gels, excised and eluted the central most intense band from the gel, and used the DNA in the band as the substrate for the relaxation enzyme. This DNA was restored to a heterogeneous population of species identical to the original distribution. The result demonstrated that a superhelical substrate is not a condition for relaxation activity.

Only recently has experimental evidence been obtained which seems to bear directly on the nature of the thermally induced fluctuations at the level of the duplex. Teitelbaum and Englander (114, 115) have conducted proton exchange experiments with synthetic adenine and cytosine-containing double helices and found that even at 0°C, the average base pair is open about 1 percent of the time. The opening reaction involves breaking hydrogen bonds between base pairs and re-establishing them with water but it does not involve a significant degree of unstacking. These authors proposed a trapped loop model to

account for the high degree of unpairing of the bases at temperatures well below the melting temperature of the DNA. They suggest that the normal helical twist in the highly mobile looped-out region is either backtwisted or supertwisted so that the open loop is trapped, kinetically speaking, in the open state. According to their model, base pair unstacking occurs only at the ends of the loops. Models which require a high degree of base unstacking are untenable because hyperchromicity is not observed and the high unfavorable enthalpy change which occurs when bases are unstacked would imbue the equilibrium constant for the opening and closing reaction with a high temperature dependence (114). The analysis performed on the limit products of the ligation temperature experiment tends to support this model. If the equilibrium constant for the opening and closing reaction was significantly influenced by temperature, it might be expected that the breadth of the distributions observed on gels would increase with increasing temperature since large departures from the ground state would become more probable as the temperature is raised. The analysis of the distributions, however, indicated that the equilibrium distribution of species is not substantially influenced by temperature shifts because the values obtained for the coefficient A at the different temperatures were all within the limits of error for the determination (0.16 ± 10 percent). Teitelbaum and Englander also point out that each base pair

added to the loop subdivides the end unstacking ΔG° and contributes its own unfavorable ΔG° for hydrogen bond breakage. As the loop size increases, the effective reaction free energy per base pair decreases and approaches the value of ΔG° for hydrogen bond breakage asymptotically. However, the size of the loop is limited by the probability of finding an unstacking within the loop region. The generation of internal unstacking would tend to sever the loop and allow the two component segments to separate. An interesting feature of the model is that total loop opening and closing is not important in determining base pair opening and closing behavior. Rather, it is the random, non-directional loop migration and the oscillations at the loop edge which are the determining factors.

Evaluation of the Free Energy of Superhelix Formation

The principal advantage to the electrophoretic analysis performed in this study is that it involved a direct measurement of relative concentrations of component species in the distributions and did not require a knowledge of the unwinding angle of ethidium bromide.

The quantity k determined in this study is a molecular weight independent term which can be used to compute the coefficient A for any closed circular DNA ($k = AN$). Its magnitude was found to be 1.6×10^3 with an error of about 10 percent.

Depew and Wang (41), using a similar mode of analysis, determined a comparable value with a magnitude of about 2.3×10^3 while Pulleyblank et al. (40) reported a value of $2.06 \pm 0.14 \times 10^3$. The variability of the results from such a straightforward method of analysis is somewhat surprising. There are, however, three major sources of error. First and most important, is the establishment of an accurate baseline. Since it is not the absolute amount of DNA in each band which is determined but rather, the amount relative to the theoretical amount at $\tau = 0$, a misplaced baseline has a pronounced influence on the resulting ratios. Experience has shown that, following electropohresis and staining, there is occasionally a faint trail of fluorescent material in the sample channel which overlapped the pattern of DNA components. If this is not taken into account, the background level which is established by tracing between channels is not the true baseline, and ignoring this correction results in lower calculated values of $-\ln N_i/N_o$. Attempts were made in this study to minimize this problem by beginning the trace at a point behind the Form II band, tracing the series of bands, and then returning to the same point to begin the background scan. The densitometer was zeroed at this point so that each trace started from the same level of optical density. This procedure effectively subtracted the residual fluorescence from the sample channel and gave more uniform results.

A second source of error arises from a nonlinear film response. This generally had a more profound effect on the least intense bands in the distributions. In this study, this was considered to be a minor source of error since the film response was checked routinely. Variations in the film exposure time and developing time had an effect on the film response but these effects were considered minor in comparison to the problems encountered in establishing the baseline.

A third source of error resulted from exposure of the gels to ultraviolet light. Exposure to ultraviolet light for periods of time exceeding 5 to 10 minutes resulted in a lessening of the fluorescence intensity of the bands and, in some instances, caused the bands to disappear entirely. This was accompanied by an overall increase in the level of background fluorescence in the gel. The gels could be restored to their original state by resoaking for 45 minutes in staining solution.

A source of error unique to this investigation but not to others is non-uniformity of band shape. This may be attributed directly to a non-uniform migration rate of a particular species through gels containing ethidium and was particularly noticeable when the superhelical winding number of the DNA species under electrophoresis conditions was near zero but large enough to render it separable from Form II. Under

conditions where the mobility was not uniform, such as instances when ethidium scavenging occurred, these bands appeared broader and more diffuse. The suspected cause of the broadening effect is the possibility that the sense of supercoiling changes during electrophoresis. Bands representing DNA species which are sufficiently coiled do not seem to be affected. During the course of these experiments, gels with patterns containing species showing visible signs of non-uniform mobilities were discarded and the separation repeated following an adjustment in the ethidium concentration.

This latter problem was not encountered by Depew and Wang (41) because the transfer of the DNA from ligase reaction conditions to electrophoresis conditions induced a sufficient number of superhelical turns in the covalently closed relaxed product to make its components clearly visible under standard electrophoresis conditions. Pulleyblank et al. (40) alleviated this problem by performing the electrophoresis at 4°C in the presence of magnesium. Under their conditions, the product of the relaxation enzyme reaction when electrophoresed in standard buffer migrated as if the average τ of the population was very close to zero since relatively few bands were visible. This limited change in τ of the population of species upon transfer to the electrophoresis environment is due to the opposing effects of lowering of the temperature from reaction conditions (37°C) to the electrophoresis temperature (23°) and

the decrease in the ionic strength from essentially 0.2 M salt to a buffer with an ionic strength equivalent to about 15 mM NaCl. Although the necessity of including ethidium in the gel presented its problems, this method of exposing the components of the distributions was preferred because it offered a more sensitive means of controlling the positions of the patterns in the gel.

A measure of confidence in the densitometric analysis reported in this study was provided by an independent method of DNA quantification. The results obtained when bands were excised from gels and counted gave values of 0.153, 0.157, and 0.163 in three determinations using SV40(I'). This close correspondence with the densitometric analysis is interpreted as indicating that the densitometric analysis is fundamentally sound.

It is useful to compare the results of the result of this study with the results reported by Hsieh and Wang (47) in which they expressed the free energy of superhelix formation in terms of ν_c (see equation 11). Like the electrophoretic analysis, their approach did not require a knowledge of the duplex unwinding angle of ethidium. However, a valid comparison requires that both results be examined using equivalent values of either ν_c or τ. Equation 11 can be rearranged to express G_τ in terms of τ^2.

$$G_\tau = \frac{-aRT(360)^2\tau^2}{2N\phi^2} \qquad (27)$$

Using Wang's reported value for a of -11.0, a value of 26° for ϕ, and a value of -25 for τ, Wang's equation yields for the free energy of superhelix formation for SV40 DNA a value of about 40 kcal/mole which is about 30 percent lower than the value found in this study. The value of k as calculated from Wang's results is about 1.06×10^3. A reasonable objection which may be raised at this point is that the conversion of Wang's expression in terms of ν_c to an equivalent expression in terms of τ requires a knowledge of the duplex unwinding angle of ethidium. The value of τ of -25 for SV40 DNA under electrophoresis conditions was calculated from ν_c as determined by dye titrations on gels, using a 26° unwinding angle for the calculation. The value of ν_c of 0.035 determined by the gel titration technique may be substituted directly into Wang's equation to obtain a value for the free energy of superhelix formation independent of ϕ. This substitution results in a value of 35 kcal/mole which is about 60 percent of that determined by the electrophoretic method. In light of the discussion which follows on dye titrations, the close correspondence of the G_τ values obtained from ν_c and τ indicate that ϕ is very close to 26°.

The theoretical analysis given by Bauer and Vinograd (28) when modified to reflect the 26° duplex unwinding angle of

ethidium bromide yields a value of k of 1.78×10^3. This is in very close agreement with the results found in this study using the electrophoretic analysis. Although Hsieh and Wang (47) point out that certain assumptions in the four components thermodynamic theory employed by Bauer and Vinograd may introduce a significant error into the calculation of ν from buoyant density, they also indicate that any molecular parameters related to the bending or twisting of the DNA will introduce a source of error into their method. Hsieh and Wang (47) presented sedimentation data which suggested that these molecular parameters between the DNA and the DNA:dye complex may not be identical. The decrease in the relative spacing between adjacent bands in populations of DNA molecules electrophoresed in the presence of ethidium bromide as reported in this investigation may also be a reflection of this difference.

Superhelical DNA and Base Pair Stability

The results presented in this study allow an estimation of the influence of supercoiling on the stability of a base pair in a covalently closed molecule. To address this question, a modification of the derivation provided by Davidson (48) is employed. The derivation is based on the assumption that, while G_τ is an extensive quantity dependent on the length of the DNA, $g(\sigma)$, the free energy per 10 base pairs, is an intensive quantity independent of the size of the DNA and is equal

for all covalently closed molecules having equivalent superhelix densities. This statement is summarized by the expression

$$G_\tau = \beta^\circ g(\sigma) \tag{28}$$

The free energy released by a small change in the number of superhelical turns ($\Delta\tau$) is

$$\frac{\Delta G_\tau}{\Delta\tau} = \beta^\circ \frac{dg}{d\sigma}\frac{d\sigma}{d\tau} = \beta^\circ g'(\sigma)\frac{d\sigma}{d\tau} \tag{29}$$

It follows from the definition of σ that

$$\beta^\circ \frac{d\sigma}{d\tau} = 1 \tag{30}$$

so that

$$\frac{\Delta G_\tau}{\Delta\tau} = g'(\sigma) \tag{31}$$

From the results of this investigation

$$G_\tau = \frac{kRT}{N}\tau^2 \tag{32}$$

The intensive quantity $g(\sigma)$ is given as

$$g(\sigma) = \beta^\circ\frac{kRT}{N}\sigma^2 \tag{33}$$

Since $\beta^\circ = N/20$ and $k = 1.6 \times 10^3$, equation 33 reduces to

$$g(\sigma) = 80RT\sigma^2 \tag{34}$$

From the above, it follows that

$$g'(\sigma) = 160RT\sigma = \Delta G_\tau/\Delta\tau \tag{35}$$

As first determined by Hsieh and Wang (47) if the denaturation of one base pair unwinds the primary helix by 0.1 turns ($\Delta\tau = 0.1$), the free energy change involved in this unwinding in a molecule with a superhelix density of 0.050 is calculated to

be on the order of 480 calories. If the free energy change involved in denaturing a single A-T base pair in an A-T segment of DNA is taken to be 1 kcal, the destabilizing effect of supercoiling in a molecule with a superhelix density of 0.050 is not enough to cause the base pair to open. At 25°C, a superhelix density of 0.11 or greater would be required to open an A-T base pair.

The influence of the free energy of superhelix formation on the stability of the duplex in a negatively supercoiled molecule will also have a predictable effect on the binding of ligands. As pointed out by Davidson (48), if K_I represents the equilibrium constant for the binding of a ligand to a supercoiled molecule and K_{II} for a nicked circular DNA, and the binding of the ligand causes an unwinding of $\Delta\tau$, then

$$K_I = K_{II} \exp [-g'(\sigma)\Delta\tau/RT] \qquad (36)$$

The ratio K_I/K_{II} is independent of the length of the DNA molecule under consideration but is directly influenced by the magnitude of the superhelix density. As long as the ligand causes an unwinding of the duplex, its binding will be favored by the negatively supercoiled DNA. This property of supercoiled DNA could represent an important control mechanism <u>in vivo</u>. If, for example, it is necessary to increase the synthesis of a particular gene product, the binding of RNA polymerase to the promotor will be facilitated if the DNA in the region of the promotor is supercoiled. Saucier and Wang (116) and Wang (117)

have demonstrated that the transcriptional template activity of a covalently closed DNA is dependent on its degree of superhelicity. Considering that in vivo the DNA of eukaryotes and probably prokaryotes is stably associated with beads, only the superhelix density of the region being transcribed need be taken into account. There is no requirement that the structural organization of the entire chromosome be disrupted. Since equation 36 is an exponential function, the affinity of the polymerase for the promotor can be dramatically affected simply by altering the superhelicity of the DNA containing the binding site.

Evaluation of Topological Parameters by Gel Electrophoresis

The electrophoretic dye titration technique described in the RESULTS section offers a sensitive means by which the extent of supercoiling of a closed DNA molecule can be assessed rapidly and conveniently. In addition, by permitting the simultaneous titration of several DNAs, a direct comparison of the superhelix densities of the DNAs is possible under identical conditions (118).

The major source of error in this method when the titration is performed as described lies in the conversion of critical free dye concentrations to values of ν_c. The accuracy of the result is strongly dependent upon the accurate knowledge of the association constant for the EtBr:DNA binding reaction. Unfortunately, Hinton and Bode (29) have shown that this association

constant is very sensitive to small changes in ionic strength, particularly when the ionic strength is very low. Since the ionic strength of buffers which can be used for electrophoresis must be low to minimize the rate of heat production, small differences in buffer concentration from titration to titration will have a measurable effect on the affinity of the dye for the DNA. The addition of 20 mM NaCl to the standard electrophoresis buffer used for the titrations helped to reduce the sensitivity of the association constant to small variations in ionic strength.

The association constant of $2.0 \pm 0.2 \times 10^6$ 1/mole which was estimated by extrapolation from the data of Hinton and Bode (29) was determined from a conductance measurement of the ionic strength of electrophoresis buffer containing 20 mM NaCl. It should be noted, however, that the assumption has necessarily been made that the ionic strength within the gel is the same as that of the buffer. While this assumption is justifiable, the presence of charged groups within the agarose matrix will increase the effective ionic strength and lower the association constant.

The experiment designed to obtain an estimate of the association constant demonstrated that the association constant extrapolated from the data of Hinton and Bode (29) is reasonable. The accuracy of the determination could theoretically be improved by increasing the difference in the temperatures at which the

two DNAs were relaxed. However, the upper temperature limit for relaxation activity is approached at temperatures above 43°C and the limit product of the reaction is relaxed or positively supercoiled under electrophoresis conditions when the reaction temperature was much below 32°C. The experiment demonstrated, in addition to providing an estimate of K_a, that two DNAs differing only slightly with respect to superhelix density can be differentiated by the titration technique if small enough increments in dye concentration are used. The calculated difference in superhelix density between the two DNA samples used in this experiment is only 0.004, corresponding to only 1.8 superhelical turns in SV40 DNA. A 10 ng/ml increment in dye concentration was chosen for the titration of the native DNAs because it offered an acceptable degree of accuracy in light of the uncertainties in K_a.

The results of the titrations can be compared to previously reported values only when appropriate corrections are applied to compensate for differing ionic environments. This correction factor amounts to an increase in $\sigma_{o,12}$ of between 0.005 and 0.009 when the DNA is transferred from a low salt to a high salt environment ($\sigma_{o,26}$ = 0.011 to 0.020). When these corrections are applied to the data presented in Table 5, the resulting superhelix densities are all nearly comparable to the data obtained from sedimentation velocity-dye titrations and buoyant density measurements made in high salt (9,22).

Fortunately, there exists a direct method for determining the reliability of K_a and the resulting values of σ_o. Keller (58) and more recently, Shure and Vinograd (51), have taken advantage of the resolving power of agarose gels to count the number of superhelical turns in several native DNAs. Their methods depend on preparing superhelix density intermediates ranging from $\tau = 0$ to $\bar{\tau}$, the mean superhelical winding number of the population of native Form I components. By the direct band counting procedure, Keller found that SV40 DNA at a temperature of 37°C possessed -24 ± 2 superhelical turns (58). This figure corresponds well to the -25 ± 2 figure obtained by dye titration in this investigation at 23°C when temperature corrections are applied. Shure and Vinograd (51), using a similar but non-identical procedure, reported a value of $\bar{\tau}$ for SV40 DNA of -26 ± 0.5. These authors also applied the same analysis to Minicol DNA (pVH51) for which they reported a $\bar{\tau}$ of -19 ± 0.5 turns. This compares quite favorably with the $\tau_{o,26}$ value of -20 ± 2 obtained in this study by dye titration. The close correspondence reflects the accuracy of the values of ϕ and K_a used in the calculations.

In agreement with results of previous investigations (21, 31), the superhelix densities of all of the native DNAs are reasonably constant, regardless of whether the DNA was isolated from a eukaryotic or prokaryotic source. This seems to suggest that similar modes of coiling exist in eukaryotes and prokaryotes.

Griffith has reported that SV40 DNA has associated with it 21 ± 1 beads. Using the titration data obtained in this investigation and applying a moderate salt correction, the number of turns per bead must be on the order of about 1.3 to 1.5. Similar values are determined for prokaryotic DNA. This number, however, represents the sum of the winding per bead and the extent of quaternary coiling per bead. In light of the neutron diffraction studies of Bram et al. (93), the contribution of quaternary coiling could be significant. The extent of DNA coiling within a single bead is expected to be about 1 turn although this value need not be integral.

One additional point which should be made in connection with the titration data regards the overall shape of the titration curves and the free energy of superhelix formation associated with the DNA molecule. The titration curves obtained were never symmetrical. When $\nu < \nu_c$, the free energy of superhelix formation favors the dye binding reaction because the intercalation of ethidium unwinds the duplex and reduces supercoiling. However, when ν exceeds ν_c, the free energy of superhelix formation opposes the binding reaction because superhelical turns are being introduced. For this reason, the amount of ethidium required to affect a given change in τ will always be greater when ν is greater than ν_c.

Distributions of Native DNA Components

For each of the DNAs examined in this study, the distribution of species in the native form of a DNA was always broader than that observed when the same DNA had been treated with enzymes which alter the topological winding number. The broadened native distributions are interpreted as being the result of two distributions, a primary one arising from thermally induced fluctuations in the conformation of the DNA, and a second arising as a result of a Gaussian distribution of the fundamental units of chromosome structure. The probability function describing this latter distribution is independent of DNA length and is assumed to be responsible for the downward displacement of the Form I plot by 0.1 in the A versus 1/N plot shown in Figure 21 and for the nonlinearity of the plot of observable Form I species versus $N^{1/2}$ shown in Figure 22. The combination of the two distributions which influence the distribution of Form I components is highly degenerate. If, for example, the assumption is made that the amount of winding per chromosomal repeating unit, whether it is a eukaryotic nucleosome or a prokaryotic equivalent, is a single negative superhelical turn, then it is impossible to decide whether a given DNA molecule at the center of a Form I distribution represents a molecule with an average number of beads, $\bar{n}$, and no turns introduced as a result of thermal fluctuations, a molecule with $\bar{n} + 1$ beads and a positive turn

from thermal fluctuations, or any other combination of compensating events. From the graph shown in Figure 22, it is clear that, compared to the number of Form I' species observable, the number of Form I species observable is a function of a higher order of N. The number of observable species, a measure of the breadth of the distribution, should be influenced by the variability in the number of chromosomal subunits per unit length of DNA as well as the extent of winding per subunit.

The primary significance of the study of native DNAs is that it suggests a very close structural similarity between the intracellular arrangement of DNA in eukaryotes and prokaryotes. Griffith has demonstrated quite convincingly that SV40 DNA is associated with beads or nucleosomes which are indistinguishable from those associated with the cellular DNA (71). Similarly, Germond *et al.* (89) have used SV40 DNA as a model for chromatin studies in eukaryotes. Because of the close proximity of the value of A for SV40(I) to the A *versus* 1/N plot for native bacterial DNAs, this finding suggests very strongly that plasmid DNAs in *E. coli* and the intracellular replicative form of ØX174 DNA are coiled into structures which are nearly identical in terms of their DNA winding and distribution to the nucleosomes associated with SV40 DNA. In spite of the fact that the basic composition of the beads in prokaryotes must be quite different from those in eukaryotes, the data seems to indicate that the fundamental structure has been preserved.

There is a very limited amount of information concerning the organization of the prokaryotic chromosome. As discussed in the introduction, Griffith was able to observe the beads associated with E. coli and lambda DNA by electron microscopy (96). However, because of their fragility, special precautions had to be taken in order to preserve them during sample preparation. A criticism which has been raised in relation to Griffith's report is that the electron photomicrographs do not provide conclusive evidence that the beads actually exist since the technique is subject to the generation of artifacts. The results of the analysis of the Form I distributions supports Griffith's findings.

An additional line of supportive evidence which has been known for some time is that closed circular DNAs from prokaryotes and eukaryotes have similar superhelix densities. This similarity is apparent from the dye titration data. There are, however, many ways to generate a superhelix density of 0.050 to 0.060 in a molecule. For example, ligation of Form II in the presence of a suitable amount of ethidium followed by extraction of the dye will result in a covalently closed molecule with a superhelix density in this range. The distribution of species observed on gels, however, will not be substantially different from Form I' generated in the absence of the duplex unwinding agent. Keller showed that this was true for SV40 DNA relaxed in the

presence of ethidium (58). Thus, a dye titration is a coarser method of analysis and does not provide the same degree of detail seen in the densitometric analysis of native distributions performed in this investigation.

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