PROPERTIES OF CLOSED CIRCULAR DNA

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ABSTRACT

A method for the measurement of the superhelix density, σ_0, of a closed circular DNA from the separation between fluorescent buoyant bands of nicked and closed circular forms in an ethidium bromide-CsCl equilibrium gradient is presented. Nicked and closed circular DNA's are banded together in a gradient formed by centrifuging a CsCl solution of density 1.56 gm/ml. containing 330 μg/ml ethidium bromide at 20°C for 48 hours in the preparative ultracentrifuge. The separation between the bands, normalized by the separation between the nicked and closed circular forms of a DNA of known superhelix density, is shown to be linearly related to the difference in superhelix density between the DNA's of known and unknown superhelix densities according to the equation

$$\Delta\sigma_0 = (0.115 \pm 0.005)(\Omega_c - 1)$$

where

$$\Delta\sigma_0 = \sigma_0 - \sigma_0^* \quad \text{and} \quad \Omega_c = \frac{\Delta r}{\Delta r^*} \frac{\bar{r}}{\bar{r}} \frac{(\bar{v}_1\theta^* - 1)^2}{(\bar{v}_1\theta - 1)^2}$$

Δr is the separation between the bands, $\bar{r}$ is the average distance of the two bands from the center of rotation, θ is the buoyant density of the DNA and $\bar{v}_1$ is the partial specific volume of water. The asterisk refers to the reference DNA. A molecular weight dependence of the intercept is seen when $\lambda b_2 b_5 c$ DNA (molecular weight 25×10^6) is banded against a SV40 DNA (3×10^6) standard. The relationship was obtained

by measuring the separations for DNA's whose superhelix densities were determined by sedimentation velocity-dye titrations. Native SV40 viral DNA with a superhelix density of -0.039 was used as a standard in all cases. DNA's with altered superhelix densities were prepared by closing nicked circular DNA's with polynucleotide ligase under various conditions.

Ten closed SV40 DNA's with superhelix densities ranging from -0.007 to -0.085 have been prepared. This family of DNA's has been used to examine the effects of superhelix density on the sedimentation velocity behavior of closed SV40 DNA. The sedimentation coefficient increases as the absolute value of the superhelix density rises from a low value to 0.019, then decreases to a local minimum at 0.035 and finally increases steadily as $|\sigma_0|$ rises to 0.085.

The sedimentation velocity-ethidium bromide titrations of these DNA's have been converted from the primary $s^0_{20,*}$ versus c data, in which $s^0_{20,*}$ is the standard sedimentation coefficient still uncorrected for the buoyant effect of bound ethidium chloride and c is the free ethidium bromide concentration, to the more meaningful $s^0_{20,w}$ versus σ_0 form, with the aid of coefficients in the expression for the free energy of superhelix formation. The resultant curves form a family that is approximately superimposable on the curve for $s^0_{20,w}$ versus σ_0 in the absence of ethidium bromide.

The dependence of the sedimentation coefficient of selected SV40 DNA's upon ionic strength, the nature of the cation, and temperature is consistent with the previously reported effects of these variables on the

rotation angle of the base pairs along the helix axis.

Separations between open and closed circular DNA's in buoyant CsCl gradients containing the ethidium bromide analogue, propidium di-iodide, are shown to be 1.8 times larger than in ethidium bromide.

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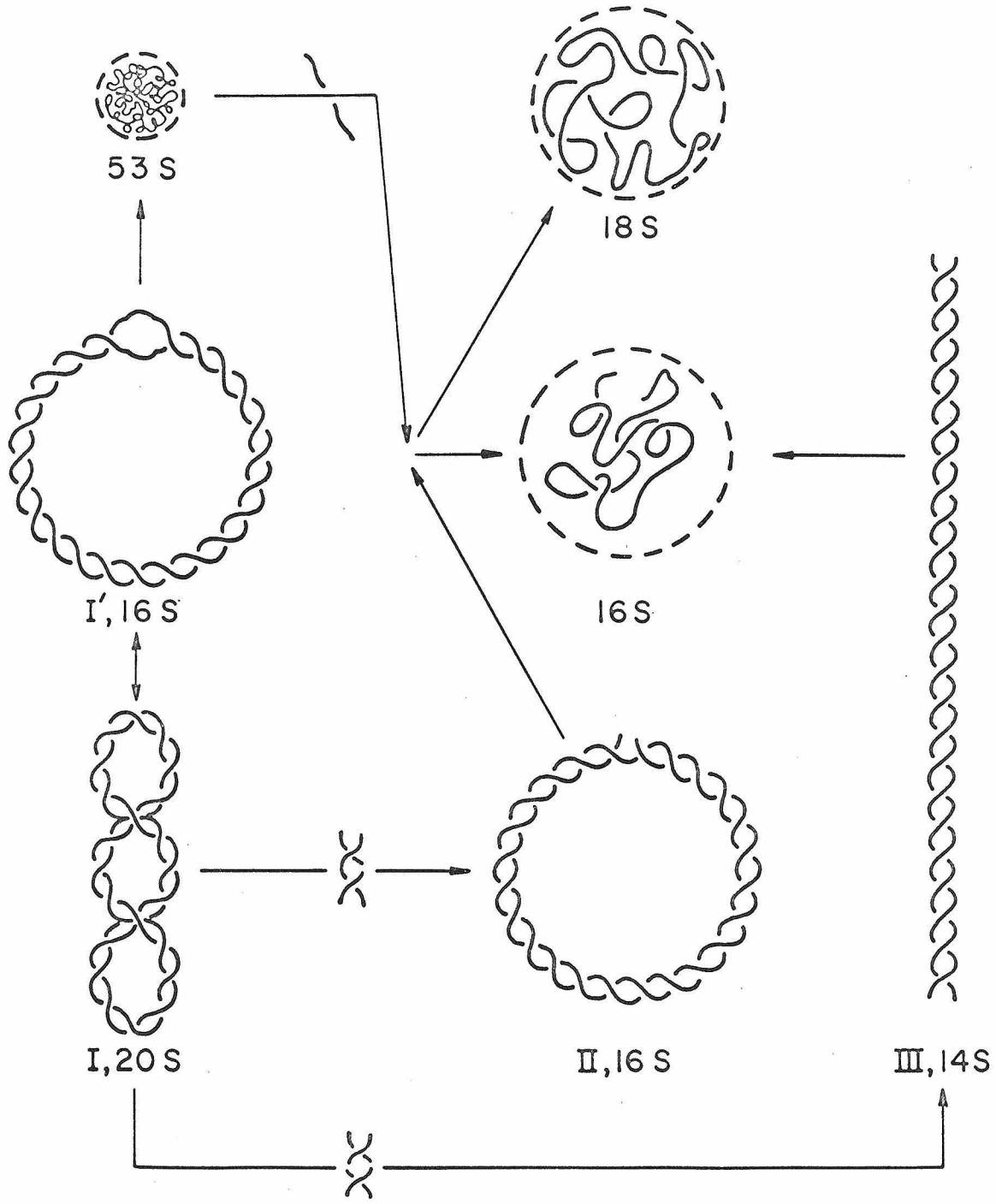
INTRODUCTION

Closed circular duplex DNA's have been found to occur in a wide variety of organisms including viruses, intracellular replicative forms of viruses, animal mitochondria and cytoplasm, bacterial episomes and plasmids and kinetoplasts of protoza (1, 2). These DNA's range in molecular weight from 500,000 daltons for small closed circles from HeLa cells (3) to 140×10^6 daltons for the sex factor F′(galλ_cI857bio) from E. coli (4).

The most commonly observed characteristic of closed circular DNA is the existence of superhelical or tertiary helical turns. These arise from the constraint in covalently closed circular DNA molecules which fixes the number of times one strand must go around the other. Under any particular specified conditions, the Watson-Crick double helical structure will absorb the major part of these crossovers. However any excess or deficit of crossovers not absorbed in the Watson-Crick structure will appear as superhelical or tertiary turns in a twisted circular molecule (form 1). Figure 1 shows a diagramatic representation of the various forms of polyoma DNA (5, 6), a closed circular DNA of molecular weight 3×10^6 daltons. The sedimentation coefficients of the forms in neutral or alkaline NaCl solutions are shown below the forms. Other closed DNA's have comparable forms but with different sedimentation coefficients depending on their molecular weight and the number of superhelical turns in the molecule.

Closed circular molecules have characteristic properties which distinguish them from other DNA molecules. Two of these characteristics which were noted early in studies of closed circular DNA are:

FIG. 1. Diagramatic representation of the various forms of polyoma viral DNA. The number indicate the sedimentation coefficients in either neutral or alkaline NaCl.


53 S
18 S
I', 16 S
16 S
I, 20 S
II, 16 S
III, 14 S

(1) the DNA very rapidly renatures after heat denaturation at 100°C in 0.001 M salt implying incomplete strand separation (7) and (2) upon denaturation in alkaline CsCl, the majority of the DNA sediments as a fast compact form approximately three times as fast as would be expected for a single stranded linear form of one-half the molecular weight of the intact molecule (7). These properties are consistent with the proposed twisted circular model. Upon denaturation these molecules remain topologically linked and do not strand separate. Thus they can rapidly renature and exist as a compact form in alkali (53S form in Fig. 1). Upon reaction with DNase or chemical nicking agents, the molecules are converted to a relaxed circular form (form II) which sediments more slowly in neutral solvents than does the twisted circular form (5). This occurs with the introduction of only one single strand scission per molecule. No further change in the sedimentation coefficient occurs with further nicking until large numbers of scissions have been made, converting the nicked circular form to a linear form (form III). This occurs when two scissions occur on opposite strands close enough to each other that the hydrogen-bonds of the base pairs between them are broken by thermal energy. The same form may be generated by treatment of the closed circular form with E. coli endonuclease I (5), which makes a double strand scission in DNA.

In denaturing solvents, the singly nicked circular form sediments as two distinct species, a single stranded circular form (18S) and a single stranded linear form (16S).

Upon partial denaturation of the closed circular form, a relaxed

circular form (I′) is obtained which has sedimentation properties equivalent to the nicked circular form (6). This transition occurs with milder denaturation conditions than are necessary for the denaturation of the nicked circular form. This is due to the positive free energy of superhelix formation (8). As a small number of base pairs are denatured, the strain of the superhelical turns may be released by unwinding the denatured region. Further denaturation of the closed circular form requires more extreme denaturation conditions than are necessary for the nicked circular form as the molecule must now wind up again.

Bauer and Vinograd (9) have proposed an equation expressing the topological constraint in closed circular DNA.

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

α is the winding number or topological linking number, β is the number of Watson-Crick turns in the DNA when there is no constraint and τ is the number of superhelical turns. An intensive quantity, the superhelix density, σ, has been defined as the number of superhelical turns divided by β_0, one-tenth the number of base pairs in the molecule. Right-handed duplex turns are taken to be positive and thus α and β are both positive numbers. On the basis of studies with the intercalating dye, ethidium bromide, the naturally occurring superhelical turns have been assigned a negative direction (9).

Electron microscopy of closed circular DNA is consistent with the interpretation of experimental results (5). Closed circular DNA

appears as a twisted circular molecule with a number of crossovers. Molecules which have single strand scissions appear as open circles with few or no crossovers.

The binding of the intercalating dye, ethidium bromide (EB), to closed circular DNA has been shown by Bauer and Vinograd (9) to initially remove superhelical turns and convert the molecule to the relaxed circular form which has a sedimentation coefficient equivalent to that of the nicked circular form with the same amount of bound EB. As further dye is bound, the closed circular molecules wind up with superhelical turns of the opposite direction and the sedimentation coefficient increases.

The affinity of the dye for the closed circular form is initially greater than that for the nicked circular form as the binding of the dye releases the strain of superhelical turns on the molecule by unwinding the Watson-Crick turns and simultaneously removing superhelical turns. At the equivalence point where the closed circular molecule is relaxed, the affinity of the dye is the same for the closed and nicked molecules. As further dye is bound the affinity of the EB for DNA is greater for the nicked circular form than for the closed form. Here the binding of dye is introducing more superhelical turns into the closed molecule. The existence of the equivalence point and a knowledge of the unwinding angle of EB (10) provide a method for determining the number of superhelical turns present in a closed circular DNA molecule.

Two methods have been previously used to measure this equivalence point. The first method is to determine the dye concentration at

which the sedimentation velocities of the closed and nicked DNA's are equivalent (9, 11). The second method makes use of the buoyant effect of bound dye on the buoyant density of the DNA (9). Since bound EB has a partial specific volume of 1.02 ml/gm, the binding of EB decreases the buoyant density of the DNA. At the relaxed state where closed and nicked DNA's have the same affinity for EB, the buoyant densities of the nicked and closed circular DNA's are equal. From the amount of dye bound at this point, the superhelix density may be determined.

A new method for the measurement of the superhelix density from the separation between buoyant bands of open and closed circular DNA at high EB concentrations is presented in Chapter I of this thesis. The amount of EB bound by a superhelical DNA at high dye concentrations is limited by a balancing of the negative free energy of dye binding and the positive free energy of superhelix formation. Nicked circular DNA's do not have the restriction and will thus bind larger amounts of dye and band at lower densities. DNA's with low negative superhelix densities which reach the relaxed form and begin to form positive turns at low dye concentrations will bind less dye and have a larger separation than DNA's with large negative superhelix densities which bind considerable amounts of dye before reaching the relaxed form. The separation between bands of nicked and closed circular DNA normalized by the separation for DNA of a known superhelix density is shown to be linearly related to the superhelix density of the unknown DNA.

Chapter II of this thesis is a study of the relationship between

the superhelix density and the sedimentation properties of a closed circular DNA and the dependence of the superhelix density of a closed circular DNA on the environmental conditions of the DNA.

Chapter III is a publication (12) presenting a partial study of an EB analogue, propidium di-iodide, which gives larger separation between buoyant bands of open and closed circular DNA than does ethidium bromide.

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CHAPTER I

A Buoyant Method for the Determination of the Superhelix Density of Closed Circular DNA

1. Introduction

It has become clear recently that naturally occurring closed circular DNAs may differ among themselves in superhelix density (the number of superhelical turns per ten base pairs). The superhelix densities range from -2×10^{-2} for mitochondrial DNA from SV40-transformed mouse cells in culture to -5×10^{-2} for the DNA from the bacterial virus PM2 (Espejo & Canelo, 1968a,b). Moreover, the superhelix densities of intracellular closed forms of SV40 DNA (Eason & Vinograd, 1971) and PM2 DNA (Espejo & Sinsheimer, 1971) are significantly different from the superhelix densities of the corresponding DNAs isolated from the viruses. Mitochondrial DNAs from cells grown in media containing the intercalating dye ethidium bromide also have altered absolute superhelix densities which may be as high as 12×10^{-2} (Smith, Jordan & Vinograd, 1971).

Four methods have been previously used to estimate the superhelix density of closed circular DNAs: sedimentation velocity-dye titrations (Crawford & Waring, 1967; Bauer & Vinograd, 1968; Wang, 1969a,b), buoyant density-dye titrations (Bauer & Vinograd, 1968), buoyant density-base titrations (Vinograd, Lebowitz & Watson, 1968), and viscometric-dye titrations (Révet, Schmir & Vinograd, 1971). The sedimentation velocity-dye titration procedure with the intercalating dye ethidium bromide has been the most commonly used method.

We present here a new and simpler method for determining the superhelix density of closed circular DNAs. In this procedure we measure the distance between bands of closed and nicked circular DNA at equilibrium in a buoyant ethidium bromide-CsCl gradient in a

preparative ultracentrifuge (Radloff, Bauer & Vinograd, 1967). The separations are measured relative to the separation in the same experiment for a DNA of known superhelix density in order to eliminate the effects of variables such as temperature and angular velocity. A description of the theoretical basis for this method is presented elsewhere (Bauer & Vinograd, 1970b).

The buoyant density of a closed DNA-ethidium bromide complex at an arbitrary high ethidium bromide concentration depends on the superhelix density of the closed DNA in the absence of ethidium bromide (Hudson et al., 1969; Bauer & Vinograd, 1970a). It is shown in this study, in agreement with the theoretical analysis of Bauer & Vinograd (1970b), that the relative buoyant separations between the nicked and closed forms are linearly related to the superhelix density of the closed DNA in the absence of dye. The superhelix densities of the closed SV40 DNAs used to calibrate this method were measured by the sedimentation velocity-ethidium bromide titration under standard conditions in 2.83 M-CsCl at 20°C (Upholt, Gray & Vinograd, 1971). A linear relationship with a different intercept, but the same slope, was found for higher molecular weight $\lambda b_2 b_5 c$ and F-factor DNAs.

2. Materials and Methods

(a) Preparation of DNAs

Viral SV40 DNA was obtained by phenol extraction of purified virus as described by Bauer & Vinograd (1968), or by sodium dodecylsulfate extraction of the virus. The latter procedure was essentially the same as that described for the extraction of DNA from mitochondria by Hudson & Vinograd (1967). Polyoma DNA was obtained from the virus by phenol extraction. The closed DNA was obtained by banding in ethidium bromide-CsCl (Radloff, Bauer & Vinograd, 1967).

PM2 virus, which contains closed circular duplex DNA with a molecular weight of about 6×10^6 daltons, was grown and isolated as described by Espejo & Canelo (1968a,b) and Espejo, Canelo & Sinsheimer (1969). The DNA was extracted from the virus with phenol and the closed circular DNA was isolated from an ethidium bromide-CsCl buoyant density gradient.

Phage $\lambda b_2 b_5 c$ was grown in *Escherichia coli* K12 W3110 and isolated by phase separation in 8% polyethylene glycol-0.05% dextran sodium sulfate, 0.35 M-NaCl (Sedat & Sinsheimer, 1964). The phage was then purified by buoyant banding in CsCl. The DNA was released from the virus by the addition of EDTA followed by osmotic shock or by phenol extraction. An additional sample of $\lambda b_2 b_5 c$ DNA was the gift of J. C. Wang.

F-factor DNA was grown in *E. coli* W1485 obtained from D. Freifelder. The bacteria were lysed by treatment with lysozyme and sarkosyl (Bazaral & Helinski, 1968). The DNA was isolated by selective alkaline denaturation of nicked circular and linear DNA, followed by adsorption of single-stranded DNA by nitrocellulose (Cohen & Miller, 1969). F-factor

DNA of altered superhelix density was prepared by growing the bacteria in media containing 1.5 µg/ml. ethidium bromide. Further increases in ethidium bromide concentration drastically decreased the yield of closed circular DNA with no significant increase in superhelix density. F-factor closed DNA was purified by banding in buoyant ethidium bromide-CsCl.

(b) Polynucleotide ligase

The polynucleotide ligase used was the preparation described by Hudson, Upholt, Devinny & Vinograd (1969).

(c) Closure of DNA by ligase

SV40 DNA I was converted to SV40 DNA II by pancreatic DNase I, and closed by polynucleotide ligase in the presence of varying amounts of ethidium bromide as described by Hudson et al. (1969). The enzymatically closed preparations designated SV40 DNA L1 and SV40 DNA L2 were those of the above authors. An additional preparation, SV40 DNA L3, was obtained by ligase action at an SV40 II concentration of 62 µg/ml. in the presence of 6.7 µg/ml. ethidium bromide at 30°C.

Lambda b_2b_5c DNA was cyclized at 56°C for 2 hr in 2.0 M-NaCl, 0.01 M-EDTA as described by Wang & Davidson (1966). The DNA was concentrated by dialysis against dry Biogel P300. The hydrogen-bonded circular duplexes were closed by DNA ligase at 30°C at the following ethidium bromide and DNA concentrations, respectively: L1, 0.0 and 31.8 µg/ml.; L3, 2.3 and 15.4 µg/ml.; and L2, 1.67 and 24.3 µg/ml.

(d) Chemicals

The ethidium bromide was supplied by Boots Pure Drug Company, Ltd., Nottingham, England. The ethidium bromide analog referred to as propidium diiodide is that described by Hudson et al. (1969). Optical grade CsCl was obtained from Harshaw Chemical Company. Calf thymus DNA with an absorbance ratio of $A_{260}/A_{235} = 2.3$ was obtained from Sigma Chemical Company and was used without further purification.

(e) Analytical ultracentrifugation

The band sedimentation velocity experiments were performed with 12 mm, double-sector, type I band-forming centerpieces (Vinograd, Radloff & Bruner, 1965). The Spinco model E ultracentrifuge was equipped with photoelectric scanning system and multiplex unit to permit multicell runs. All experiments were performed at 20°C. The sedimentation solvent was 2.83 M-CsCl, 0.01 M-Tris-HCl, pH 7.5 to 8.0, density 1.35 g/ml. A stock solution of ethidium bromide (~100 μg/ml.) in the same solvent was added to the sedimentation solvent with a Hamilton microliter syringe of the proper capacity. Ethidium concentrations were assayed with a Cary 14 or 15 spectrophotometer. A reciprocal extinction coefficient of 81.6 μg/ml. per absorbance unit at 487 nm was used [see section (h) under Materials and Methods]. The sedimentation solvent was measured with a Manostat digital pipet.

Concentration gradients of ethidium bromide in the ultracentrifuge cell were minimized by adding ethidium bromide to the DNA in the sample well. The amount of ethidium bromide required to give a free ethidium bromide concentration in the sample well equal to that in the sedimentation

solvent was estimated with the aid of the binding isotherms for SV40 I and II (at 25°C) of Bauer & Vinograd (1968) for the sedimentation of SV40 I and II and polyoma I and II. The required amount of ethidium bromide in experiments with other DNAs was calculated with the Scatchard (1949) equation, using the binding constant and the maximum value of ν measured spectrophotometrically by Wang (1969a) for calf thymus DNA in 3 M-CsCl, 0.01 M-EDTA at 20°C. In most of the experiments, the sample solutions contained 2 M-CsCl, in order to reduce the magnitude of the refractive index gradient formed when the sample was layered onto the sedimentation solvent. The total lamellar volumes were 5 to 15 µl. and contained 0.1 to 0.5 µg of DNA. Increasing the amount of DNA to 0.6 µg had no effect upon the value of the sedimentation coefficient of SV40 DNA. The lower amount of DNA above was used in the case of the lambda DNA. The solutions were mixed in the sample well by repeatedly drawing the liquid into a piece of polyethylene tubing attached to a 50-µl. Hamilton syringe.

Sedimentation coefficients were evaluated with the aid of a computer from least-squares analysis of the log(r) versus t plots. The results were corrected to standard conditions, and expressed as $s^{\circ}_{20,*}$, for the Na form according to Bruner & Vinograd (1965). The ratio of standard sedimentation coefficients of Cs and Na DNAs was taken to be 1.31 (Bruner & Vinograd, 1965). The effect of bound dye on the buoyant density was neglected in the calcuations of the $s^{\circ}_{20,*}$. Buoyant densities of the DNAs measured at 25°C were corrected to 20°C with the temperature coefficient of Vinograd, Greenwald & Hearst (1965).

Densities of CsCl solutions were determined refractometrically according to the equation

$$\rho^{25} = 10.2402\ n_D^{25} - 12.6483,\ 1.00 < \rho < 1.38 \qquad (1)$$

(Bruner & Vinograd, 1965) and corrected to 20°C values (International Critical Tables, 1933).

(f) Determination of the solubility of ethidium bromide in CsCl solutions

Supersaturated solutions of ethidium bromide in CsCl solutions of various densities were prepared by mixing concentrated CsCl solutions with ethidium bromide solutions, 1,300 to 2,000 µg/ml. The ethidium bromide solutions contained Tris-HCl-EDTA in amounts appropriate to give final concentrations of 0.01 M-Tris-HCl, 0.001 M-EDTA, pH 8.0, in the CsCl solutions. Solid ethidium bromide was added to provide seed crystals for precipitation. The solutions were placed in a 20°C water bath in the dark. Samples were removed from the bottom of tubes and diluted with 2.83 M-CsCl, 0.01 M-Tris-HCl, 0.001 M-EDTA, pH 8.0, to approximately 24 to 120 µg/ml. for spectral measurements. Spectra were measured daily for 17 days until no further decrease in the concentration was observed. The concentrations of ethidium bromide were calculated as described in section (h).

(g) Preparative ultracentrifugation

DNA samples (0.5 to 1 µg each of closed and open forms) in 3 ml. of CsCl solutions, density 1.56 to 1.58 g/ml., containing 330 µg/ml. of

ethidium bromide or propidium diiodide were centrifuged in the Spinco model L or L2-65B ultracentrifuge for 24 to 48 hr at 20°C. The lower density above was employed with propidium diiodide. The speeds were 43,000 and 40,000 rev./min for the SW50 and SW50.1 rotors, respectively. A reference tube containing SV40 I and II was present in all experiments.

(h) Photography and measurement

The fluorescent DNA bands were photographed and the separations between bands were measured as described by Hudson et al. (1969). Most of the tubes were photographed with a 2.5 x 3.5 inch sheet film bellows-type camera mounted on a frame and focused on a tube holder mounted on the same frame (Watson, Bauer & Vinograd, 1971). The tube holders accept three ultracentrifuge tubes and are interchangeable for different tube sizes. Ultraviolet illumination (365 nm) from behind the tubes is provided by a light-box mounted on the frame next to the tube holder. Exposures of Kodak Royal Pan sheet film were at f9 for approximately 15 sec through a Wratten 16 filter. Magnification factors were determined from calibration marks cut into the tube holders and filled with fluorescent paint.

(i) Spectrophotometric binding measurements

The binding of ethidium bromide to calf thymus DNA was measured spectrophotometrically as described by Waring (1965). In this procedure, the absorbance of ethidium bromide is measured as a function of DNA concentration at constant ethidium bromide concentration. The Cary 14 spectrophotometer was equipped with a specially constructed, thermostatted

copper cell holder which accommodates the 5-cm and 1-cm spectrophotometer cells. The temperature was 20.0 $\pm$ $\sim$0.1°C and the solvent was 2.83 M-CsCl, 0.02 M-Tris, pH 8.2 to 8.4.

Solutions for spectral measurements were prepared by mixing stock solutions of ethidium bromide and EB-DNA of equal ethidium bromide concentrations to give different binding ratios at constant ethidium bromide concentrations. These two stock solutions were prepared by adding equal volumes of concentrated ethidium bromide solution from a calibrated Manostat digital pipet to weighed amounts of DNA and buffer solutions of known densities. Solution densities were measured with a calibrated 500-µl. pipet. Other volume measurements were made with calibrated Manostat pipets or calibrated Hamilton syringes. These procedures avoid drainage errors in pipeting viscous DNA solutions. The spectra of the EB-DNA solutions were corrected for the small absorbance of the solvent and the calf thymus DNA solutions.

The absorbances at 460, 470, and 480 nm were used to calculate the fraction of the ethidium bromide bound to DNA (Waring, 1965). The extinction coefficients of the bound dye were determined by extrapolating plots of the molar extinction versus reciprocal DNA concentration to infinite DNA concentration. Reciprocal extinctions of 313, 232, and 178 µg/ml. per absorbance unit were obtained for the bound dye at 460, 470, and 480 nm, respectively.

The extinction coefficient and the wavelength of maximum absorption of the free dye were observed to depend on the dye concentration (Table 1) as previously reported by Wang (1969a). Our extinction coefficients at 460 nm were within 2% of those quoted by Wang (1969a) for

Table 1

Absorbance of ethidium bromide in 2.83M-CsCl solutions, 20°C

EB	E_{460}	E_{470}	E_{480}	λ_{max}	E_{max}
µg/ml.	µg/ml. per absorbance unit			nm	µg/ml. per absorbance unit
107.4	101.8	89.0	82.4	486†	81.4
53.7	98.5	86.9	81.3	485	80.6
26.6	93.1	83.0	78.3	483	77.6
10.6	93.3	81.8	78.1	482	77.9

† W. Bauer (private communication) obtained a reciprocal extinction coefficient of 78.9 µg/ml. per absorbance unit at 487 nm for a dye concentration approximately 100 µg/ml. in 5.25M-CsCl.

concentrations between 22 and 100 μg/ml. He observed no change in the extinction coefficient at 460 nm at concentrations below 20 μg/ml. ethidium bromide. There was a weight loss of approximately 2% upon drying ethidium bromide powder in a vacuum for extensive periods at room temperature. This loss is not included in the reciprocal extinction coefficients used here.

The binding isotherm was determined with two pairs of solutions. The first set of data was obtained with an ethidium bromide concentration at 18.5 μg/ml. and a maximum DNA concentration of 1334 μg/ml. in a 1-cm cell. The second set was obtained with an ethidium bromide concentration of 107.4 μg/ml. and a maximum DNA concentration of 760 μg/ml. in a 5-cm cell. The calf thymus DNA concentrations were determined spectrophotometrically with a reciprocal extinction coefficient of 50 μg/ml. per absorbance unit at 260 nm. The free ethidium bromide concentration in each solution was determined by an iterative procedure using the spectral data and a plot of the data in Table 1.

The effect of pH on the spectra of the initial ethidium bromide and EB-DNA solutions was examined because a difference of 0.25 pH units was observed between the two solutions. No spectral effect was observed when the pH was varied between pH 7.5 and 8.4.

3. Results

(a) Sedimentation velocity-dye titrations

Sedimentation velocity-dye titrations with the exception of those for $\lambda b_2 b_5 c$ (Wang, Baumgarten & Olivera, 1967; Wang, 1969a,b) have been performed by boundary sedimentation with both the closed and open circular DNAs present in the same cell. This method suffers from the disadvantage that the separate boundaries cannot be distinguished near the region of the minimum and an artificially widened minimum region results. The DNA, moreover, binds a significant portion of the input dye. In order to calculate ν_c, the molar ratio of bound dye per DNA nucleotide at the minimum of the titration, either the free-dye concentration must be determined from spectral measurements of the free-dye concentration centripetal to the sedimenting boundary in the centrifuge (Bauer & Vinograd, 1968), or the amount of dye bound must be determined from spectrophotometric measurements of the metachromic effect in the solutions containing the dye and the two DNA components (Crawford & Waring, 1967). The first method requires a careful calibration of the optical scanning system of the centrifuge.

Sedimentation velocity-dye titrations in this work were performed with the band sedimentation method and with single purified components. The amount of dye bound by the small amount of DNA present in the band does not significantly affect the free dye concentration in the sedimentation solvent. The free dye concentration is, therefore, the same as the initial dye concentration.

Superhelix densities, σ_o, of several naturally and enzymatically

closed circular DNAs were determined by the sedimentation velocity-dye titration procedure. Titrations of two SV40 DNAs with different superhelix densities are presented in Figure 1 along with the sedimentation velocity titration of nicked SV40 DNA, form II. The sedimentation coefficient of the nicked form decreases slowly as the ethidium bromide concentration is increased to 15 μg/ml. The curve for the closed DNA isolated from the virus contains a minimum at 5.0 μg/ml. and a maximum at about one-tenth this concentration. The minimum in the curve obtained with SV40 L1 DNA, prepared by nicking and closing purified viral DNA <u>in vitro</u>, is at a much lower free dye concentration, 1.0 μg/ml. The superhelix densities corresponding to the above free dye concentrations are -3.9×10^{-2} and -0.9×10^{-2} for SV40 I and SV40 L1, respectively (Table 2). These values were calculated with the binding parameters determined as described in section (b) of Results and the relation of Bauer & Vinograd (1968)

$$\sigma_o \equiv \tau/\beta^o = -0.67\ \nu_c \qquad (2)$$

where σ_o is the superhelix density in the absence of dye, τ is the number of superhelical turns, β^o is one-tenth the number of base pairs in the molecule, and ν_c is the number of moles of ethidium bromide bound per mole nucleotide at the minimum of the sedimentation velocity-dye titration curve.

The sedimentation velocity-dye titrations of two SV40 DNAs with still higher superhelix densities exhibit minima at higher free dye concentrations (Fig. 2). The local maximum seen in the viral form

FIG. 1. Sedimentation coefficient as a function of free ethidium bromide concentration for SV40 L1 (Δ), SV40 I (o), and SV40 II (□) DNA's. Error bars represent the 95% confidence interval in the slope of the ln r versus t plots. The absence of an error bar indicates that the error was smaller than the symbol.

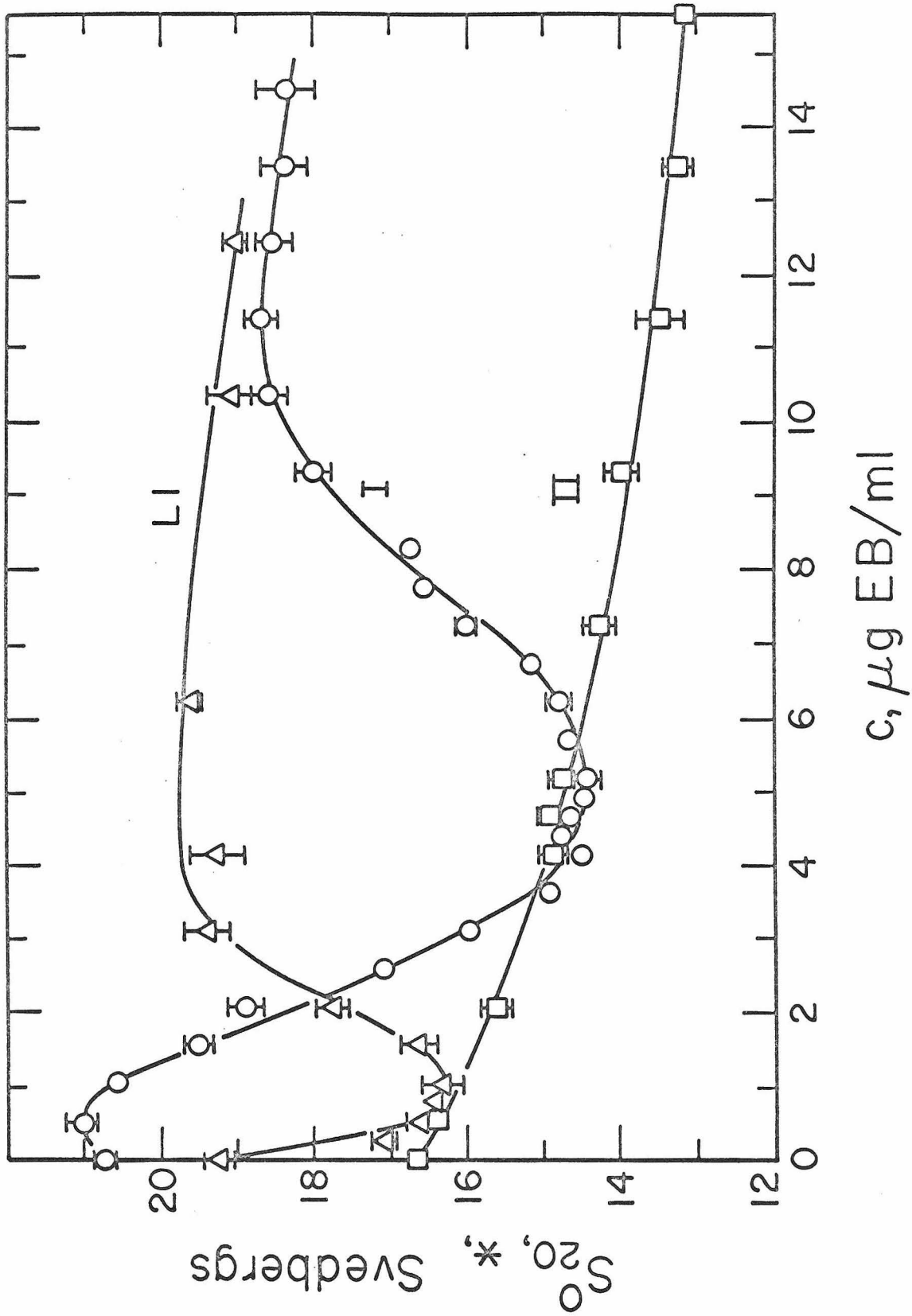

S^0_{20}, *, Svedbergs
20
18
16
14
12
0
2
4
6
8
10
12
14
c, μg EB/ml
LI
I
II

TABLE 2

The critical dye concentration and the superhelix density of native and enzymatically closed viral DNAs as determined by the sedimentation velocity-dye titration procedure

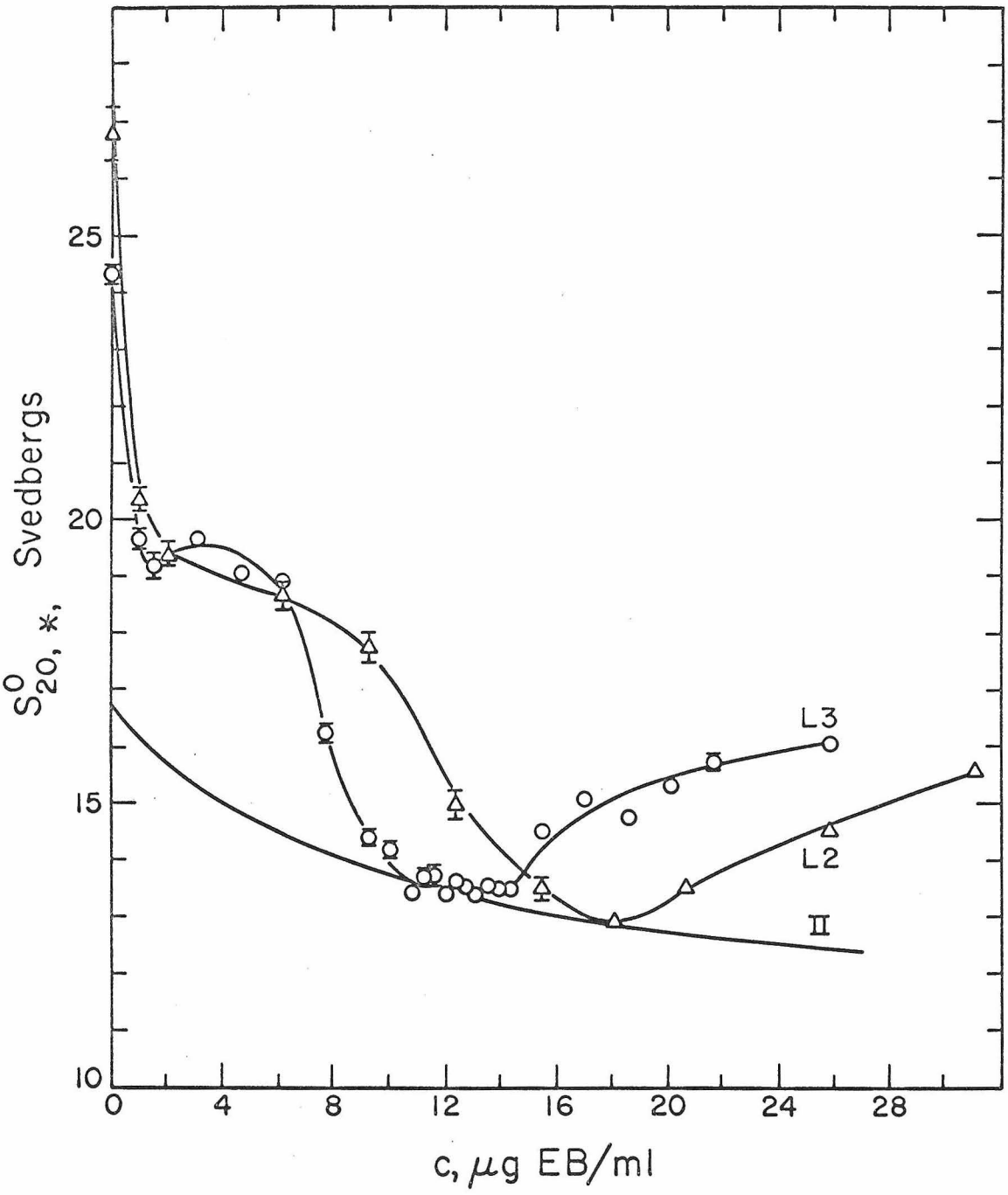
DNA	EB at minimum c' μg/ml.	Superhelix density $-\sigma_o \times 10^2$	Superhelical turns $-\tau$ †
Polyoma I	4.2 ± 0.4	3.3 ± 0.3	16 ± 2
SV40 L1	1.0 ± 0.2	0.9 ± 0.2	5 ± 1
SV40 I	5.2 ± 0.3	3.9 ± 0.2	19 ± 1
SV40 L3	12.6 ± 0.8	7.1 ± 0.5	35 ± 2
SV40 L2	18.0 ± 0.9	8.5 ± 0.4	42 ± 2
PM2 I	8.0 ± 0.5	5.3 ± 0.3	51 ± 3
$\lambda b_2 b_5 c$ L1	0.75 ± 0.2	0.72 ± 0.2	27 ± 7
$\lambda b_2 b_5 c$ L2	3.2 ± 0.3	2.6 ± 0.2	95 ± 7
$\lambda b_2 b_5 c$ L3	11.4 ± 0.2	6.7 ± 0.2	244 ± 7
F-factor I	4.5 ± 0.2	3.5 ± 0.2	320 ± 18
F-factor I-EB	5.4 ± 0.3	4.0 ± 0.3	385 ± 29

† The values of τ were calculated with the relation, $\tau = \sigma_o M/10\,\overline{M}$, where M is the molecular weight of the DNA and $\overline{M}$ is the average molecular weight of the Na form of a nucleotide pair. The

Table 2, continued...

molecular weights of polyoma, SV40, PM2, $\lambda b_2 b_5 c$, and F-factor DNAs (3.3, 3.3, 6.3, 25 and 60 megadaltons, respectively) were calculated from sedimentation coefficients of the nicked circular forms.

FIG. 2. Sedimentation coefficient as a function of free ethidium bromide concentration for SV40 L3 (o), SV40 L2 (Δ), and SV40 II (solid line with no data points) DNA's.


25
20
15
10
0
4
8
12
16
20
24
28
$S^0_{20,*}$, Svedbergs
c, μg EB/ml
L3
L2
II

(Fig. 1) is again evident in the curve for SV40 L3 with a superhelix density of -6.8×10^{-2}. A local minimum is also seen in this curve in which S falls sharply from 24 S to 19 S as ethidium bromide is added to a concentration of 2 μg/ml. The sedimentation coefficient of the SV40 DNA with the highest degree of supercoiling prepared in this study, -8.5×10^{-2}, drops from 28 S to a shoulder at 19 S upon addition of 2 μg/ml. ethidium bromide. A concentration of 17.5 μg/ml. ethidium bromide was required to relax this DNA.

The curves in Figure 3 show that the absolute value of the superhelix density, σ_o, of the viral DNA from the mouse virus, polyoma, is slightly smaller ($3.3 \pm 0.3 \times 10^{-2}$) than the DNA from the related monkey virus, SV40 ($3.9 \pm 0.2 \times 10^{-2}$). The sedimentation velocity-dye titration of PM2 DNA obtained from PM2 virus on infection of a marine bacterium, Pseudomonas Bal 31, again shows an early minimum and a local maximum as the molecule is unwound from a high negative superhelix density to an intermediate superhelix density (Fig. 4). The superhelix density of viral PM2 DNA is $-5.3 \pm 0.3 \times 10^{-2}$, the highest so far obtained for a native closed circular DNA.

Figure 5 presents the results for a sample of enzymatically closed $\lambda b_2 b_5 c$ DNA, a material with a molecular weight of 25 megadaltons and a superhelix density of $-6.7 \pm 0.2 \times 10^{-2}$. The early minimum and the local maximum also occur with this material. Partial dye titrations in the minimum region alone were performed with L1, L2, and F-factor DNAs of two different superhelix densities. The results of these titrations are shown in Table 2.

FIG. 3. Sedimentation coefficient as a function of free ethidium bromide concentration for polyoma I (o) and II (□) DNA's. The curve for SV40 I (---) is included for comparison.

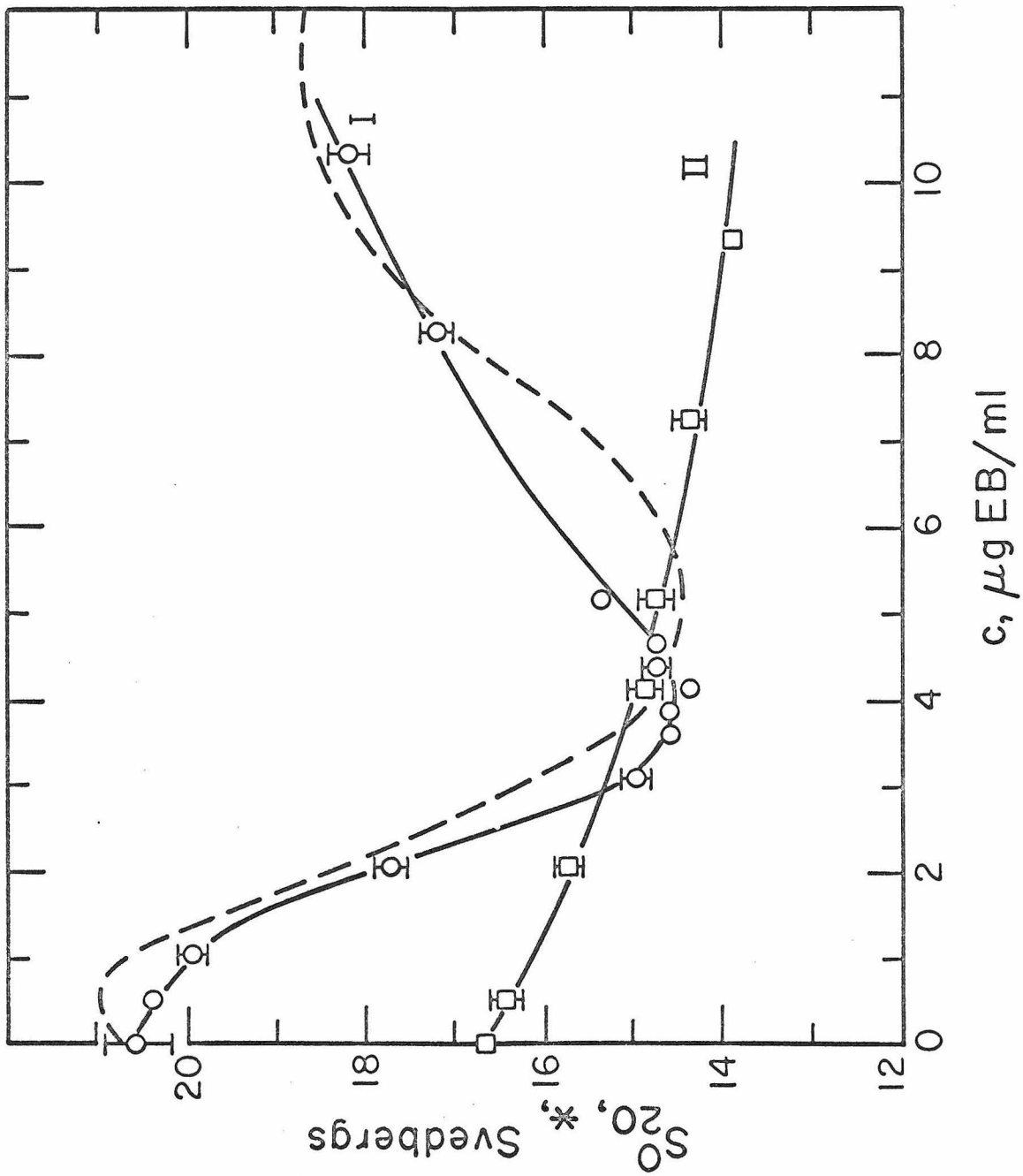

I
II
S_{20}, * Svedbergs
20
18
16
14
12
0
2
4
6
8
10
c, μg EB/ml

FIG. 4. Sedimentation coefficient as a function of free ethidium bromide concentration for PM2 I (o) and II (□) DNA's.

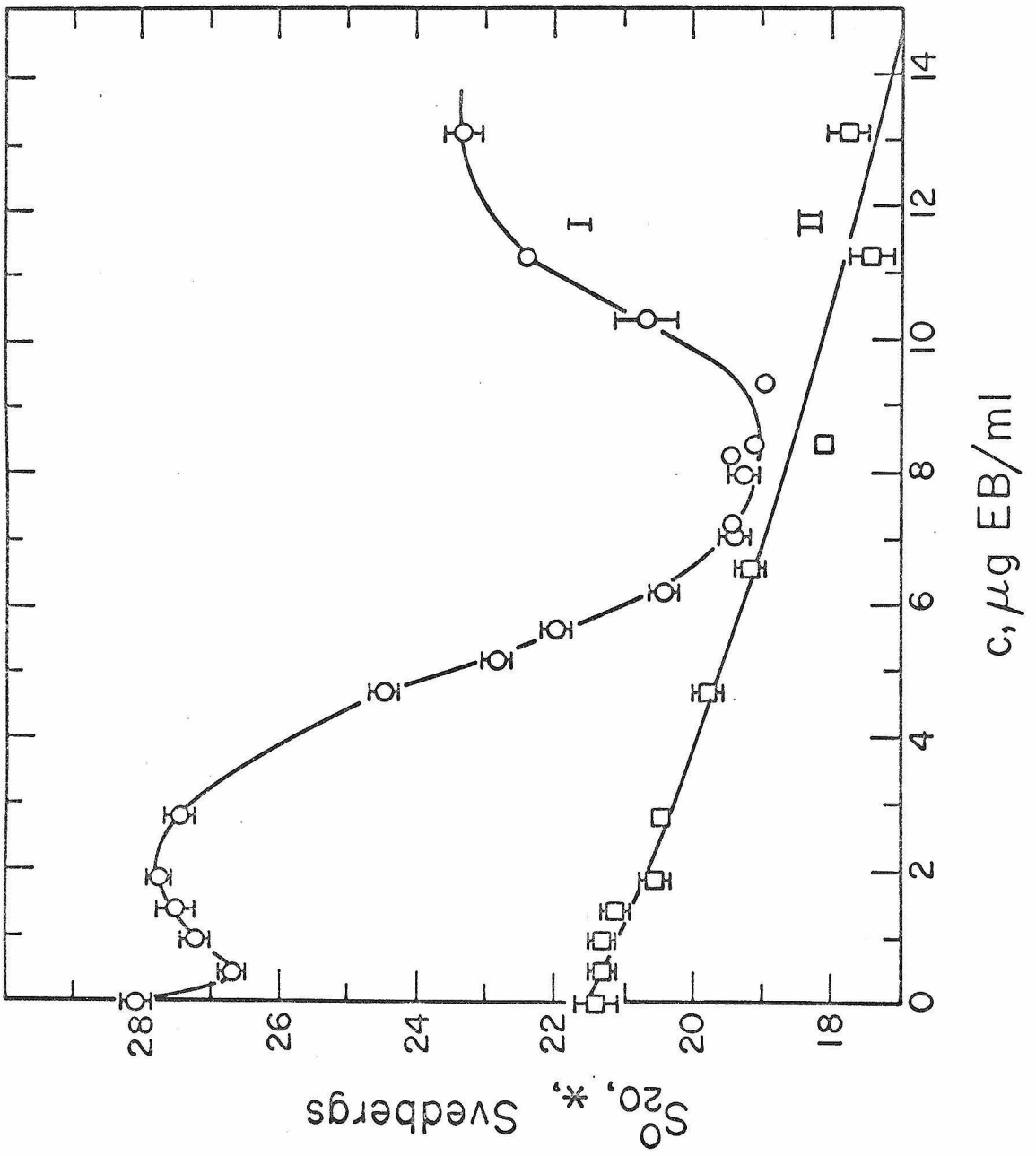
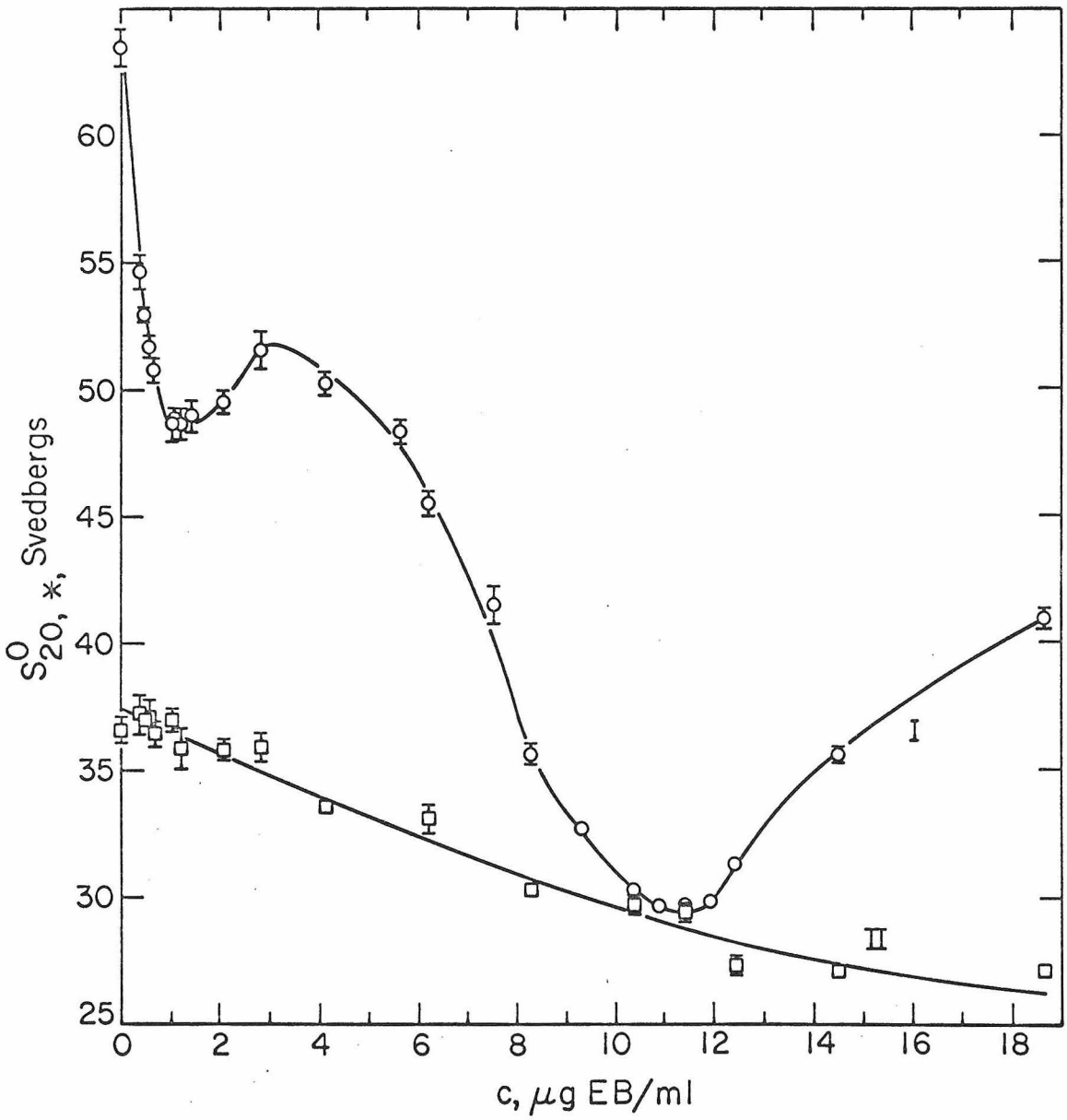

S^0_{20}, *, Svedbergs
28
26
24
22
20
18
I
II
0
2
4
6
8
10
12
14
c, μg EB/ml

FIG. 5. Sedimentation coefficient as a function of free ethidium bromide concentration for the $\lambda b_2 b_5 c$ L3 (o) and nicked $\lambda b_2 b_5 c$ (□) DNA's.


60
55
50
45
40
35
30
25
0
2
4
6
8
10
12
14
16
18
$S^0_{20,*}$, Svedbergs
c, μg EB/ml
I
II

(b) Spectrophotometric determination of the binding parameters

Spectrophotometric binding data determined as described by Waring (1965) are plotted in Figure 6 according to the independent binding equation of Scatchard (1949)

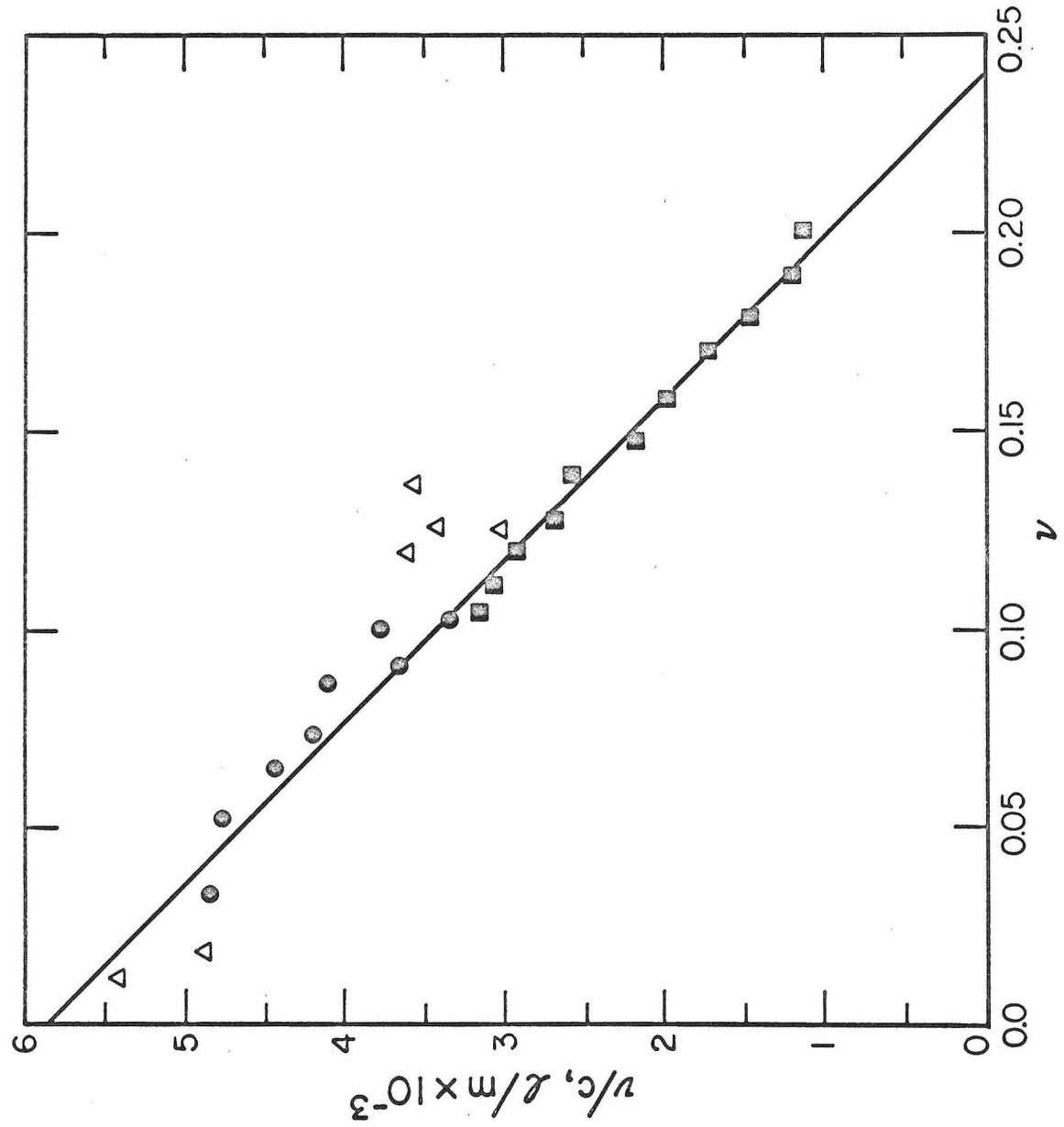
$$\nu/c = \underline{K}(\nu_m - \nu) \qquad (3)$$

where ν is the molar ratio of bound dye to nucleotide, c is the free dye concentration, $\underline{K}$ is the intrinsic binding constant, and ν_{max} is the number of binding sites per nucleotide. Unreliable data points obtained in the extremes of ν in one set of measurements were not used in the least-square determination of $\underline{K}$ and ν_{max}. The line corresponding to the least-squares values for $\underline{K}$, $2.45 \pm 0.08 \times 10^4$ l./mole, and for ν_{max}, 0.242 ± 0.008 moles ethidium bromide/mole nucleotide, is drawn in Figure 6. The binding parameters are in good agreement with those determined by Wang (1969a), who performed similar measurements with calf thymus and $\lambda b_2 b_5 c$ DNAs in 3 M-CsCl, 0.01 M-EDTA, 20°C. He obtained 2.66×10^4 l./mole and 0.23 for $\underline{K}$ and ν_{max}, respectively. Bauer & Vinograd (1970a) found that their data at high values of ν were fit better by an excluded site model (Crothers, 1968) in which one potential intercalative binding site is excluded for every ethidium bromide molecule bound. Our data are fit better by the Scatchard (1949) model.

(c) The relative separation between DNA bands in dye-CsCl gradients

Samples of closed circular DNA and the corresponding nicked circular form were banded together in dye-CsCl gradients in preparative ultracentrifuges. The separations between the bands were measured on the photographs

FIG. 6. Values of ν/c, from the spectrophotometric measurement of the binding of ethidium bromide to calf thymus DNA, plotted as a function of ν. Measurements were made in 2.83 M-CsCl, 0.01 M-Tris at 20°C in either a 5-cm cell with total ethidium bromide concentration of 19.0 μg/ml. (●) or a 1-cm cell with total ethidium bromide concentration of 107 μg/ml. (■). The least-square straight line was calculated omitting unreliable data points (Δ) as described in the text.


v/c, ℓ/m x 10⁻³
ν
0.0
0.05
0.10
0.15
0.20
0.25
0
1
2
3
4
5
6

of the fluorescent bands with a Nikon 6 projection comparator at a ten-fold magnification. The optimum amount of DNA per band is about 1 to 2 μg. Less than 0.5 μg gave poor contrast, while larger amounts of material formed wider bands which reduced the accuracy of locating the centers of the bands. The separation for the higher molecular weight DNAs could be determined with greater accuracy because these DNAs formed narrower bands.

A tube containing the reference DNAs was included in each experiment to minimize differences in the separations due to differences in rotor speed or temperature. Rotor speed is the more significant of these two variables. A 1% change in rotor speed changes the separation by 2%.

The difference in the amounts of dye bound to the open and closed circular DNA is linearly related to the superhelix density (Bauer & Vinograd, 1970b). The measured relative separation between bands must, therefore, be corrected for any factors which affect the relative separation other than the difference in the amount of dye bound. A constant amount of ethidium bromide bound to two DNAs of different initial buoyant densities causes a different buoyant shift because of changes in the preferential solvation by water. To correct for this effect, the value of the normalized buoyant separation, $\Delta r/\Delta r^*$, where Δr^* indicates the separation between the open and closed reference DNAs, is multiplied by the reciprocal of the factor in equation (9) of Bauer & Vinograd (1970b)

$$f^{-1} = (\bar{v}_1\theta^* - 1)^2/(\bar{v}_1\theta - 1)^2 \qquad (4)$$

where $\bar{v}_1$ is the partial specific volume of water set equal to unity, and θ and θ^* are the buoyant densities of the unknown and reference DNAs in the absence of dye.

The measured separation is also dependent on the value of the density gradient, which in turn is proportional to the distance from the center of rotation. Any change in the mean position of the bands in different tubes will affect the relative separation. The normalized buoyant separations were therefore multiplied by $\bar{r}/\bar{r}^*$, the ratio of the average distances from the axis of rotation for the unknown and reference DNAs, respectively. The final quantity which characterizes the relative differences in the amount of ethidium bromide bound is

$$\Omega_c = f^{-1} \frac{\bar{r}}{\bar{r}^*} \frac{\Delta r}{\Delta r^*} \qquad (5)$$

Table 3 gives the values of Ω_c for the DNAs studied here, and also for some of those studied by Hudson et al. (1969). The errors in Ω_c are the standard deviation of the several measurements of the distances between bands in a single experiment.

(d) The ethidium bromide gradient and the solubility of ethidium bromide in CsCl solutions

Figure 7 presents the dye and density distributions in a CsCl solution of initial density 1.565 g/ml. and initial ethidium bromide concentration of 330 μg/ml., centrifuged for 48 hours in an SW-50.1 rotor at 40,000 rev./min, 20°C. The solubilities of ethidium bromide in selected CsCl solutions with densities between 1.46 and 1.61 g/ml.

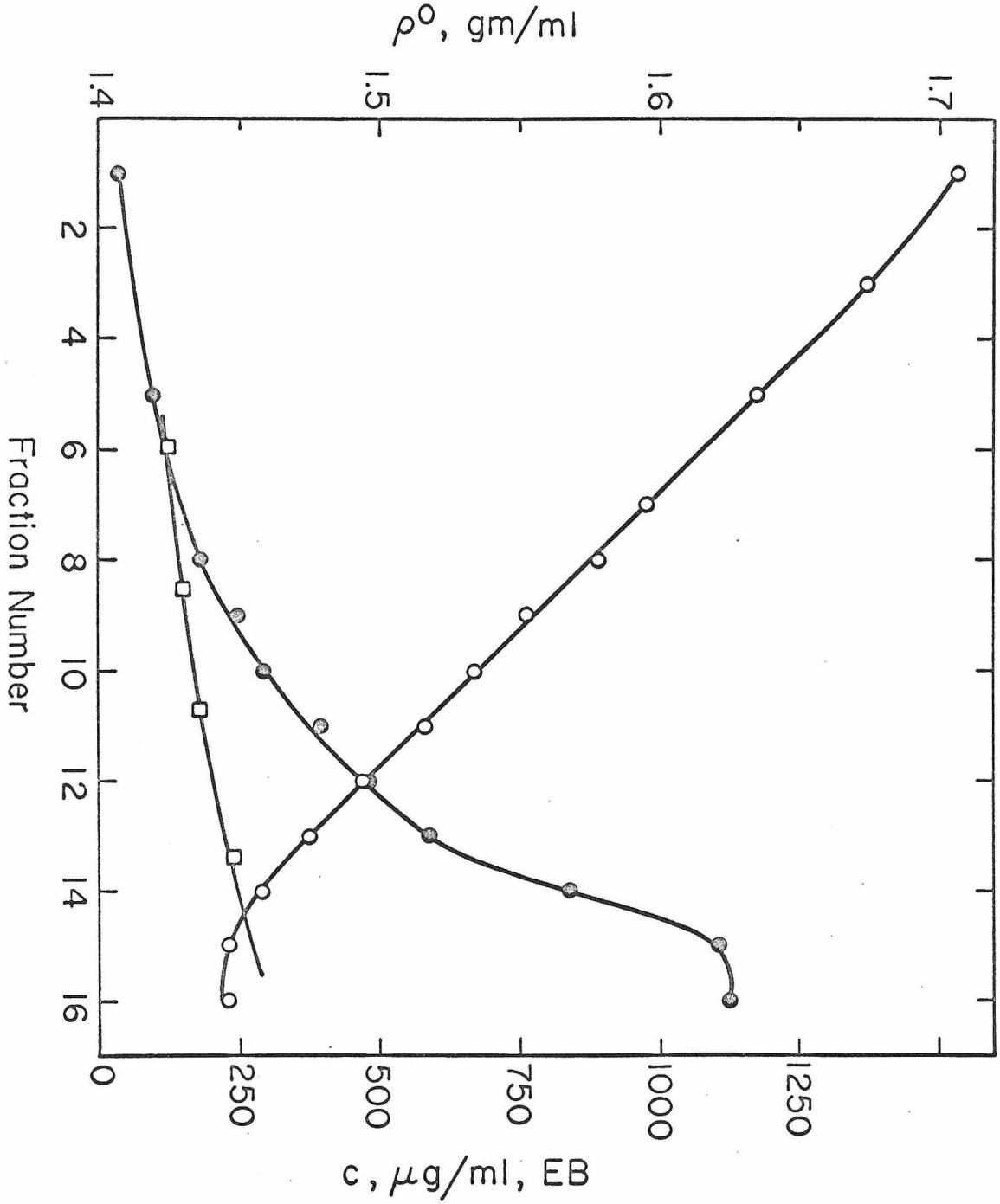
Table 3

Superhelix densities of viral and F-factor DNAs. Calibration of the buoyant separation method with data obtained from ethidium bromide-sedimentation velocity titrations

DNA	Relative buoyant separation Ω_c	Superhelix density $-\sigma_o \times 10^{-2}$ Sed. vel. titration	Equation
			Equation (6)
SV40 L1	1.26 ± 0.01	0.9 ± 0.2	0.9 ± 0.2
Polyoma I	1.02 ± 0.03	3.3 ± 0.4	3.7 ± 0.3
SV40 I	1.00 ± 0.01+	3.9 ± 0.3+	3.9 ± 0.3
PM2 I	0.86 ± 0.01	5.3 ± 0.4	5.5 ± 0.1
SV40 L3	0.75 ± 0.02	7.1 ± 0.5	6.8 ± 0.2
SV40 L2	0.60 ± 0.02	8.5 ± 0.4	8.6 ± 0.2
			Equation (7)
$\lambda b_2 b_5 c$ L1	1.21 ± 0.03	0.7 ± 0.2	0.5 ± 0.3
" L2	0.99 ± 0.04	2.7 ± 0.2	3.0 ± 0.3
" L3	0.67 ± 0.02	6.7 ± 0.3	6.7 ± 0.2
F-factor I	0.94 ± 0.03	3.5 ± 0.2	3.6 ± 0.3
" I-EB	0.92 ± 0.02	4.0 ± 0.3	3.8 ± 0.3

+This DNA was used as a standard for the determination of the relative buoyant separations.

FIG. 7. Density (o) and dye (●) distributions in a 3.0 ml. ethidium bromide-CsCl gradient centrifuged for 48 hr at 40,000 rev./min in a SW50.1 rotor at 20°C. Initial density and ethidium bromide concentrations were 1.565 g/ml. and 330 μg/ml., respectively. The squares indicate the solubilities of ethidium bromide at the corresponding concentrations of CsCl.


ρ°, gm/ml
1.4
1.5
1.6
1.7
Fraction Number
2
4
6
8
10
12
14
16
0
250
500
750
1000
1250
c, μg/ml, EB

are also presented in Figure 7. The solubility data reveal that the top 45% of the gradient is supersaturated with respect to ethidium bromide. Moreover, the solubility in the initial homogeneous solution containing 330 μg/ml. is only 150 μg/ml.

We have found that the supersaturated solutions are quite stable at rest and during centrifugation, if precipitation of ethidium bromide is avoided during preparation of the solutions. If precipitation occurs during the preparation of the final solution, a variable amount of ethidium bromide (which may be as much as 50% of the total) precipitates and deposits at the top of the tube during the centrifugation. Initial precipitation could be observed in experiments in which the concentrated ethidium bromide, 10 mg/ml., was layered onto the concentrated CsCl solution having a density of about 1.59 g/ml. If unstirred, the lamella became turbid. Although the solution appeared to clarify after agitation with a vortex mixer, small crystals or nuclei must have remained which then acted as seeds for further crystallization during ultracentrifugation. The ethidium bromide losses from the final solution increased when the solutions were chilled prior to centrifugation, and tended to be insignificant if the final solution were warmed to approximately 50°C. Initial precipitation was avoided by diluting the added ethidium bromide with the buffer and the DNA solutions to a final concentration of about 900 μg/ml. or 400 μg/ml., followed by addition of CsCl solution with a density of 1.88 g/ml. or solid CsCl, respectively. The concentrated CsCl solution or the solid CsCl was added in

stages with intermediate mixing as a precautionary measure. Similarly, all solutions except those containing DNA were passed through Millipore filters.

(e) Relationship between the corrected relative separations between bands in ethidium bromide and the superhelix density

A plot of the corrected relative separation between bands of nicked and closed circular DNAs versus superhelix density as determined by sedimentation velocity-dye titration is shown in Figure 8. The superhelix densities are plotted in the form $\sigma_o - \sigma_o^*$, where σ_o^* is the superhelix density of the reference DNA, SV40 I, and is taken to be -3.9×10^{-2} (Table 2).

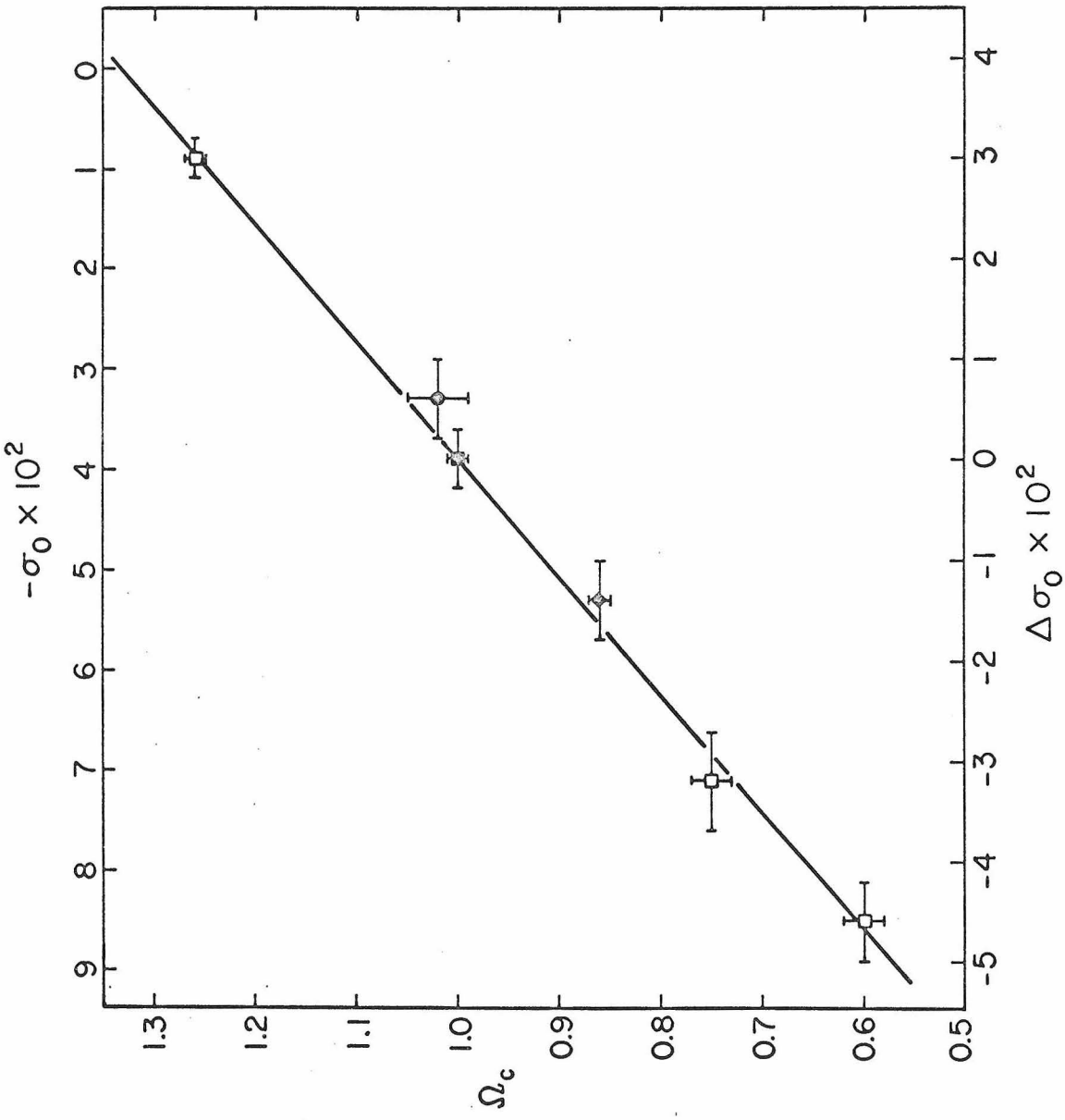
The line in Figure 8 is the least-square fit to the data shown. Each point used in the least-squares procedure was weighted according to its error. The intercept is necessarily unity and was assigned this value. The least-squares procedure, appropriately modified for the assignment of the intercept, was applied twice: once with the errors assigned to $\Delta\sigma_o$, and once with the errors assigned to Ω_c. The slopes of the two least-squares lines differed by only 0.1%. The slopes and their errors were averaged to give the relationship

$$\Omega_c = 1 + (8.66 \pm 0.34)\Delta\sigma_o \qquad (6a)$$

$$\Delta\sigma_o = (0.115 \pm 0.005)(\Omega_c - 1) \qquad (6b)$$

Equation (6a) may be compared with the theoretical results of Bauer & Vinograd (1970b) by substituting k, the binding constant for

FIG. 8. The corrected relative buoyant separation, Ω_c, in CsCl density gradients containing 330 μg/ml. ethidium bromide as a function of superhelix density (upper abscissa) or as a function of the difference in superhelix density (lower abscissa). Filled symbols represent native closed DNA forms; unfilled symbols represent closed DNA's obtained from enzymatic closure of nicked DNA: SV40 DNA (□); polyoma DNA (o); PM2 DNA (◇). The least-square line was obtained as described in text.


$-\sigma_0 \times 10^2$
0
1
2
3
4
5
6
7
8
9
Ω_c
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
$\Delta\sigma_0 \times 10^2$
4
3
2
1
0
-1
-2
-3
-4
-5

the excluded site binding model (Crothers, 1968; Bauer & Vinograd, 1970a), and the value of c (330 μg/ml., 8.4×10^{-4} mole/l.) into equation (13) of Bauer & Vinograd (1970b) and combining the result with their equation (16). A value for k of 1.18×10^{4} l./mole was calculated from the limit of ν/c at $\nu = 0$ from our binding data with the relationship $\lim_{\nu \to 0} \nu/c = 0.25k$ (Crothers, 1968). The slope in their equation (16) which, except for a higher order term, has the form of equation (6a), becomes 9.24. This slope is 7% higher than the experimentally observed value. The higher-order terms become significant only at high negative superhelix densities and reduce the discrepancy between the two equations in that region.

The data for the $\lambda b_2 b_5 c$ and F-factor DNAs in Figure 9 clearly give a line displaced from the line for the lower molecular weight DNAs. The weighted least-squares treatment was applied to the high molecular weight data and the relation

$$\Omega_c = 0.91 \pm 0.03 + (8.9 \pm 0.1)\Delta\sigma_o \qquad (7a)$$

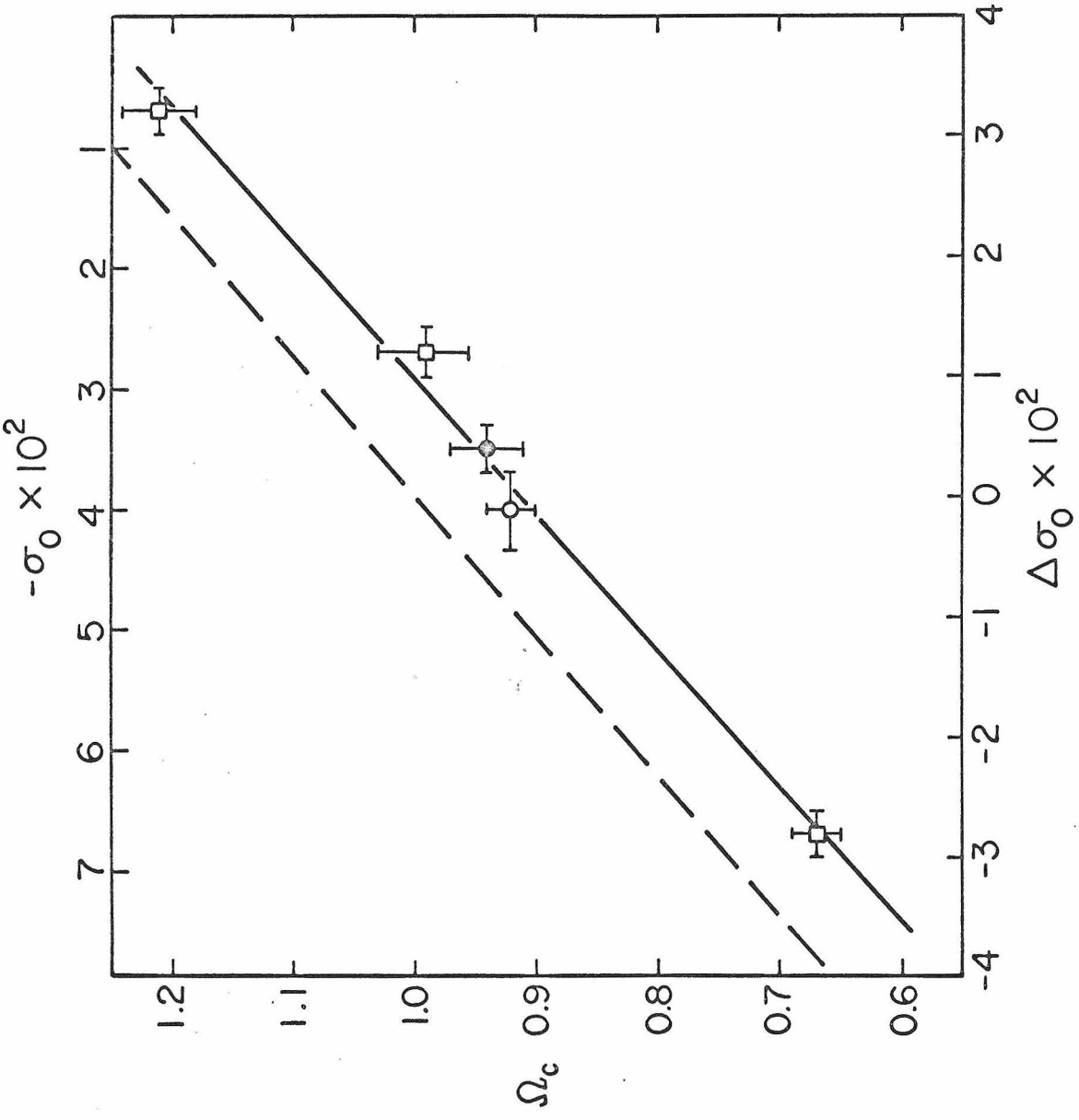
$$\Delta\sigma_o = (0.112 \pm 0.001)(\Omega_c - 0.91 \pm 0.03) \qquad (7b)$$

was obtained by averaging the slopes and intercepts of the two least-squares treatments. The errors are the mean deviations between the two treatments.

(f) Relationship between relative separations in propidium diiodide and superhelix density

Relative separations were also measured for some of the DNAs in the

FIG. 9. The corrected relative buoyant separation, Ω_c, in ethidium bromide-CsCl gradients with initial ethidium bromide concentrations of 330 μg/ml. as a function of σ_0 and $\Delta\sigma_0$ for $\lambda b_2 b_5 c$ (□) and F-factor (o) DNA's. The least-square line was obtained as described in the text. The dashed line is the least-square line presented in Fig. 8 for 3 to 6 megadalton DNA.


$-\sigma_0 \times 10^2$
$\Delta\sigma_0 \times 10^2$
Ω_c
1
2
3
4
5
6
7
-4
-3
-2
-1
0
1
2
3
4
1.2
1.1
1.0
0.9
0.8
0.7
0.6

presence of the dye propidium diiodide. This analog of ethidium bromide was shown by Hudson et al. (1969) to enhance the separation between open and closed circular DNA forms by a factor of approximately 1.8 over that observed in the presence of ethidium bromide. The values of and Ω_c^{PDI}, the corrected relative separation for propidium diiodide, are shown in Table 4. A plot of Ω_c^{PDI} versus $\Delta\sigma_o$ is given in Figure 10. The equation of the line calculated as described for equation (6) is

$$\Omega_c^{PDI} = 1 + (10.5 \pm 0.6)\Delta\sigma_o \qquad (8a)$$

$$\Delta\sigma_o = (0.095 \pm 0.005)(\Omega_c^{PDI} - 1) \qquad (8b)$$

Values of $-\sigma_o$ calculated from equation (8) appear in Table 4.

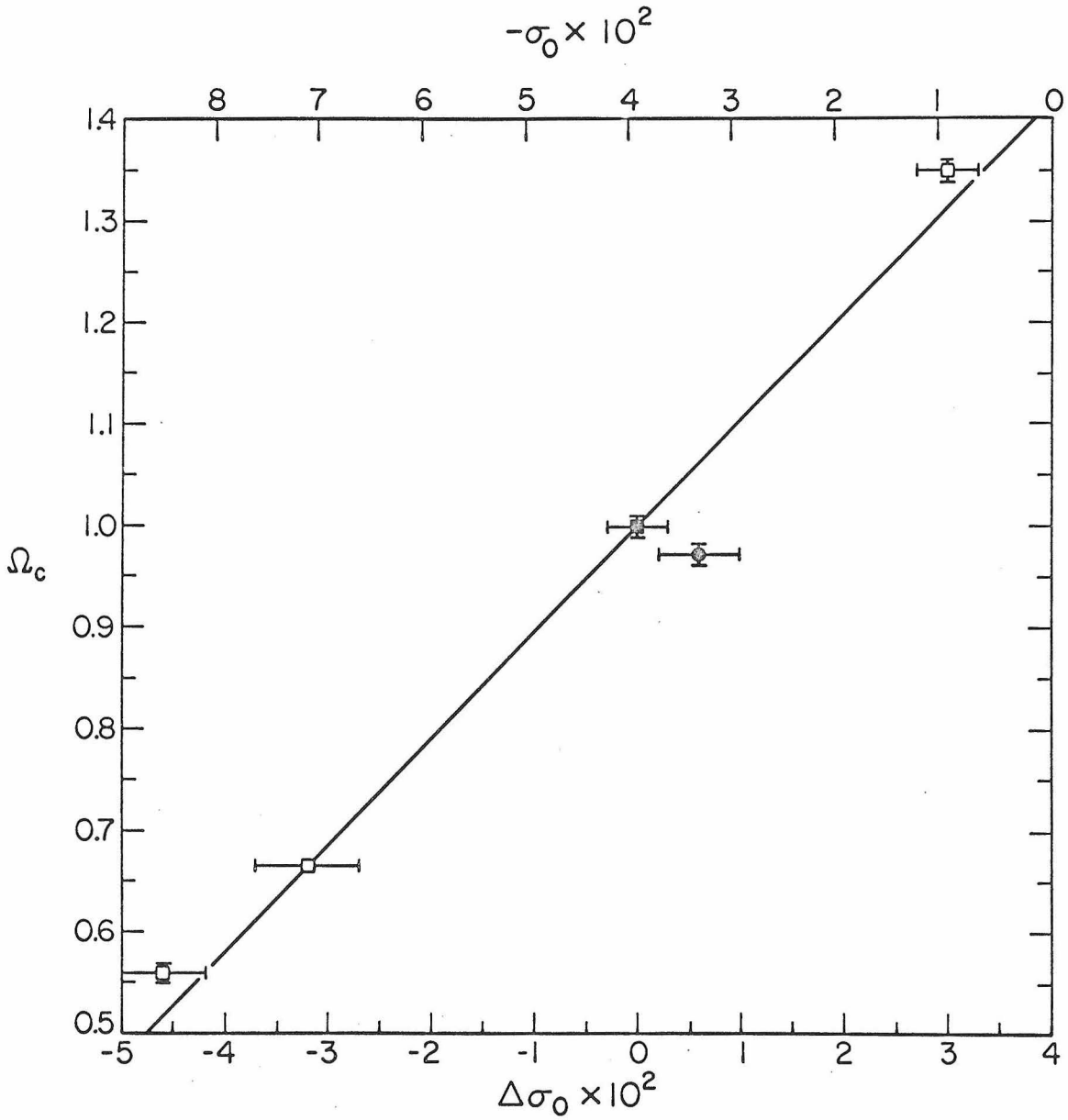
The derivation of the factor f of equation (4) which corrects for variations in the initial buoyant density requires a knowledge of $\bar{v}_4$, the partial specific volume of the bound dye (Bauer & Vinograd, 1970b). The partial specific volume of the bound ethidium chloride was measured by the above authors and found to be 1.02 ml./g. The value was set equal to unity in the derivation of the expression for f (Eq. 4). The value of $\bar{v}_{4,PDCl}$, was estimated with the aid of the partial specific volume for ethidium chloride, the molar volumes for nitrogen, CH_2, and CH_3 groups quoted by Langridge et al. (1960), and the molar volume for the Cl^- ion calculated from data for CsCl by Ifft & Williams (1967). The resulting estimate for $\bar{v}_{PDCl}$ was 0.981 ml./g. We have examined the approximations made by Bauer & Vinograd (1970b) in the development of equation (4) with the above value for $\bar{v}_{PDCl}$ and the extreme values of Γ_o', the preferential solvation by water, encountered in this work

Table 4

Relative buoyant separations between the closed and open forms of viral DNAs in propidium diiodide-CsCl gradients

DNA	Relative buoyant separations Ω_c	Superhelix density $-\sigma_o \times 10^2$	
		Equation 8	EB-sed.vel.titration
SV40 L1	1.35 ± 0.01	0.5	0.9 ± 0.2
SV40 I	1.00 ± 0.01	3.9	3.9 ± 0.3
Polyoma I	0.97 ± 0.01	4.2	3.3 ± 0.4
SV40 L3	0.666 ± 0.006	7.1	7.1 ± 0.5
SV40 L2	0.56 ± 0.01	8.1	8.5 ± 0.4

FIG. 10. The corrected relative buoyant separation, Ω_c^{PDI}, in CsCl gradients containing 330 μg/ml. of propidium di-iodide as a function of σ_0 and $\Delta\sigma_0$. The symbols represent various DNA's as described in the legend to Fig. 7. The least-squares line was obtained as described in text.


$-\sigma_0 \times 10^2$
8
7
6
5
4
3
2
1
0
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
Ω_c
-5
-4
-3
-2
-1
0
1
2
3
4
$\Delta\sigma_0 \times 10^2$

and have found that the resulting equation is still valid.

4. Discussion

(a) Precision and accuracy of the method

Equation (6b) may be used to determine the superhelix density of DNAs in the molecular weight range 3 to 6 megadaltons, with an error in σ_o of $\pm$ 0.2 x 10^{-2} associated with the experimental determination of Ω_c. The error in the slope of equation (6b) contributes a smaller error, about 4%, in σ_o.

The repeatability of the method was demonstrated in five experimental determinations of the superhelix density of PM2 DNA in three separate centrifugations. The results ranged from -5.2 x 10^{-2} to -5.5 x 10^{-2}, with a mean of -5.4 x 10^{-2} and a standard deviation of $\pm$ 0.13 x 10^{-2}.

Measurement of the superhelix densities of two mouse mitochondrial DNAs with a molecular weight of 10 megadaltons were performed by Smith, Jordan & Vinograd (1971) by the method described here and by the viscometric-ethidium bromide titration method (Révet, Schmir & Vinograd, 1971). Their results fell directly on the line for the 3 to 6 megadalton DNAs. The superhelix densities were -11.4 x 10^{-2} and -1.7 x 10^{-2}.

The measurements reported in this study were made with CsCl solutions that had initial densities of either 1.56 or 1.58 g/ml. The theoretical equations of Bauer & Vinograd (1970b) and the experimental data for the ethidium bromide and CsCl gradients shown in Figure 7 were examined together, in order to estimate the error involved in

using an equation defined at initial density of 1.56 g/ml. for measurements made in solution of initial density of 1.58 g/ml. The coefficient in equation (6a) decreases by 2%, giving an error in σ_o of -0.06×10^2 for a DNA with a zero superhelix density and an error of 0.1×10^{-2} when the σ_o is -8.6×10^{-2}. These errors are within the experimental errors of the method.

(b) Effect of molecular weight

Equation (7b) should be used for DNAs in the molecular weight range 25 to 60 megadaltons. The uncertainties in the intercept and the slope will combine to yield an uncertainty of $\pm 0.4 \times 10^{-2}$ units in σ_o. The slope of this equation is not significantly different from that of equation (6), but the intercept is 0.91 instead of unity as in equation (6). The reason for this shift in the intercept with molecular weight is not understood.

The two DNAs used to determine this line have guanine + cytosine contents of 51% ($\lambda b_2 b_5 c$) and 46% (F-factor) estimated from their buoyant densities. These are both higher than the GC content of SV40 (41%) which was used to determine the equation at low molecular weight. Polyoma (3 megadaltons, 49% GC) and PM2 (6 megadaltons, 41% GC) DNAs fall very close to the SV40 line. If they are included in the least-square analysis, the slope of equation (6a) increases from 8.6 to 8.7.

Bauer & Vinograd (1970b) have reported an approximate 30% increase in the intrinsic binding constant for ethidium bromide as the GC content increases from 31% for Clostridium perfringens to 71% for Micrococcus

lysodeikticus DNA. A larger effect was observed by Waring (1965), who regarded the result to be within his limits of error. LePecq & Paoletti (1967) and Wang (1969a) did not detect any variations in the intrinsic binding constant with base composition.

We have considered the effect of an increase in K of 10% for $\lambda b_2 b_5 c$ DNA and 5% for F-factor DNA on the values of σ_o estimated from the sedimentation velocity-dye titrations. When these new values of σ_o are used, a new slope of 8.5 and a new intercept of 0.94 are obtained in equation (7a). These changes bring the two sets of data into closer agreement, but only account for approximately one-third of the observed discrepancy in the intercept.

Inasmuch as polyome DNA with close to the same base composition as $\lambda b_2 b_5 c$ DNA falls close to the SV40 line, it appears that the major effect is related to molecular weight rather than to base composition. The PM2 point falls slightly off the SV40 line in the direction of the line for $\lambda b_2 b_5 c$ and F-factor DNAs as would be expected for a molecular weight effect. F-factor and $\lambda b_2 b_5 c$ fall on the same line and there appears to be no significant change in Ω_c with an approximately two-fold increase in molecular weight above 25 megadaltons. For DNAs with molecular weights between these two values, superhelix densities may be estimated with an error of approximately $\pm$ 0.4 x 10^{-2} by interpolating between the two lines.

(c) The superhelix densities of polyoma and SV40 viral DNAs

Polyoma and SV40 DNA are similar sized closed circular DNAs that are

contained in structurally similar oncogenic viruses of the papova group. The negative superhelix density of SV40 DNA, 3.9×10^{-2}, is 20% greater than the $3.3 \pm 0.3 \times 10^{-2}$ value for polyoma DNA (Table 2). Although this discrepancy appeared to be outside the estimated errors of 5% and 10% for SV40 and polyoma, respectively, we performed a set of sedimentation velocity experiments to check the differing etihidium bromide-sedimentation velocity titrations shown in Figure 3. Both DNAs were sedimented in paired cells in a single rotor at 3 and 6 μg/ml. ethidium bromide. At these ethidium bromide concentrations the sedimentation coefficients of SV40 DNA and polyoma DNA differ by about 7% and -3%, respectively. It was found that SV40 DNA sedimented 7% faster than polyoma DNA at 3 μg/ml. ethidium bromide and 3% slower than polyoma DNA at 6 μg/ml. ethidium bromide, in accord with the expectation of the two data sets presented in Figure 3.

Previously reported values of the superhelix density of polyoma viral DNA are in agreement with our value of -3.3×10^{-2}. Crawford & Waring's (1967) value, $-2.5 \pm 0.4 \times 10^{-2}$ obtained in an ethidium bromide-sedimentation velocity titration at 20°C in 0.05 M-Tris buffer becomes $-3.2 \pm 0.5 \times 10^{-2}$, when corrected for the effects of counterion and ionic strength with the data for the magnitude of these effects obtained by Wang (1969a) and Upholt, Gray & Vinograd (1971). Vinograd, Lebowitz & Watson (1968) reported a value of $-3.2 \pm 0.2 \times 10^{-2}$ obtained by the alkaline buoyant shift method in 6 M-CsCl at 25°C. The correction for the effect of temperature is -0.07×10^{-2}. In this range of CsCl

concentration, 2.8 to 6 M, it is reasonable to assume that the effect of ionic strength is negligible since the ionic strength dependence of the superhelix density has been found to level off between 2 and 3 M-CsCl. The agreement within 3% among the three values for the superhelix density of polyoma DNA may be taken as corroborative evidence for the validity of the 12° unwinding angle for ethidium bromide (Fuller & Waring, 1964).

Two values of the superhelix density of SV40 DNA have been reported by Bauer & Vinograd (1968; 1970a). Their value, $-3.3 \pm 0.7 \times 10^{-2}$, obtained by the ethidium bromide-sedimentation velocity titration at 20°C in 1 M-NaCl becomes $-3.9 \pm 0.7 \times 10^{-2}$ when corrected to standard conditions for the effects of ionic strength and counterion. While this result is in agreement with the value obtained here, it is noted that the error range is large. The second value, $-3.1 \pm 0.3 \times 10^{-2}$, obtained by the buoyant shift method in ethidium bromide-CsCl density gradients when corrected for temperature becomes $-3.2 \pm 0.3 \times 10^{-2}$, a value that is just significantly lower than the value obtained by the ethidium bromide-sedimentation velocity titration method.

The principal unproven assumption in the ethidium bromide-CsCl buoyant shift method is that the preferential binding of water by CsDNA is unaffected at constant water activity by the binding of ethidium. We have calculated that a -4% change in the preferential hydration induced by the ethidium bromide bound to the relaxed closed molecule would account for the 20% discrepancy between the values of

σ_o for SV40 DNA noted here. In this study and in a companion paper (Upholt, Gray & Vinograd, 1971) we have chosen the $-3.9 \pm 0.2 \times 10^{-2}$ value as the standard value for the superhelix density of SV40 DNA.

(d) Comparison of the empirical and theoretical relations for the dependence of the relative buoyant separation upon the superhelix density

The slope in the theoretical equation of Bauer & Vinograd (1970b) modified as described in Results, section (e), agrees with our experimental data for SV40 DNA within 7%. This agreement may be in part adventitious because of certain approximations made in deriving the theoretical equation and the accuracy of the experimental data used in its development. The excluded site binding model (Bauer & Vinograd, 1970a; Crothers, 1968) is an integral part of the derivation; and the binding data used to fit this model were obtained by the buoyant shift method in 4 to 6 M-CsCl at 25°C in the analytical ultracentrifuge. Our values for σ_o were determined from sedimentation velocity-dye titrations and Scatchard binding parameters obtained from spectrophotometric data measured in 2.83 M-CsCl at 20°C. We obtained a superhelix density for native SV40 DNA which differs significantly from that obtained by Bauer & Vinograd (1968). Moreover the measured dye gradient (Fig. 7) indicates that the free dye concentrations at the centers of the bands of nicked and closed DNAs are substantially different. This difference, assumed to be small in the derivation of the equation, might have a significant effect on the theoretical slope.

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CHAPTER II

Sedimentation Velocity Behavior of Closed Circular SV40 DNA as a Function of Superhelix Density, Ionic Strength, Counterion and Temperature

SUMMARY

Ten closed SV40 DNA's with superhelix densities (superhelical turns per ten base pairs) ranging from -0.007 to -0.085 have been prepared. This family of DNA's has been used to examine the effects of superhelix density on the sedimentation velocity behavior of closed SV40 DNA at high ionic strength in cesium chloride and sodium chloride solutions. The sedimentation coefficient increases as the absolute value of the superhelix density, $|\sigma_0|$, rises from a low value to 0.019, then decreases to a local minimum at 0.035, and finally increases steadily as $|\sigma_0|$ rises to 0.085. Examination of several of these DNA's in the electron microscope has suggested a plausible explanation for the observed non-monotonic variation of s with σ_0.

The sedimentation velocity-ethidium bromide titrations of these DNA's (Gray, Upholt & Vinograd, manuscript in preparation) have been converted from the primary $s^0_{20,*}$ *versus* c data, in which $s^0_{20,*}$ is the standard sedimentation coefficient still uncorrected for the buoyant effect of bound ethidium chloride and c is the free ethidium bromide concentration, to the more meaningful $s^0_{20,w}$ *versus* σ form, with the aid of the coefficients in the expression for the free energy of superhelix formation developed by Bauer & Vinograd (1970a). The resultant curves form a family that is approximately superimposable on the curve for $s^0_{20,w}$ *versus* σ_0 in the absence of ethidium bromide. Similarly transformed sedimentation velocity-dye titrations for viral PM2 DNA (6×10^6 daltons) and a high superhelix-density $\lambda b_2 b_5 c$ DNA (25×10^6

daltons) have the same general character and contain both a local maximum and a local minimum.

The results of a study of the dependence of the sedimentation coefficient of selected SV40 DNA's upon ionic strength, the nature of the cation, and temperature are consistent with the previously reported effects of these variables (Wang, 1969a) on the rotation angle of the base pairs along the helix axis.

1. Introduction

It was proposed by Vinograd, Lebowitz, Radloff, Watson & Laipis (1965) that the closed circular DNA isolated from polyoma virus contains superhelical turns, and that these turns are removed when the duplex is slightly unwound in the early stages of an alkaline titration monitored by sedimentation velocity experiments. The sedimentation coefficient declines to the value for the nicked DNA and then rises as the titration proceeds and superhelical turns of the opposite sign are introduced. Sedimentation velocity titrations of this type have since been carried out with the intercalating dye ethidium bromide as the titrant (Crawford & Waring, 1967; Bauer & Vinograd, 1968; Wang, 1969a, b). This dye has been reported to unwind the duplex as it binds (Fuller & Waring, 1964). The critical molar binding ratio ν_c, moles EB† per mole nucleotide at the minimum in the titration curve, may be calculated for the relaxed closed DNA with the binding parameters obtained with linear DNA. The superhelix density of the dye-free molecule σ_0, an intensive quantity defined as the number of superhelical turns per ten base pairs, can then be calculated with the relation (Bauer & Vinograd, 1968)

$$\sigma_0 = -0.67\ \nu_c \qquad (1)$$

The latter authors measured the binding isotherm for native viral SV40 DNA by a buoyant method, and were then able to convert the s versus c EB titration curve to a more meaningful s versus σ curve. It became

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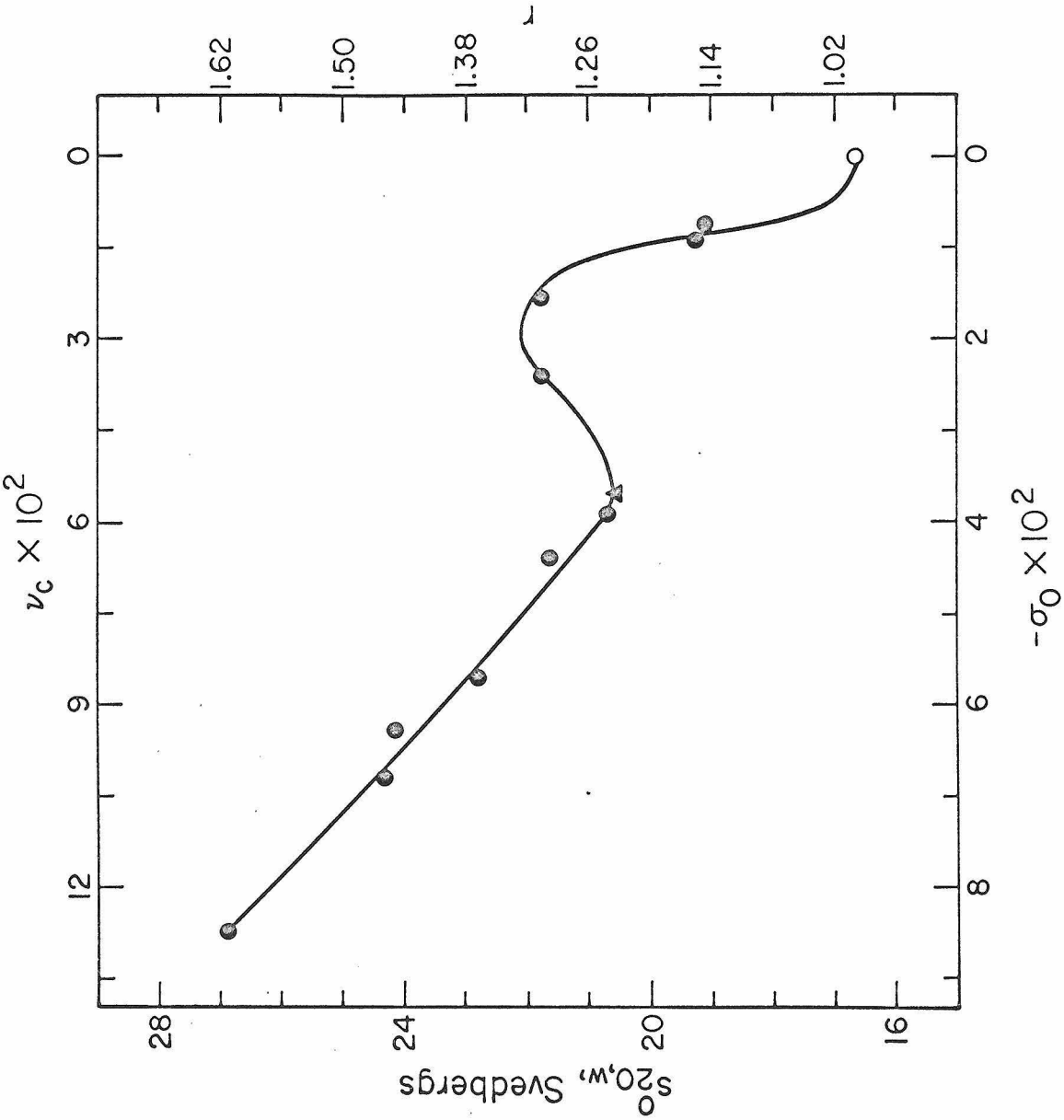
† Abbreviation used: EB, ethidium bromide.

apparent that the sedimentation coefficient was a sensitive index of the superhelix density in the range of 0 to - 0.01 tertiary turns per ten base pairs, but was an insensitive index in the range between - 0.01 to - 0.03.

With the object of extending our understanding of the effects of tertiary turns on the configuration of a closed DNA, we have prepared a set of ten closed SV40 DNA's with superhelix densities ranging from - 0.007 to - 0.085 and have studied the sedimentation velocity behavior of these DNA's in the absence, as well as in the presence, of ethidium bromide. The DNA's were closed enzymatically with polynucleotide ligase in the presence of different concentrations of EB (Wang, 1969a; Hudson, Upholt, Devinny & Vinograd, 1969).

The main result of this investigation is the finding that s, in the absence of EB, depends upon σ_0 in a complex way (Fig. 1). The sedimentation coefficient first rises sharply from 17 S at a zero value of σ_0 to a maximum of 22 S at - 0.02, then declines to a local minimum value of 21 S at - 0.04, and finally rises steadily to 27 S at - 0.08. This non-monotonic behavior indicates that at least three kinds of configurations are encountered as increasing numbers of superhelical turns are introduced. Electron micrographs of DNA's suggest that the first rise in s with $|\sigma_0|$ is the result of a steady reduction in the radius of gyration of an essentially random-coil structure. The compact form with, on the average, a spherical symmetry then becomes elongated as a tight interwound structure is formed at intermediate superhelix densities. At higher superhelix densities, the interwound structure becomes increasingly branched and the sedimentation coefficient correspondingly rises.

FIG. 1. Sedimentation coefficient, $s^{o}_{20,w}$, of closed circular DNA's as a function of superhelix density. Both quantities were measured in 2.83 M-CsCl, 0.01 M-Tris, pH 8.0 at 20°C. (o) SV40 DNA; (Δ) polyoma DNA; (o) nicked circular SV40 DNA. The right-hand ordinate, r, represents the sedimentation coefficient of the closed DNA normalized by the value for the nicked DNA.


r
1.62
1.50
1.38
1.26
1.14
1.02
$\nu_c \times 10^2$
0
3
6
9
12
$-\sigma_0 \times 10^2$
0
2
4
6
8
$s^0_{20,w}$, Svedbergs
28
24
20
16

We have converted the $s^{o}_{20,*}$ versus c curves obtained in the sedimentation velocity-ethidium bromide titrations of these DNA's to $s^{o}_{20,w}$ versus ν or σ curves in order to obtain further data on the effect of σ on the frictional coefficient. The quantity $s^{o}_{20,*}$ is the standard sedimentation coefficient still uncorrected for the buoyant effect of bound ethidium chloride. The dye bound per nucleotide, ν, was calculated with the aid of our isotherm for the binding of EB to linear DNA as modified by the Bauer & Vinograd (1970a) treatment of the effects of the free energy of superhelix formation. The resultant curves, $s^{o}_{20,w}$ versus σ, form a family that is approximately superimposable on the curve for $s^{o}_{20,w}$ versus σ_0 in the absence of ethidium bromide.

We have also measured the sedimentation coefficient of SV40 DNA's of different superhelix densities as a function of the ionic strength, the nature of the cation, and the temperature. When the results are considered in terms of the data for $s^{o}_{20,w}$ versus σ_0, it is apparent that σ is altered slightly by changes in any of these three variables, in agreement with the conclusions derived by Wang (1969a) in his study of closed λ DNA.

2. Materials and Methods

(a) Preparation of DNA

Viral SV40 DNA was obtained as described by Gray, Upholt & Vinograd (manuscript in preparation). The polyoma DNA was obtained from purified large plaque virus grown on mouse kidney primaries (Vinograd, Lebowitz & Watson, 1968). The DNA was isolated by phenol extraction. The $\lambda b_2 b_5 c$ phage DNA was a gift from J. C. Wang.

Enzymatically closed SV40 DNA's with superhelix densities of -0.009, -0.085, and -0.068 are the materials previously referred to as L1, L2, and L3, respectively, by Hudson et al. (1969). The lambda DNA with a superhelix density of -0.067 is the DNA referred to as λL3 by Gray, Upholt & Vinograd (manuscript in preparation). Six additional closed SV40 DNA's were prepared as described by Hudson et al. (1969). The DNA and the EB concentrations in the incubation mixtures are listed in Table 1.

(b) Polynucleotide ligase

The ligase was the preparation described by Hudson et al. (1969).

(c) Chemicals

The ethidium bromide was a gift from Boots Pure Drug Co. Ltd., Nottingham, England. Optical grade cesium chloride was obtained from the Harshaw Chemical Company, Cleveland, Ohio. The D_2O was obtained from Bio-Rad Laboratories, Richmond, California.

TABLE 1

DNA and ethidium bromide concentrations used in the preparation of closed circular DNA's with different superhelix densities

σ_0	EB μg/ml.	DNA μg/ml.
-0.007	0.0	70
-0.009†	0.0	24
-0.015	1.5	60
-0.024	3.0	60
-0.052	5.2	60
-0.057	6.3	60
-0.063	7.4	60
-0.068†	6.7	62
-0.085†	6.8	26
-0.067‡	2.3	15

† These DNA's are, in order, L1, L3, and L2 SV40 DNA's previously described by Hudson et al. (1969) and by Gray, Upholt & Vinograd (manuscript in preparation).

‡ Lambda b_2b_5c DNA. This DNA is λL3 of Gray, Upholt & Vinograd (manuscript in preparation). All others are SV40 DNA's.

(d) Analytical ultracentrifugation

The sedimentation velocity experiments were performed by the band procedure (Vinograd, Bruner, Kent & Weigle, 1963) in a Beckman model E ultracentrifuge equipped with a photoelectric scanner. The results were analyzed as described by Gray, Upholt & Vinograd (manuscript in preparation). Double-sector, 12-mm, type I, band-forming centerpieces were used in the experiments with CsCl as the sedimentation solvent. When NaCl was used, the experiments were performed with 30-mm centerpieces to reduce the DNA concentration and the extent of hydrodynamic interaction. The chamber heater was turned off and the refrigeration readjusted to avoid thermal convection in experiments at low ionic strength. The NaCl was dissolved in 51.6% D_2O to provide an additional stabilizing density gradient in experiments at NaCl concentrations below 1 M. Experiments below 20° C were performed in a black anodized rotor.

In order to perform experiments at high temperatures, the heating circuit was modified to allow greater heating of the rotor. The 4-volt transformer in the heating circuit was replaced with a transformer with an output of 12 volts. A 3-amp Powerstat was placed in the primary circuit of this transformer to allow control of the heating voltage. The 1.2-ohm heating wire was replaced by a heavier wire with a resistance of 4.4 ohms. This system makes available a variable wattage with a maximum of 33 watts compared to the previous constant 13.3 watts.

(e) Preparative ultracentrifugation

Separations of open and closed SV40 DNA forms were determined in CsCl-EB buoyant gradients and measured from photographs (Watson, Bauer & Vinograd, 1971) as described by Gray, Upholt & Vinograd (manuscript in preparation).

(f) Electron microscopy

Specimens for electron microscopy were prepared by the modified procedure of Kleinschmidt & Zahn (1959), as described by Clayton & Vinograd (1967). The DNA, in 1.0 M-CsCl, 25 μg/ml. cytochrome c, 0.01 M-EDTA and 0.005 M-Tris, pH 8.0, was spread onto 0.15 M-NH_4OAc, pH 8.0. The film was picked up on parlodion-coated grids immediately after spreading. Leaving the DNA in the film for a longer time resulted in a gradual relaxation of the superhelical turns. Specimens were examined in a Philips EM300 electron microscope.

3. Results

(a) Dependence of the sedimentation coefficient of closed SV40 DNA on superhelix density in 2.83 M-CsCl

Figure 1 presents the standard sedimentation coefficient of closed SV40 DNA in 2.83 M-CsCl, density 1.35 g/ml., as a function of σ_0, the superhelix density in the same solvent. The superhelix densities were calculated from the magnitudes of buoyant separations observed in buoyant CsCl-EB density gradients with the empirical relationship obtained by Gray, Upholt & Vinograd (manuscript in preparation). We will show later, in corroboration of the findings of Wang (1969a, b), that the rotation angle of the bases along the duplex varies slightly with the ionic strength, the nature of the counterion, and temperature. In this work we will consider 2.83 M-CsCl at 20° C to be the standard solvent for the measurement of superhelix density and designate this quantity as σ_0. In the past, we have used σ_0 to indicate the superhelix density in the absence of ethidium bromide without specifying the temperature of the solvent.

The frictional coefficient of closed SV40 DNA obviously depends upon the superhelix density in a complex way. The sedimentation coefficient, which is inversely proportional to the frictional coefficient, first rises from 16.7 S to a local maximum value of 22.0 S at $\sigma_0 = -0.019$. It then decreases by 1.4 Svedberg units between -0.019 and -0.038. The sedimentation coefficient again rises to 26.9 S as the negative superhelix density increases further to 0.085. This curve is qualitatively similar to the curves obtained by Gray, Upholt & Vinograd

(manuscript in preparation) in sedimentation velocity-dye titrations of SV40 DNA's with $|\sigma_0| > 0.045$, with PM2 viral DNA, and with a closed λ DNA with a superhelix density of -0.067. The value of s indicated by the open circle at zero superhelix density in Figure 1 is the sedimentation coefficient of nicked SV40 DNA.

(b) Sedimentation velocity-dye titrations. A method for calculating the dependence of the fully corrected sedimentation coefficient upon the superhelix density of the DNA-dye complex

Sedimentation velocity-dye titrations plotted as $s^0_{20,*}$ versus c curves contain, in a distorted form, information about the variation of the frictional coefficient with superhelix density. We have converted the dye titration curves obtained by Gray, Upholt & Vinograd (manuscript in preparation) to $s^0_{20,w}$ versus ν curves in order to compare them with the $s^0_{20,w}$ versus σ_0 curve obtained in the absence of ethidium bromide. Our procedure is first to calculate the molar binding ratio ν at each free dye concentration and then to reduce the sedimentation coefficient to standard conditions by the procedure of Bruner & Vinograd (1965), with due regard for the buoyant density of the DNA-dye complex.

Bauer & Vinograd (1970 a, b) have derived an equation for the binding isotherm which takes into account the effects of the free energy of superhelix formation on the affinity for the binding of ethidium bromide by a closed circular DNA. Their modified Scatchard relation (Bauer & Vinograd, 1970 a)

$$\nu = kc(\nu_m - \nu) \exp - [a(\nu - \nu_c) + b(\nu - \nu_c)^2] \qquad (2)$$

was used to calculate ν as a function of c with the values of the experimentally determined parameter ν_c. The quantity k in equation (2) is the intrinsic binding constant of the relaxed DNA; ν_m, the number of binding sites expressed as sites per nucleotide; ν_c, the critical molar binding ratio; and a and b, the free-energy coefficients. We have used the values 2.45×10^4 l./Mole and 0.241 for k and ν_m (Gray, Upholt & Vinograd, manuscript in preparation), and 23 and -100 for a and b, respectively (Bauer, 1968). The latter coefficients were obtained by means of a computer-generated method to obtain the best fit to equation (2) of experimentally determined binding of ethidium bromide to native viral SV40 DNA at 25° C in approximately 5 M-CsCl solution.

Inasmuch as equation (2) cannot be solved explicitly for ν, we prepared plots of ν versus c for each of the DNA's titrated by Gray, Upholt & Vinograd (manuscript in preparation). The plots were then used to transform the $s^0_{20,*}$ versus c curves (Fig. 2(a)) to $s^0_{20,*}$ versus ν curves (Fig. 2(b)). This conversion substantially expands the abscissa at negative values of σ and contracts it at positive values. The well at the principle minimum becomes more symmetrical.

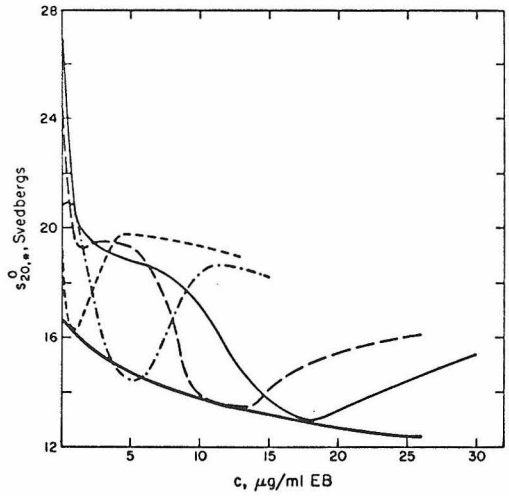
The sedimentation coefficients, $s^0_{20,*}$, in Figures 2(a) and (b) were obtained by the procedure of Bruner & Vinograd (1965) with $\theta_{20° C}$,

FIG. 2. Sedimentation velocity-dye titrations of four closed circular SV40 DNA's in 2.83 M-CsCl, 0.01 M-Tris, pH 8.0. Superhelix densities: (----) -0.009; (-·--·-) -0.039; (————) -0.068; (——) -0.085. The heavy line is for nicked circular SV40 DNA.

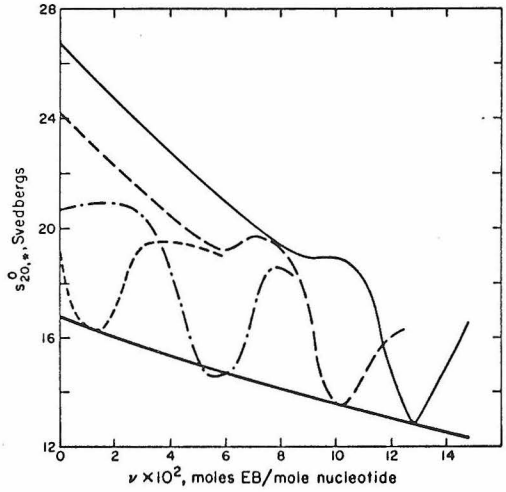
(a) $s^{0}_{20,*}$, standard sedimentation coefficient uncorrected for the buoyant effect of bound ethidium versus free dye concentration, c.

(b) $s^{0}_{20,*}$ versus ν, moles of ethidium bound per mole nucleotide.

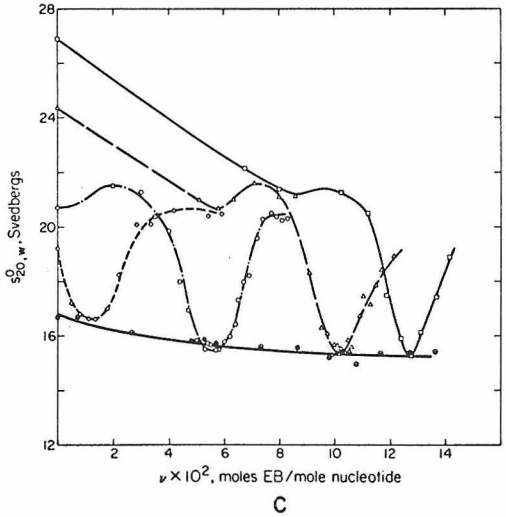
(c) $s^{0}_{20,w}$, standard sedimentation coefficient corrected for the buoyant effect of bound ethidium versus ν.


28
24
20
16
12
$s^0_{20,w}$, Svedbergs
5
10
15
20
25
30
c, μg/ml EB

a


28
24
20
16
12
$s^0_{20,w}$, Svedbergs
0
2
4
6
8
10
12
14
ν ×10², moles EB/mole nucleotide

b


28
24
20
16
12
$s^0_{20,w}$, Svedbergs
2
4
6
8
10
12
14
ν ×10², moles EB/mole nucleotide

c

the buoyant density, taken to be a constant with a value of 1.692 g/ml. In order to obtain $s^{o}_{20,w}$, we have evaluated θ as a function of ν from Figure 13 in Bauer & Vinograd (1968), and have recalculated the sedimentation coefficients in Figures 2(a) and (b). The final results are presented in Figure 2(c). The correction becomes larger as the dye concentration increases. The effect is seen clearly in the behavior of the curve for the nicked circular DNA (Fig. 2(b)). The sedimentation coefficient $s^{o}_{20,*}$ decreases from 16.7 S to 12.5 S as ν is increased from 0 to 0.14. On the other hand, $s^{o}_{20,w}$ is more nearly constant in Figure 2(c) and changes only from 16.7 S to 15.3 S over the same range of ν. The 8% increase in the frictional coefficient is to be attributed both to the 28% extension of the molecule, an estimate based on the assumption that each intercalated ethidium ion extends the duplex by 3.4 Å (Fuller & Waring, 1964), and to changes in the stiffness of the molecule. The net result of the data conversions is a series of curves that resemble the curve in Figure 1 for the dependence of $s^{o}_{20,w}$ on σ_o in the absence of dye. A detailed comparison is presented in Figure 10 in the Discussion.

Similar conversions for viral PM2 DNA and for a sample of enzymatically closed $\lambda b_2 b_5 c$ DNA are presented in Figures 3 and 4, respectively. The final curve for PM2 DNA with a molecular weight of 6×10^6 daltons and a superhelix density of -0.053 contains both the local minimum and the local maximum, as does the curve for $\lambda b_2 b_5 c$ DNA with a molecular weight of 25×10^6 daltons and a superhelix density of -0.067. The general features of the curves for $s^{o}_{20,w}$ <u>versus</u> σ appear to be

FIG. 3. Sedimentation velocity-dye titration of open (□) and closed (o) circular PM2 DNA in 2.83 M-CsCl, 0.01 M-Tris, pH 8.0, presented as in Fig. 2(c). The inset shows the same data plotted in the form, $s^{o}_{20,*}$ *versus* c.

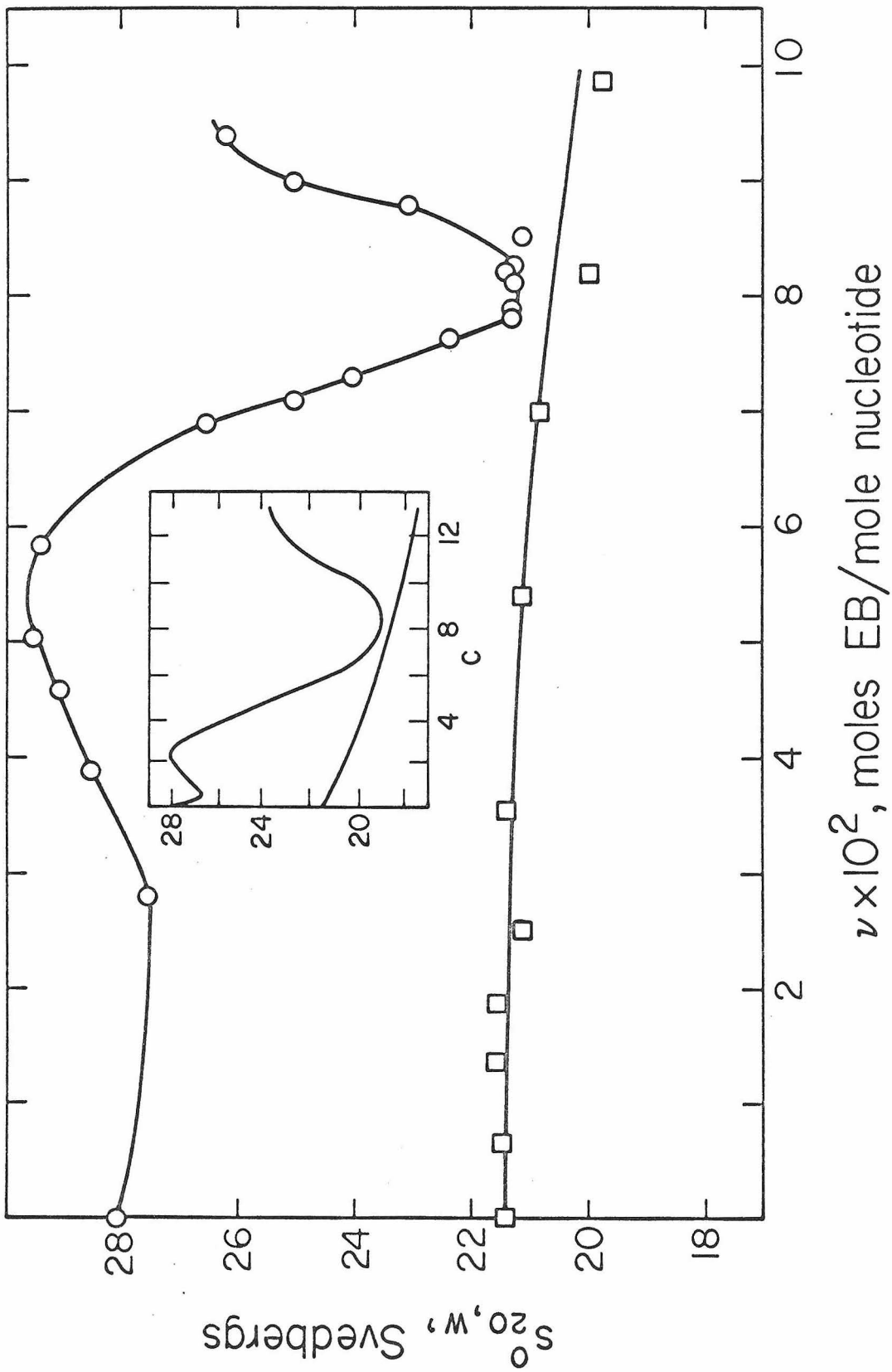
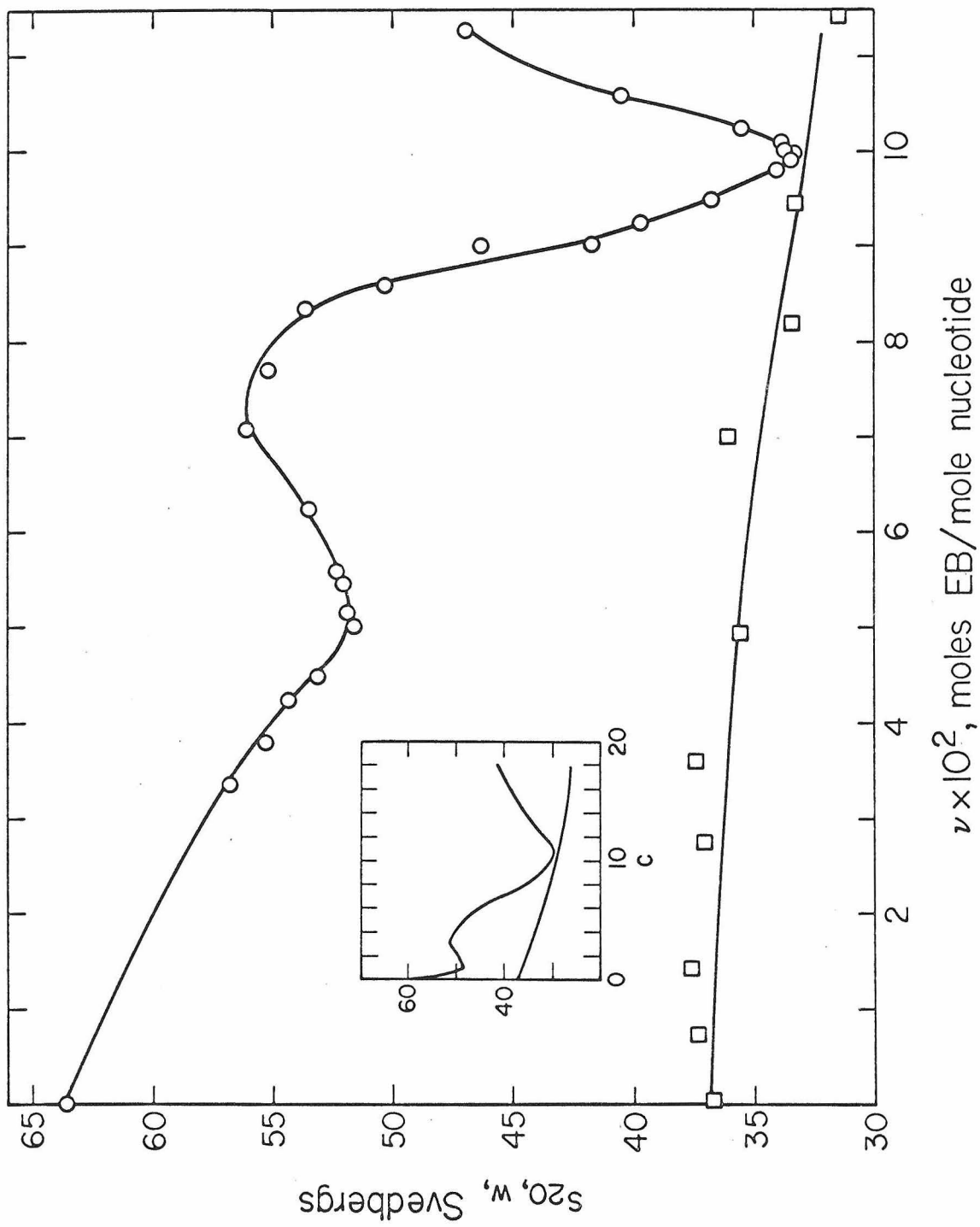

$s^0_{20,w}$, Svedbergs
28
26
24
22
20
18
2
4
6
8
10
$\nu \times 10^2$, moles EB/mole nucleotide
28
24
20
4
8
12
c

FIG. 4. Sedimentation velocity-dye titration for open (□) and closed (o) circular $\lambda b_2 b_5 c$ DNA in 2.83 M-CsCl, 0.01 M-Tris, pH 8.0, presented as in Fig. 2(c). The inset shows the same data plotted in the form, $s^{o}_{20,*}$ versus c.

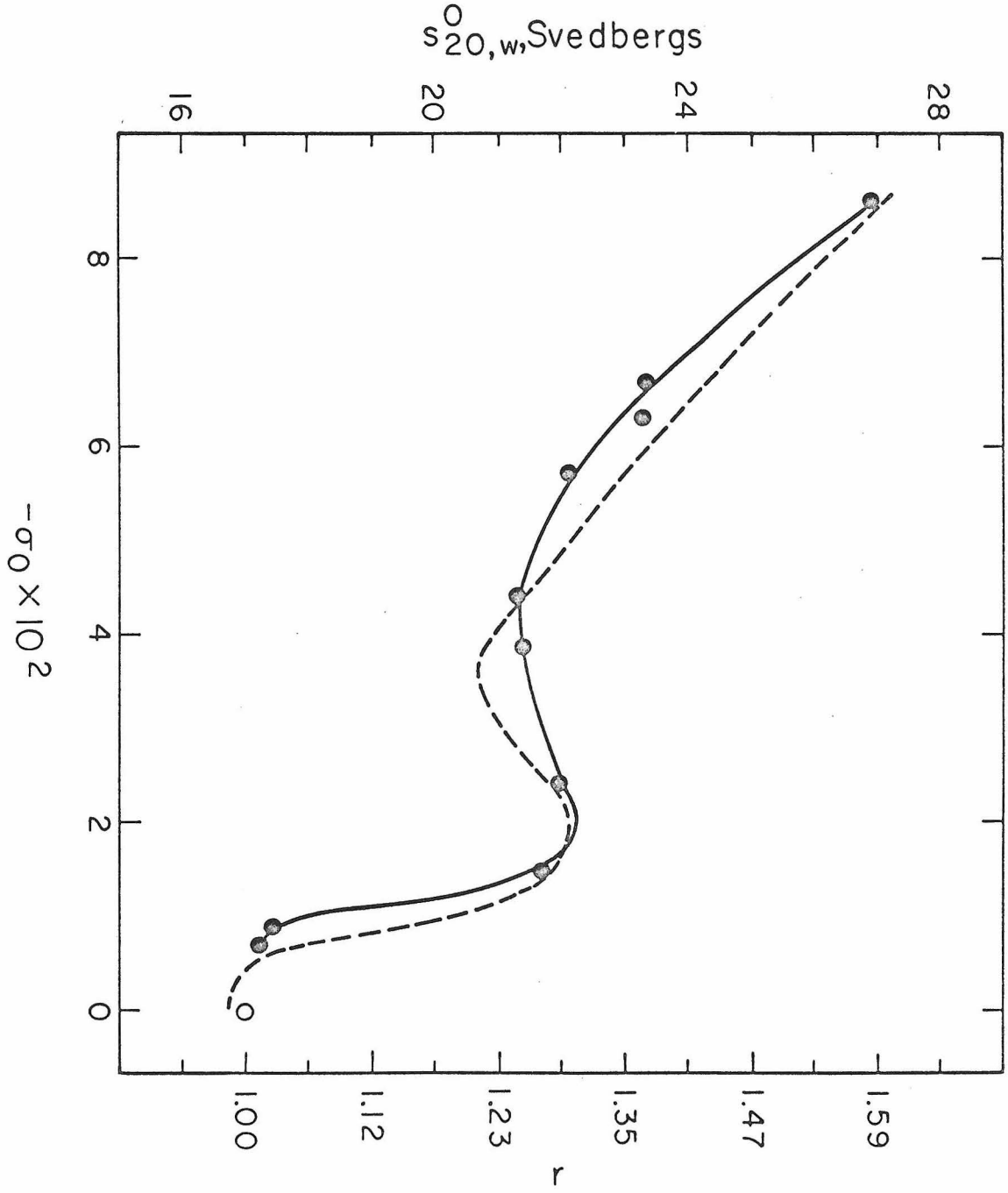

$s_{20,w}$, Svedbergs
$\nu \times 10^2$, moles EB/mole nucleotide
c

similar to those in Figure 2(c) for SV40 DNA, a result that indicates that the occurrence of at least two minima in the curve for $s^{o}_{20,w}$ versus σ is a feature of closed DNA over a range of molecular weights of at least 3 to 25×10^6 daltons.

(c) Dependence of the sedimentation coefficient of closed SV40 DNA in 1 M-NaCl upon superhelix density

Figure 5 presents the sedimentation coefficients of the set of closed circular SV40 DNA's in 1.0 M-NaCl, another sedimentation solvent commonly used in the study of DNA. The observed sedimentation coefficients were converted to standard sedimentation coefficients by the Svedberg procedure using a value of 0.556 ml./g for the partial specific volume of NaDNA (Hearst, 1962). The superhelix densities are those obtained in 2.83 M-CsCl. The data in Figure 1 for $s^{o}_{20,w}$ (NaDNA) obtained in 2.83 M-CsCl are also presented in Figure 5 for comparison. A reasonable superposition is obtained below $|\sigma_0| = 0.05$, if the NaCl curve is translated to the right by about $0.003\,\sigma$. Therefore, the combined effect of the nature of the salt (CsCl → NaCl) and the decreased ionic strength (2.83 M → 1.0 M) appears to have decreased the absolute value of the superhelix density. The small discrepancy between the sedimentation coefficients of the nicked DNA's (indicated by the open symbols in Figs 1 and 5) is a measure of our overall accuracy and precision in the sedimentation velocity experiments and in our procedures for converting observed to standard sedimentation coefficients. A larger change in σ, up to 0.005, would be required to superimpose the data in the range of σ_0 from -0.085 to -0.050. As will

FIG. 5. Sedimentation coefficient measured in 1.0 M-NaCl, 0.01 M-Tris, pH 8.0, of closed circular SV40 DNA as a function of superhelix density in 2.83 M-CsCl. The dashed line is the sedimentation coefficient in 2.83 M-CsCl. The open circle at $\sigma_0 = 0$ is the sedimentation coefficient of nicked circular SV40 DNA in 1.0 M-NaCl.


$s^0_{20,w}$, Svedbergs
16
20
24
28
$-\sigma_0 \times 10^2$
0
2
4
6
8
r
1.00
1.12
1.23
1.35
1.47
1.59

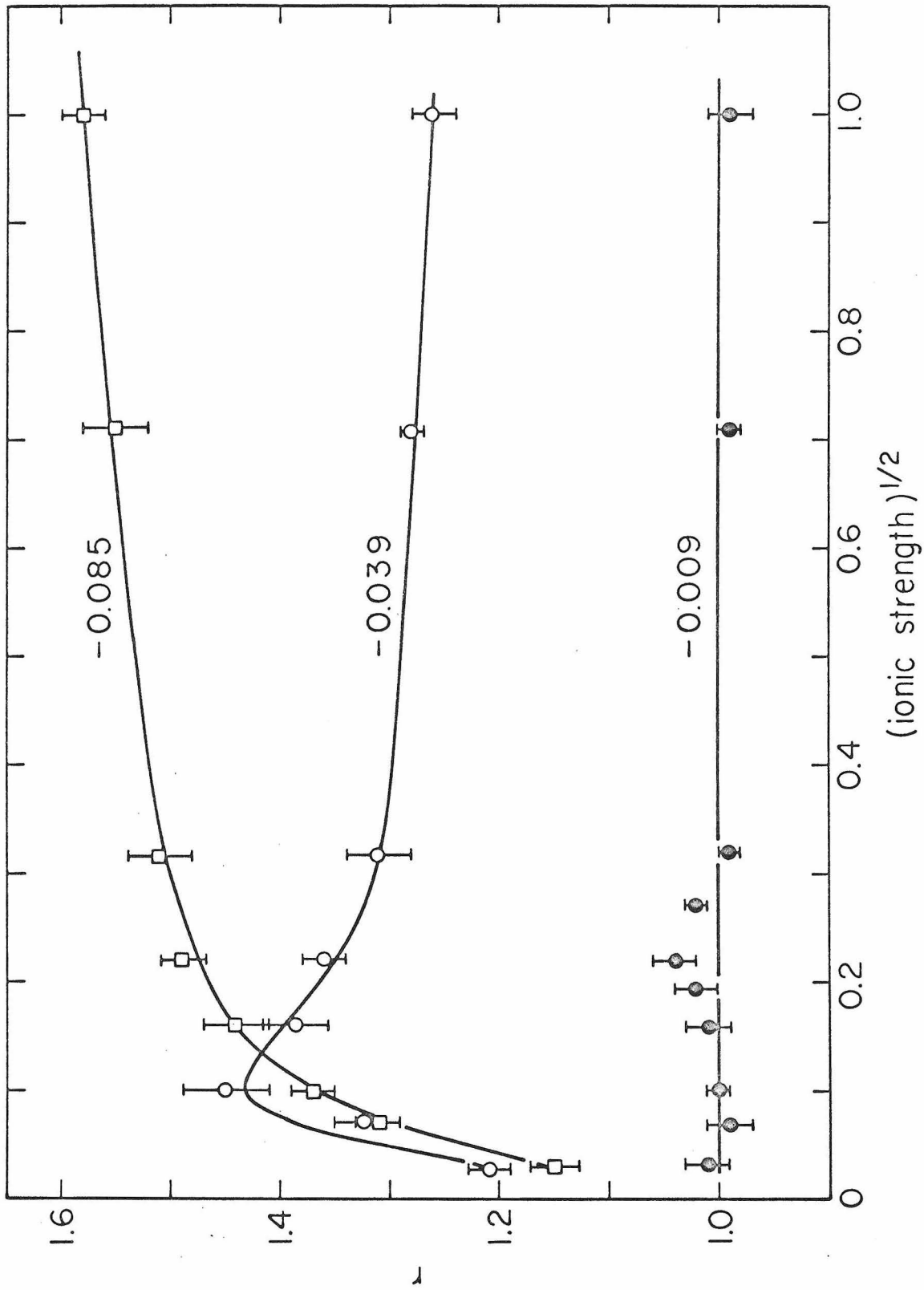
be indicated later, Wang (1969a) has presented evidence for an effect of the nature of the counterion on the rotation angle of DNA that has the same sign and approximately the same magnitude that we have noted here.

(d) Dependence of the relative sedimentation coefficient of three SV40 DNA's with different superhelix densities on the NaCl concentration between 1.0 M and 0.001 M

The effect of ionic strength on the sedimentation coefficient has been examined with DNA's of superhelix density - 0.085, - 0.039, and - 0.009 (Fig. 6). The experiments were all performed with paired samples of closed and nicked SV40 DNA and the results are given as the ratio $r = s_{obs,\,I}/s_{obs,\,II}$, where I and II stand for closed and nicked DNA. This procedure eliminates the small effects of possible variations of temperature and speed, and eliminates the need to correct the results for variations of density and viscosity of the solvent and the partial specific volume of DNA.

The r value for the DNA with the low superhelix density is substantially independent of salt concentration over the entire range, 1.0 M to 0.001 M, of concentration. The sedimentation coefficient of nicked circular DNA was substantially constant between 1.0 M and 0.02 M-NaCl and declined by 25% between 0.02 M and 0.001 M, a result similar to that reported by Rosenberg & Studier (1969) for linear T7 DNA in NaCl. In this latter range, changes in $\bar{v}$ would account for only a 2% change (Cohen & Eisenberg, 1968). It may, therefore, be concluded that primary charge effects and electrostatically induced changes in stiffness and intersegmental repulsions are operative below 0.02 M.

FIG. 6. Relative sedimentation coefficient, r, of three closed circular SV40 DNA's as a function of (ionic strength)$^{\frac{1}{2}}$ in NaCl solution in 51.6% D_2O, 10^{-4} M-Tris, pH 8.0. Superhelix densities are indicated in the figure. The sedimentation coefficients have been divided by the corresponding value for the nicked circular form measured under the same conditions.


-0.085
-0.039
-0.009
1.6
1.4
1.2
1.0
r
0
0.2
0.4
0.6
0.8
1.0
(ionic strength)$^{1/2}$

The low superhelix-density closed DNA is near the principle minimum in the curve in Figure 5, and an increase in σ with decreasing ionic strength would have little effect on s. An opposite change in σ, by as little as -0.005, should have noticeably raised the sedimentation coefficient. The results obtained here are consistent with an absence of a change or with a small positive change in σ with decreasing ionic strength. Wang (1969a) observed that the superhelix density of $\lambda b_2 b_5 c$ DNA changed by 0.004 units when the solvent was changed from 3.0 to 0.1 M-CsCl.

The r value of the DNA with a high superhelix density undergoes larger changes with ionic strength, μ. It changes from 1.58 to 1.51 between 1 M- and 0.1 M-NaCl, and then drops to 1.16 at 0.001 M-NaCl. If we now assume that σ depends on μ and that the ratios of the frictional coefficients of the closed and nicked DNA's are otherwise independent of μ, we may use the r scale in Figure 5 to assess the change in σ with μ. The result between 1 M- and 0.1 M-NaCl is , accordingly, +0.006 superhelical turns per 10 base pairs. Application of this procedure for the results at lower ionic strengths is unwarranted, because the unknown electrostatic repulsions along and between chains are expected to influence the spacial disposition of the superhelical turns.

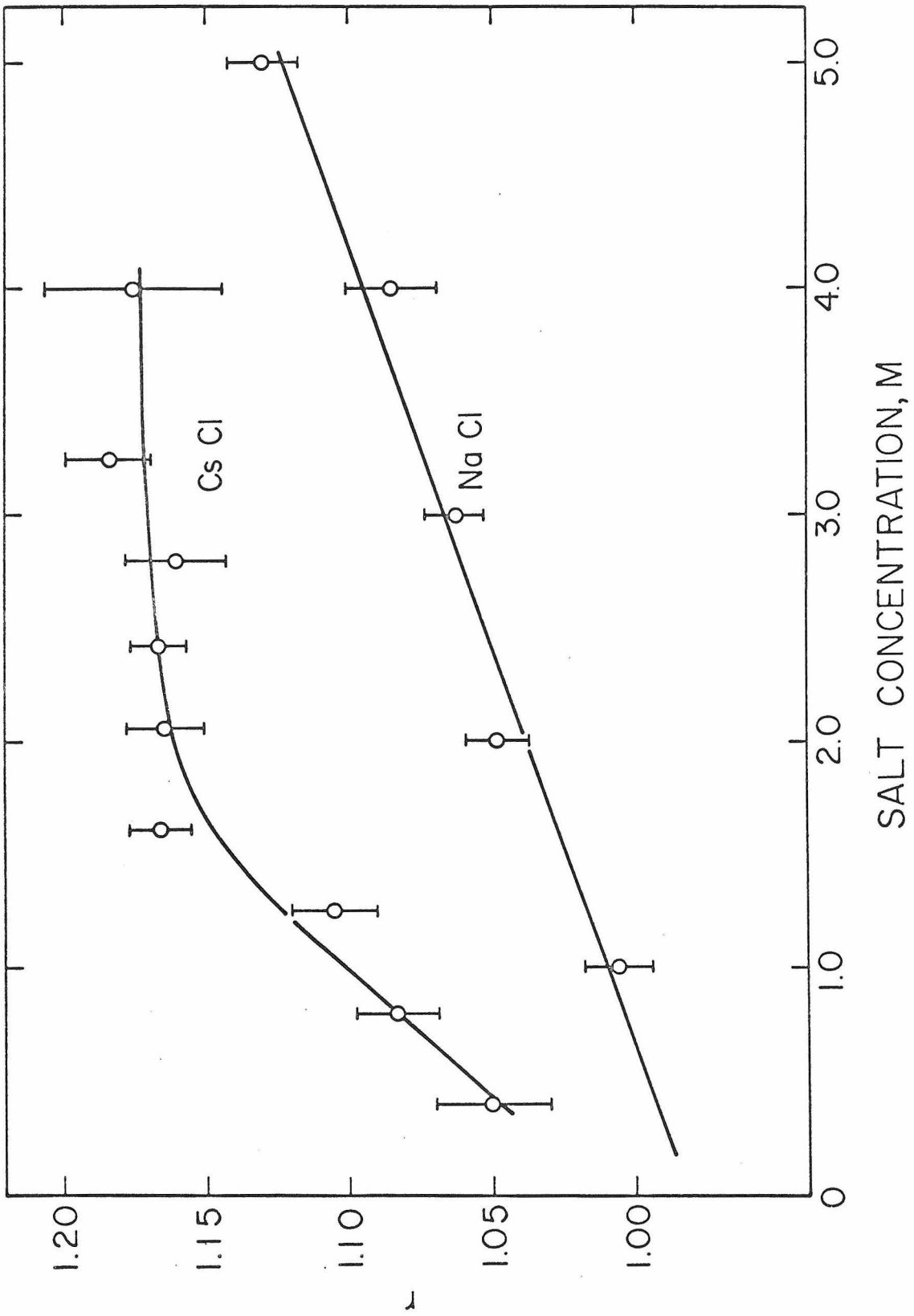
The r value of the DNA with intermediate superhelix density rises slightly from 1.26 to 1.31 between 1.0 M- and 0.1 M-NaCl. It then rises more steeply and reaches a maximum of 1.45 at 0.01 M-NaCl before it falls to 1.21 at 0.001 M. The sedimentation coefficient of this DNA is located in the secondary minimum in the s versus σ plot in

Figure 5. If we assume the positive direction for the shift in σ as was indicated in the consideration of the other two DNA's, we obtain a shift in σ of 0.018 between 1.0 M- and 0.1 M-NaCl. This estimate is of necessity inaccurate because of the weak dependence of s on σ in the region of the secondary minimum. The maximum value of r, 1.45 at 0.01 M ionic strength, is considerably larger than the value, 1.31, at the local maximum in the s versus σ curve in 1.0 M-NaCl. This indicates that configurational changes caused by effects other than changes in σ, as well as changes in σ, influence the value of r at low ionic strengths.

(e) Dependence of the relative sedimentation coefficient on ionic strength and counterion at high salt concentrations

At higher ionic strength, from 5 M- to 1 M-NaCl, the r value for closed circular DNA of low superhelix density decreases linearly from 1.12 to 1.01 (Fig. 7). Below 1.0 M-NaCl, the r value of this DNA is insensitive to μ (Fig. 6). The results at high NaCl concentrations may be interpreted as indicating that σ changes in a positive direction as the salt concentration is decreased. Reference to the r scale on Figure 5 indicates that a positive shift of σ occurs as the NaCl concentration changes from 5 M to 1.0 M. The results for CsCl may be similarly interpreted, except that r remains constant between 2 M and 4 M. A similar result was obtained by Wang (1969a). The superhelix density either no longer changes with ionic strength or some other compensating effect is occurring.

FIG. 7. Relative sedimentation coefficients of closed circular SV40 DNA with $\sigma_0 = -0.009$ as a function of NaCl and CsCl concentration in the range from 0.4 to 5.0 M.

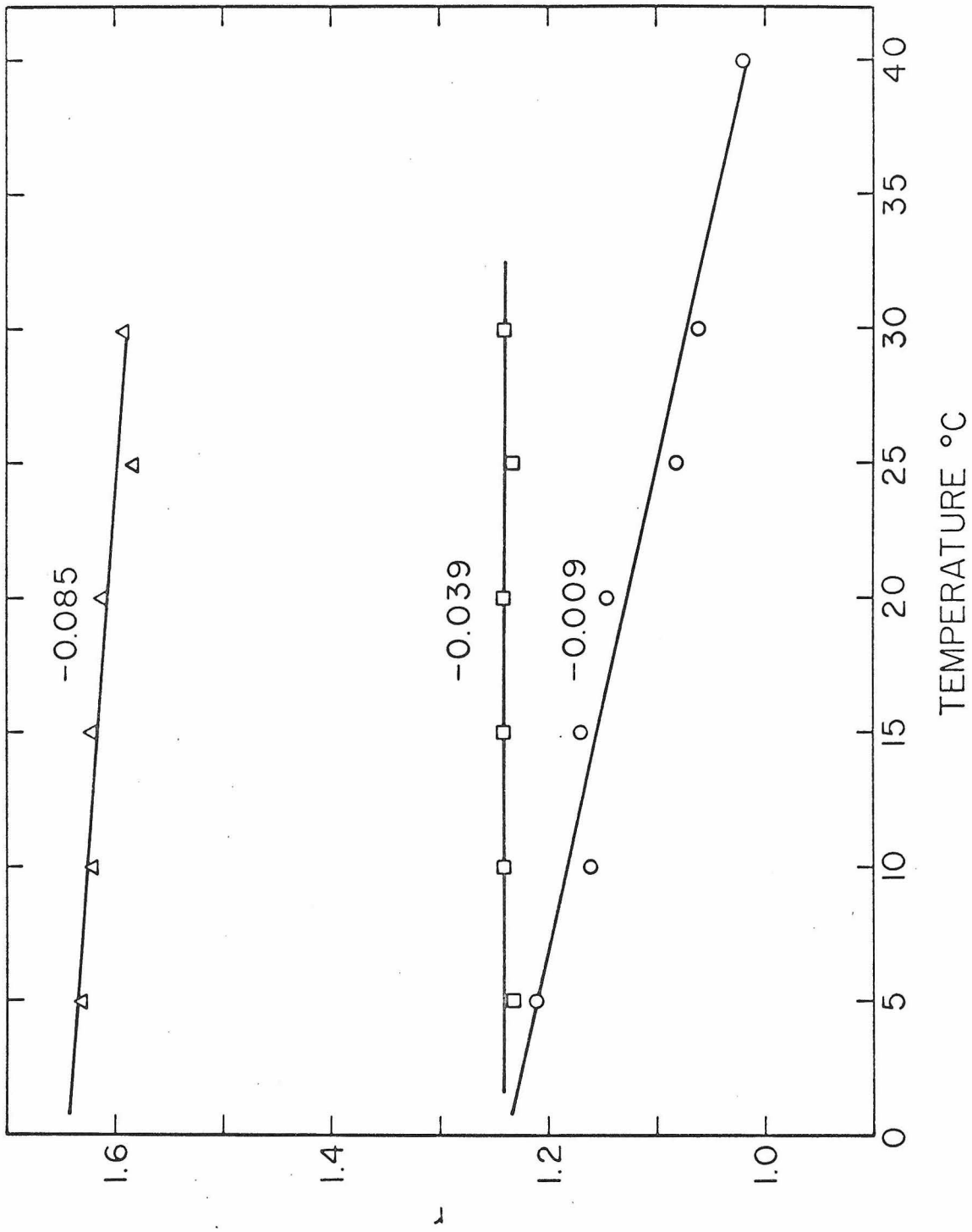

1.20
1.15
1.10
1.05
1.00
r
0
1.0
2.0
3.0
4.0
5.0
SALT CONCENTRATION, M
Cs Cl
Na Cl

The effect of the nature of the counterion can be seen by comparing the two curves in Figure 7. When values of r from Figure 7 are measured, a change in σ of +0.002 is obtained for the change from 2.0 M-CsCl to 2.0 M-NaCl. Wang (1969a) has obtained this same number.

(f) Dependence of the relative sedimentation coefficient of three SV40 DNA's with different superhelix densities on temperature

An increase in temperature over the range 5° C to 40° C has substantially no effect on the relative sedimentation coefficient in 2.83 M-CsCl for the DNA with a superhelix density of -0.039, and decreases r for both the high and low superhelix-density DNA (Fig. 8). These results are consistent with a positive value for the dependence of the superhelix density on temperature. Over a 25° C range, the value of r changes from 1.63 to 1.58 for the high superhelix-density DNA. The corresponding shift in σ (see Figure 5) is 1.5×10^{-4}/° C. Similarly, at low superhelix density r changes from 1.21 to 1.07 over a range of 25° C, with an indicated shift of 1.6×10^{-4}/° C. The intermediate superhelix-density DNA shows no shift in r, as expected from its position at the local minimum. These results confirm the direction, and are consistent with the magnitude of the effect of temperature, 1.4×10^{-4}/° C, reported by Wang (1969a, b).

FIG. 8. Relative sedimentation coefficients of three closed circular SV40 DNA's as a function of temperature in 2.83 M-CsCl, 0.01 M-Tris, pH 8.0. Superhelix densities of the DNA's are indicated in the figure.


-0.085
-0.039
-0.009
1.6
1.4
1.2
1.0
r
0
5
10
15
20
25
30
35
40
TEMPERATURE °C

4. Discussion

Several theoretical treatments of the hydrodynamic properties of circular DNA's have been developed as extensions of previous considerations of linear DNA's. Gray, Bloomfield & Hearst (1967) have extended the Hearst & Stockmayer (1962) analysis of the wormlike coil model of DNA to circular molecules and have included considerations of the effects of excluded volume. Their equation (24) for the relationship between molecular weight and sedimentation coefficient agrees well with experimental data for nicked circular DNA (Hudson & Vinograd, 1969).

Fukatsu & Kurata (1966) and Bloomfield (1966) have treated closed circular DNA's as molecules consisting of sub-rings linearly connected at fixed points by universal joints. Sedimentation coefficients predicted by these models are consistently high compared to the experimentally observed values. Gray (1967) has also applied the Kirkwood (1954) procedure to a model for a closed circular DNA of 3×10^6 daltons assumed to be a rigid double superhelix with constant curvature except at the ends which were neglected. Sedimentation coefficients in the range of the experimental values for SV40 viral DNA's of different superhelix densities were obtained with an appropriate choice of the pitch and the radius of the interwound superhelix. This approach, however, does not provide an estimate of the variation of s as a function of superhelix density in the absence of some way of specifying the variation of the pitch and the radius of the interwound superhelix with superhelix density. The rigid model is necessarily unrealistic at low superhelix densities.

It is not surprising that the configuration of closed supercoiled DNA has not yet been successfully described in theoretical studies. The problem is a difficult one in that these long chain molecules contain some degree of order superimposed on the spacial disorder induced by thermal energy. In the models of Fukatsu & Kurata (1966) and Bloomfield (1966), this order has been imposed on the model in the form of loops restricted at fixed contact points. In Bloomfield's model , the further restriction that these loops be of equal size has been imposed. Jacobson (1969) has shown that equal-sized loops are entropically unfavorable, and Fukatsu & Kurata (1966) have shown that hypothetical molecules with unequal-sized loops will have lower sedimentation coefficients than those with equal-sized loops. The restriction of loops at fixed points is unrealistic, especially in low superhelix-density DNA's in which the superhelical turns are most likely to be loosely arranged in the coiled molecule. Statistical methods which have been used successfully to describe polymer dimensions in random polymers are not applicable in a simple way to the elucidation of the quantitative aspects of the configuration of superhelical closed DNA. In the next section we discuss this problem in a qualitative way with the aid of the results of a study of the appearance of the molecule in electron micrographs.

(a) The configuration of SV40 DNA as a function of superhelix density

The portion of the s versus c or ν curve available from dye titrations of native forms of closed circular DNA's has proved to be misleading. At low values of σ, the behavior is that expected with s increasing as the number of superhelical turns increases. At higher

values of σ, in the range -0.015 to -0.04, s is relatively insensitive to σ (Fig. 1). With the preparation of DNA's of higher superhelix densities (Gray, Upholt & Vinograd, manuscript in preparation), the sedimentation coefficient was again found to be sensitive to the number of superhelical turns.

In this study we have measured the sedimentation coefficients of closed circular DNA's with different superhelix densities in the absence of dye, and thereby have avoided complications which may be associated with the binding of dye. Wang (1969a) obtained such a curve for $\lambda b_2 b_5 c$ DNA over a narrower range of σ, 0.0 to -0.026. Our local maximum (Fig. 1) occurs at a superhelix density of -0.019, compared with -0.016 in Wang's curve. Our curve then continues to a secondary minimum at -0.035. The value of s then rises again as $-\sigma_0$ increases to 0.085. It is useful at this stage to divide the s versus σ curve into three regions of σ_0: A, 0.0 to -0.019; B, -0.019 to -0.035; and C, less than -0.035 (Fig. 9).

Selected samples at various positions on the curve, as indicated in Figure 9, were examined in the electron microscope (Plate I). Native viral closed circular PM2 DNA was admixed in all preparations to check for variations in the effects of the spreading procedure on the appearance of the molecules. Electron micrographs may not be regarded to be planar projections of the three-dimensional configuration of the molecules in solution. The molecules are clearly spread by surface forces encountered in specimen preparation and lose most, if not all, of the three-dimensional coiling caused by thermal fluctuations.

FIG. 9. Sedimentation coefficient of closed circular SV40 DNA as a function of σ_0 in 2.83 M-CsCl. (a), (b), (c), and (d) indicate the DNA's shown in the electron micrographs in Plate I.

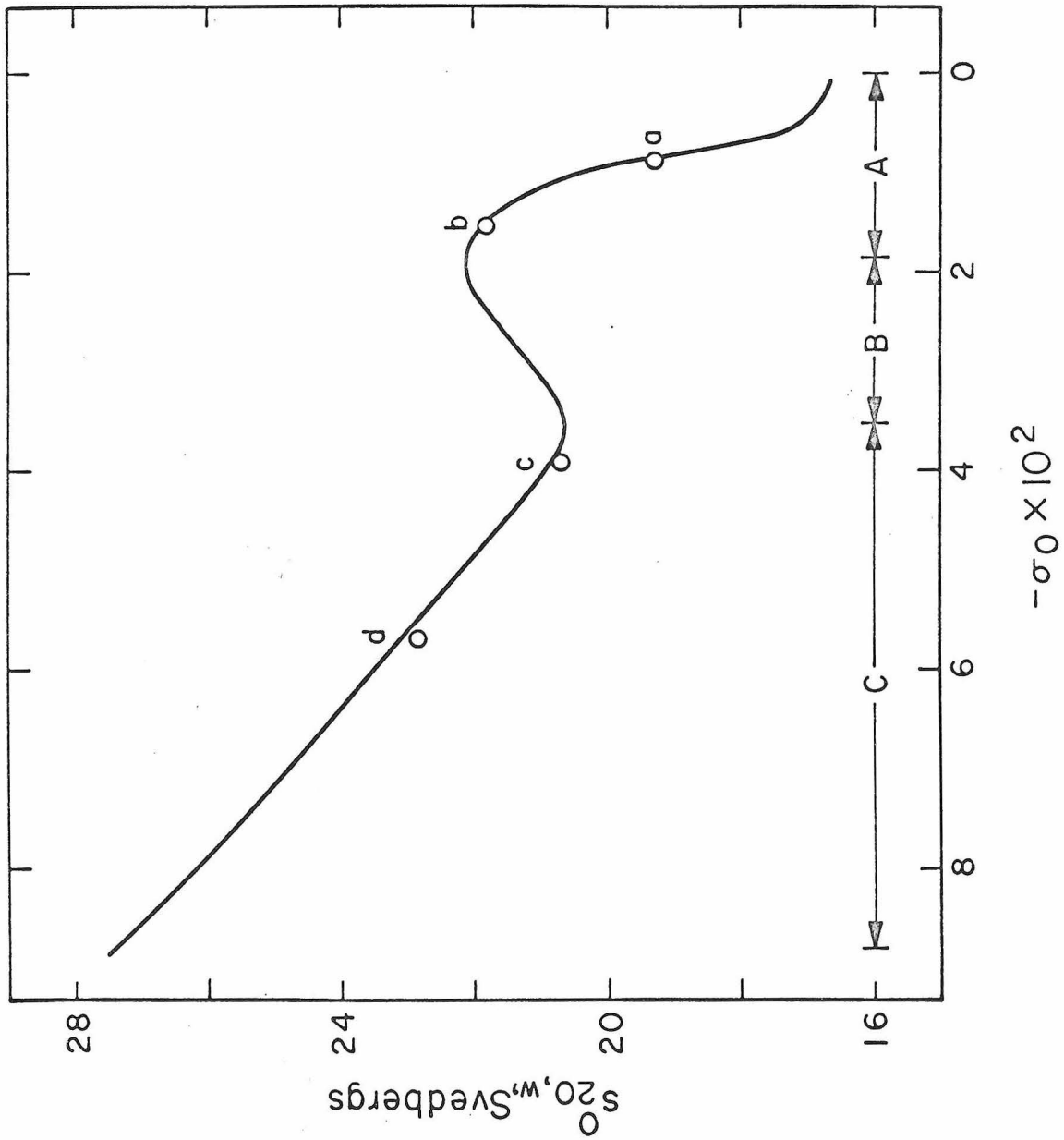
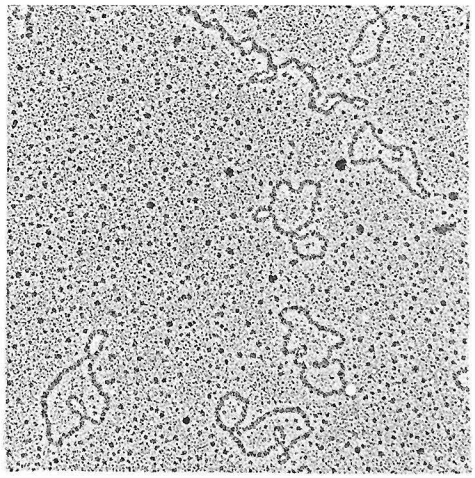
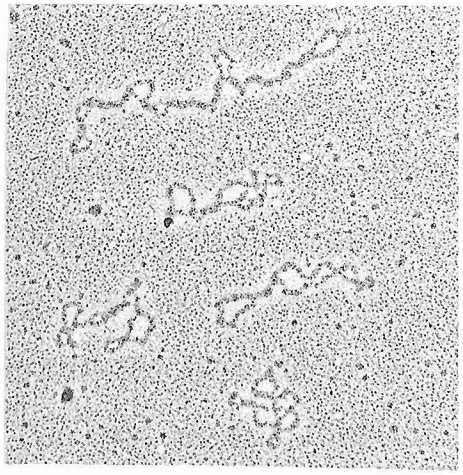

$s^0_{20,w}$, Svedbergs
28
24
20
16
a
b
c
d
A
B
C
0
2
4
6
8
$-\sigma_0 \times 10^2$

PLATE LEGEND

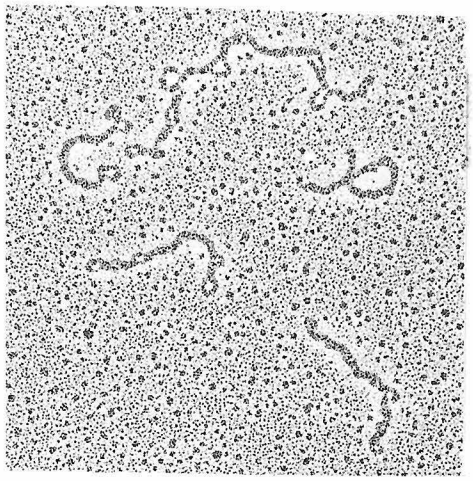
PLATE I. Electron micrographs of four closed circular SV40 DNA's with different values of σ_0: (a) -0.009; (b) -0.015; (c) -0.039; (d) -0.057. One closed circular PM2 molecule with twice the contour length of SV40 DNA is present in each photograph as a control on the possible variable effects of spreading.



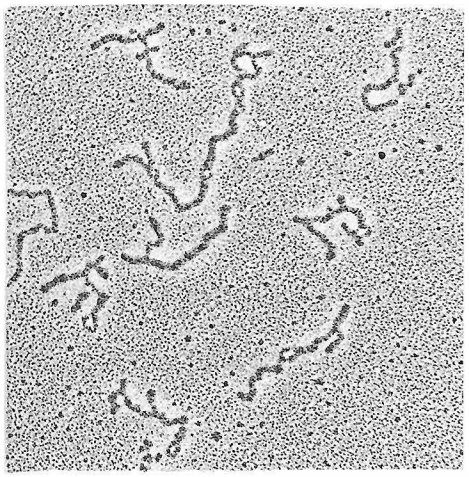
a



b



c



d

DNA a, with a low superhelix density of - 0.009, is in the initial steep region of the s versus σ_0 curve. The superhelix density may have been shifted even closer to zero by exposure to lower ionic strength during specimen grid formation. In the electron micrograph the DNA appears as an open molecule with 2 to 3 crossovers. In solution this material is coiled in three dimensions. DNA b, near the maximum between regions A and B, is more twisted with several end loops, but still has a loose appearance. DNA c, the native closed circular form, is at the local minimum between regions B and C. This form no longer has a loose appearance, but instead is twisted into an extended interwound form with few loops and very little branching. DNA d, with a high superhelix density has the appearance of a brached linear form containing essentially no loops.

A possible model for the sedimentation behavior of closed DNA has been formulated from the appearance of the electron micrographs. The closed circular DNA in region A is regarded to be in a coiled form in solution. The introduction of the first 2.5 superhelical turns (σ, 0.0 to - 0.005) appears to have little effect on the sedimentation coefficient. These first few turns are apparently accommodated in the molecule with no significant change in the equivalent hydrodynamic volume. The molecules in electron micrographs of DNA in this region have substantially the same appearance as the nicked circular molecules. As more turns are put into the molecule ($- \sigma_0$ = 0.005 to 0.018), the molecule becomes more compact and the frictional coefficient decreases. It is proposed that the superhelical turns in this region decrease the hydrodynamic

volume by reducing the radius of gyration while the molecule remains, on the average, in a spherically coiled form.

The sedimentation coefficient is at a maximum and begins to decrease as region B is entered. At the maximum, the DNA contains 10 to 12 superhelical turns. SV40 DNA contains 17 Kuhn statistical segment lengths, based on the estimate of Gray, Bloomfield & Hearst (1967) of 900 Å for the statistical segment length of DNA. This quantity is defined as the length of a segment if the behavior of the polymer molecule is approximated by a model with stiff rods connected by universal joints. As the number of superhelical turns approaches the number of statistical segments, the molecules would be expected to lose the coil character and increasingly take on a more regular and extended form. The extension is reflected in a decrease in the sedimentation coefficient in region B. As further turns are inserted, the molecule assumes a more rigid and anisotropic conformation. Electron micrographs of DNA at the minimum in this region (Plate Ic) show an extended tightly wound linear form with practically no loops or branches. The decrease in sedimentation coefficient as $-\sigma_0$ increases from 0.018 to 0.035 could be explained by a gradual shift from a coiled structure to a more rigid extended form with little branching at $\sigma_0 = -0.035$. This form would have a larger frictional coefficient and a smaller sedimentation coefficient.

As the number of negative superhelical turns further increases and $-\sigma_0$ increases beyond 0.035, the sedimentation coefficient again increases. Electron micrographs of DNA in this region show branched forms with practically no visible circular loops. Branching is expected

to increase the sedimentation coefficient, since it represents a mechanism for bringing chain segments closer together. Branched forms can be expected at high superhelix density if more turns per unit length can be absorbed without increasing the curvature. At very high superhelix density, local melting and negative changes in the rotation angle of the DNA helix may also relieve superhelical turns.

The appearance of a local maximum and a secondary minimum occurs in the s versus σ curve for all DNA's so far examined which have an adequately high absolute superhelix density. The superhelix densities corresponding to these extrema, noted in this study and in the work of others, are listed in Table 2. The table includes three data points obtained from curves of s versus σ_0 for the dye-free molecules. The remainder are from sedimentation velocity-ethidium bromide titration curves and represent the superhelix densities at the maxima and secondary minima in the titration curves. The values of σ_{max} and σ_{min} obtained with the dye-free molecules appear to be independent of molecular weight over an eight-fold range. The average values of all of the results for $-\sigma_{max}$ and $-\sigma_{min}$ are 0.019 and 0.032, respectively. The scatter in the results is largely due to the difficulty of assessing the σ values from curves containing broad and sometimes poorly defined maxima and minima.

We consider that σ_{max} represents the superhelix density at which retarding effects on s of introducing anisometric interwound structures is just equivalent to the accelerating effect of introducing tertiary turns into an approximately spherically coiled molecule. The

TABLE 2

The superhelix density of closed circular DNA at the local maximum and the secondary minimum in s versus σ curves

DNA	Mol. wt. $\times 10^{-6}$	Standard superhelix density $-\sigma_0 \times 10^2$	Local maximum† $-\sigma_{max} \times 10^2$	Secondary minimum† $-\sigma_{min} \times 10^2$
SV40 ‡	3.1		1.9 ± 0.3	3.5 ± 0.4
$\lambda b_2 b_5 c$ §	25		1.6 ± 0.3	—
SV40	3.1	8.5	2.1 ± 0.4	2.8 ± 0.4
		6.8	1.9 ± 0.3	3.0 ± 0.3
		3.9	2.4 ± 0.4	—
PM2	6.0	5.3	1.7 ± 0.3	3.6 ± 0.4
$\lambda b_2 b_5 c$	25	6.7	1.8 ± 0.3	3.2 ± 0.3
Mean ¶			1.9 ± 0.3	3.2 ± 0.5

† The error range represents the uncertainty in the choice of ν at the maxima and minima in the s versus ν curves.

‡ These data were obtained from Fig. 1 for dye-free DNA.

§ Results obtained by Wang (1969a) for dye-free DNA.

¶ Standard deviation of the tabulated values.

superhelix density at the minimum in the s versus σ curve represents the value of σ at which the accelerating effect of branching, and possibly the development of higher-order superhelices just compensates the retarding effects of the elongation into an interwound superhelix.

(b) Conversion of sedimentation velocity dye titrations to $s^{o}_{20,w}$ versus σ curves

Two previous investigators have presented the sedimentation coefficients measured in dye titrations as a function of the number of tertiary turns in the molecules rather than just as a function of the free dye concentration. Bauer & Vinograd (1968) combined sedimentation velocity data in 1.0 M-NaCl at 20° C and binding data in 5.4 to 5.6 M-CsCl at 25° C to obtain a plot of s versus ν for SV40 native closed circular DNA. Their method assumes that the free energy of superhelix formation is the same under the two different sets of conditions. Wang (1969a) measured the binding of ethidium bromide to three different closed circular $\lambda b_2 b_5 c$ DNA's using the fluorescence method to obtain s versus τ plots. The range of superhelix densities for the latter measurements was approximately -0.01 to 0.01. Both of these methods require separate binding studies for each DNA with a different superhelix density.

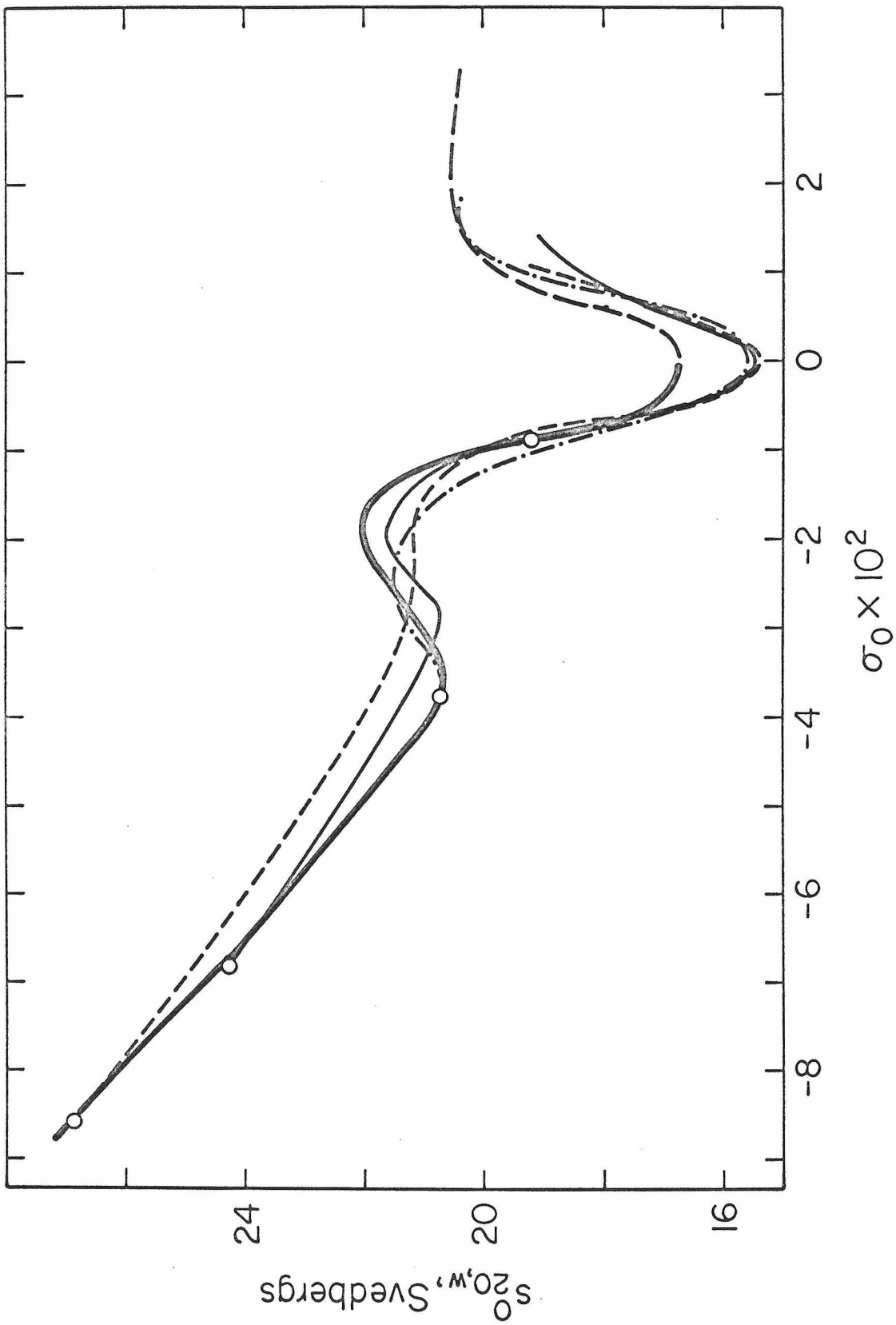
A single sedimentation velocity-dye titration potentially contains a large amount of information on the variation of s with σ. When the data from the titrations are converted to $s^{o}_{20,w}$ versus σ curves by the procedures described in the Results section, and superimposed on the s versus σ_0 curve of Figure 1, reasonable congruence of all the curves

is observed (Fig. 10). The local maxima and minima occur at approximately the same values of σ and the curves have approximately the same shapes. The correction for the buoyant effect of the bound dye does not completely raise the sedimentation coefficient to the value in the absence of bound dye. This is attributed to the effects of the bound dye on the length and stiffness of the DNA. Le Pecq & Paoletti (1967) have observed increases in the viscosity of linear DNA upon binding of ethidium bromide. Lerman (1961) has reported similar effects on the viscosity of DNA upon binding of acridines.

(c) Use of s versus σ curves to obtain changes in σ

From the work of Wang (1969a, b), it has become clear that the winding angle of DNA and, thus, the superhelix density of closed circular DNA are dependent on salt concentration, counterion, and temperature. A convenient method for measuring these effects on σ is to measure changes in the sedimentation coefficient and, from these changes, to determine the change in σ. Wang's curve of s versus σ contains a well-defined linear portion in which s or r may be used to measure changes in σ for his closed circular $\lambda b_2 b_5 c$ DNA, with the assumption that the shape of the s versus σ curve does not change under the various conditions of temperature, counterion, and ionic strength. He has used only the portion of the curve with $|\sigma_0| < 0.009$ for such determinations. To support the validity of using the s versus σ curve to obtain changes in σ from changes in r, Wang has measured changes in σ by performing dye titrations under different conditions. These dye titrations revealed the actual changes in σ, which agreed with those predicted by the

FIG. 10. Sedimentation velocity-dye titrations in the form of $s^{o}_{20,w}$ *versus* σ curves superimposed on the $s^{o}_{20,w}$ *versus* σ_o curve (——) of Fig. 1. The open circles indicate the starting points of the dye titrations of materials with initial superhelix densities of -0.009 (– – – –), -0.039 (– · – · – · –), -0.068 (— — —), and -0.085 (– – – –).


$s^0_{20,w}$, Svedbergs
24
20
16
$\sigma_0 \times 10^2$
2
0
-2
-4
-6
-8

s versus σ curve.

Two problems arise in attempts to assess changes in σ by this procedure. Very small differences in r are often obtained, and the error in r may be of the same order of magnitude as the differences. The shape of the s versus σ curve may change under the conditions considered here. Two indications of this latter problem are evident in this work. The s versus σ curves in CsCl and NaCl are not identical in shape. At low ionic strength in a region in which $|\sigma|$ is decreasing with decreasing ionic strength, the maximum value of r (1.44) for the DNA with a σ of -0.039 is considerably higher than the value (1.31) at the maximum in the s versus σ curve in 1.0 M-NaCl (Fig. 5). We do not, therefore, regard our s versus σ curves as adequate measures of the changes in σ under all conditions in our experiments. However, the curves are useful for determining the direction and approximate magnitudes of changes in σ.

(d) Effects of ionic strength, nature of the counterion, and temperature on the sedimentation coefficient of closed SV40 DNA's with different superhelix densities

The studies of Wang (1969a, b) had shown that the average rotation angle of DNA undergoes slight changes as a function of ionic strength, counterion, and temperature. A change in rotation angle θ necessarily changes the value of σ in accordance with the relation

$$\Delta\sigma = -0.028\,\Delta\theta \qquad (3)$$

where θ is measured in degrees. The negative sign indicates that an

unwinding of the duplex causes a decrease in the number of tertiary turns in a negative superhelix. We have confirmed Wang's conclusions regarding the sign and the approximate magnitude of the effects. Raising the temperature decreases θ and diminishes the absolute value of the superhelix density of negative superhelical molecules. Lowering the ionic strength and changing the counterion from Cs^+ to Na^+ have similar effects.

Our results make it clear, however, that the effects of the above variations of conditions on the sedimentation coefficient of a closed DNA depend on the superhelix density of DNA in a sensitive way. If the slope of the curve for s versus $-\sigma_0$ is negative, an environmental change that decreases θ and decreases $-\sigma_0$ slightly will cause s to become smaller. If the slope is positive, opposite changes in s occur. If the slope is close to zero, as in the regions of the two minima and the maxima, small changes in $-\sigma_0$ will have little effect on s.

The maximum in the relative sedimentation coefficient of naturally occurring closed circular DNA at 0.01 M ionic strength (Fig. 6) has been reported previously by Bode & MacHattie (1966) for intracellular closed λ DNA, and by Kiger, Young & Sinsheimer (1968) for ϕX174 replicating form DNA and intracellular closed λ DNA. We have also observed the same phenomenon with polyoma DNA. Previous explanations for the maximum suggested that more superhelical turns were present at low ionic strength as a result of a positive change in θ. However, upon elucidation of the s versus σ curve, it became clear that an increase in the sedimentation coefficient did not necessarily

imply an increase in the number of superhelical turns. The sedimentation coefficient of native SV40 DNA and of the above λ and φX174-RF DNA's should increase with either an increase or a decrease in σ. It is known from ionic strength studies in this work and from the work of Wang (1969a) that σ increases as ionic strength is lowered.

There are two separate problems associated with the sedimentation behavior at low ionic strength of closed circular DNA's with σ_0 near -0.04. The relative sedimentation coefficient increases more than can be predicted by the s versus σ curve in 1.0 M-NaCl, and the sedimentation coefficient decreases as ionic strength is lowered below 0.01 M. A portion of the increase in r is caused by an increase in σ. The remainder must be due to other effects on the conformation.

At ionic strengths below 0.02, electrostatic effects have been shown by Rosenberg & Studier (1969) to affect the sedimentation coefficient of T7 DNA. Intra- and inter-segmental electrostatic repulsions may initially affect the distribution of tertiary turns in molecules with σ_0 near -0.04 in such a way that the molecule becomes more compact than it can become in the absence of such forces.

The decrease in r below 0.01 M ionic strength may be a combination of partial early melting of the DNA duplex to remove superhelical turns and more extensive electrostatic repulsions causing a decrease in the sedimentation coefficient. An early melting effect has been observed in alkaline pH titrations of closed circular DNA's (Vinograd, Lebowitz & Watson, 1968). The early melting effect should be most significant for DNA's of high superhelix density which are correspondingly

higher energy forms.

The above interpretation of the sedimentation velocity behavior of native viral SV40 DNA at low ionic strength is consistent with the data for the high and low superhelix density DNA's. The value of r for the high superhelix density DNA decreases as the ionic strength is lowered, which is consistent with an increase in σ. The early melting would occur at a higher ionic strength for this DNA and could account for the rapid decrease in r below 0.02 M. An increase in σ would not significantly affect the value of r for the low superhelix density DNA, since it is near the principle minimum. Likewise, early melting would not occur in this DNA with substantially no superhelical turns.

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CHAPTER III

The Use of an Ethidium Analogue in the Dye-Buoyant Density Procedure for the Isolation of Closed Circular DNA: The Variation of the Superhelix Density of Mitochondrial DNA*

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*THE USE OF AN ETHIDIUM ANALOGUE IN THE DYE-BUOYANT DENSITY PROCEDURE FOR THE ISOLATION OF CLOSED CIRCULAR DNA: THE VARIATION OF THE SUPERHELIX DENSITY OF MITOCHONDRIAL DNA**

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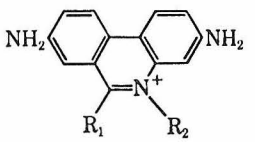
Abstract.—The separation between open and closed circular DNA in buoyant CsCl gradients containing intercalating dyes depends on the superhelix density of the closed form. These separations are about 1.8 times larger with propidium iodide than with ethidium bromide. The superhelix densities of mitochondrial DNA from HeLa cells and *Lytechinus pictus* eggs appear to be about two thirds that of mitochondrial DNA from rat and rabbit liver.

The dye-buoyant density method[1] has proved to be a reliable and efficient procedure for the detection and isolation of closed circular DNA. This communication reports the results of a study of variations in the method designed to increase the resolution of closed circular species from open species, while at the same time preserving other useful features of the method, particularly reproducibility and sensitivity for the detection of DNA by inspection or photography of fluorescent bands. The method previously described employed ethidium bromide (EB) I with $R_1 = C_6H_5$ and $R_2 = C_2H_5$ as the "dye" component. We have found that an ethidium analogue with $R_2 = C_3H_6N(C_2H_5)_2CH_3$, which we refer to as propidium iodide (PI), enhances the resolution between closed and open DNA by a factor of approximately 1.8 relative to ethidium bromide. The increased resolution appears to be independent of the molecular weight and only slightly dependent on the superhelix density of the DNA. The superhelix density σ_0 is defined as the number of superhelical turns per ten base pairs under the assumption that the angle of the duplex is the same in the closed and open forms.[2]



I

The magnitude of the separation between the closed and open (nicked or linear) DNA should be sensitive to the superhelix density of the DNA.[3] This has been found to be the case. We have concluded from the results obtained in this study that there are differences in superhelix densities between mitochondrial DNA's from various sources. The superhelix densities of mitochondrial DNA's from HeLa cells and sea urchin eggs appear to be about two thirds as large as those from rat and rabbit liver.

Materials and Methods.—Analogues of ethidium bromide: The analogues of 3,8-diaminophenanthridine used in this study were kindly provided by Dr. T. I. Watkins of Boots Pure Drug Co., Ltd. We have successfully repeated the straightforward synthesis of 3,8-diamino-5-diethylaminopropyl-6-phenylphenanthridinium iodide[4] from 3,8-dinitro-6-phenylphenanthridine. Propidium was used as the diiodide salt of the N′ methyl derivative of the above quaternary compound.

Photography and measurement: After centrifugation, the dye-CsCl gradients were illuminated with ultraviolet light as described previously[1] and photographed through a Wratten 16 filter either with a Polaroid camera and type-46L film, *ca.* 10 sec at f11, $5^3/_4$ in., or with a single-lens reflex camera and Ektachrome film, *ca.* 1 sec at f1.4, 6 in. The resulting transparencies were measured in a Nikon 6 projection comparator.[5] A separation on the film could be reproducibly measured to ±0.005 cm. The magnification factor for the fixed photographic arrangement was determined by photography of a rule and by measurement of the centrifuge tube width.

Fluorescence measurements: Fluorescence intensities were measured on a Farrand fluorimeter. The uncorrected values for different dyes were compared.

Preparation of DNA's: Viral SV40[2] and the mitochondrial DNA's[6, 7] were prepared as described elsewhere.

Polynucleotide ligase:[8–10] The enzyme was prepared from *Escherichia coli* K12 strain 1100 by a modification of the method of Gefter, Becker, and Hurwitz,[10] assayed by the adenylate binding method of Hurwitz[11] and Little *et al.*,[12] using the units of Hurwitz.[11]

Closure of SV40 DNA by ligase: Purified SV40 I DNA (54 μg/ml) was converted to SV40 II DNA with DNase I (bovine pancreas, Sigma Chemical Co.) at a concentration of 1.63×10^{-5} μg/ml in 0.015 *M* NaCl, 0.012 *M* $MgCl_2$, 0.01 *M* tris(hydroxymethyl)-aminomethane (Tris), 0.007% BSA, 0.001 *M* ethylenediaminetetraacetate (EDTA), pH 8, for 30 min at 30°. SV40 II DNA was purified by the dye-CsCl buoyant method. SV40 II DNA was closed with polynucleotide ligase in essentially the medium described by Olivera and Lehman[8] for 20 min at 30°C. SV40 L1 DNA was prepared with SV40 II DNA at 24 μg/ml with 2 units/ml of ligase. SV40 L2 DNA was prepared with SV40 II DNA at 26 μg/ml and 5 units/ml of ligase with 6.8 μg/ml of ethidium bromide added.[14] SV40 L1 DNA was purified by the dye-CsCl buoyant method and SV40 L2 DNA by sedimentation through a sucrose gradient.

Results and Discussion.—The buoyant density of a DNA species at equilibrium in a cesium chloride gradient containing a gradient of a reacting solute is approximately equal to the mass of the buoyant complex divided by its volume.[2]

$$\theta = \frac{1 + \Gamma' + \nu'}{\bar{v}_3 + \Gamma'\bar{v}_1 + \nu'\bar{v}_4}. \tag{1}$$

In equation (1), Γ' is the preferential hydration of the Cs DNA in grams water per gram Cs DNA, ν' is the mass of dye bound per gram Cs DNA, and the $\bar{v}$'s are the partial specific volume of water (1), Cs DNA (3), and the dye (4). To an approximation of about 2 per cent for the case where the partial specific volume of the dye is about equal to that of water, $\Delta\theta$, the difference in buoyant density between closed (I) and open (II) DNA is given by

$$\Delta\theta = \frac{\Delta\Gamma' (\bar{v}_3 - \bar{v}_1) + \Delta\nu' (\bar{v}_3 - \bar{v}_4)}{(\bar{v}_3 + \bar{\Gamma}'\bar{v}_1 + \bar{\nu}'\bar{v}_4)^2}, \tag{2}$$

where $\Delta\Gamma' = \Gamma_I' - \Gamma_{II}'$, and $\bar{\Gamma}' = (\Gamma_I' + \Gamma_{II}')/2$, $\bar{\nu}' = (\nu_I' + \nu_{II}')/2$, and $\Delta\nu' = \nu'_I - \nu'_{II}$. The quantity $\Delta\nu'$ is not zero at high levels of an intercalating dye because of the *restricted binding*[2] of the dye to closed circular DNA. It is this effect that gives rise to the buoyant density difference between the closed and open forms of a circular DNA.

Analogues of ethidium: A variety of analogues of ethidium were tested for an increase in $\Delta\theta$. Table 1 presents the results obtained with SV40 DNA. The individual separations have been normalized with the value for ethidium bromide (EB) measured in the same experiment in order to cancel out small variations due to time, temperature, speed, and radial distance. The ratio $\Delta r_X/\Delta r_E$ is designated χ.

TABLE 1. *Effects of substituents in the diaminophenanthridinium ring system (I) on the buoyant separation between closed and open viral SV40 DNA in dye-CsCl density gradients.*

R_1	R_2	$\chi = (\Delta r_X/\Delta r_E)$
C_2H_5	C_2H_5	0.70
ϕ	CH_3	0.92
ϕ	C_2H_5	1.00
ϕ	C_3H_7	1.01
$pNH_2\phi$	C_2H_5	1.00
ϕ	$C_3H_6N(C_2H_5)_2CH_3$	1.80

The dye concentrations were 300–500 μg/ml, and the initial density was 1.55–1.58 gm/ml. The samples were centrifuged in an SW50 rotor at 43 krpm for 48 hr at 20°.

Choice of experimental conditions: The properties of propidium iodide (PI) ($R_1 = \phi$, $R_2 = C_3H_6N(C_2H_5)_2CH_3$) relative to EB were studied in more detail. The separation between closed and open SV40 DNA in an ethidium chloride–cesium chloride density gradient is approximately constant at high dye levels. The separation becomes smaller, however, if the free dye concentration in the region of the bands falls below about 75 μg/ml. This can occur in preparative ultracentrifuges because of the large redistribution of the dye in the gradient. We have found that the originally described conditions,[1] $\rho = 1.55$ gm/ml and 100 μg/ml EB, are barely adequate to maintain the needed free dye concentration at the bands. Raising the initial EB concentration from 200 to 500 μg/ml has no observable effect on the separation of SV40 DNA components. In the case of PI, it was found that the separation increased upon raising the concentration from 100 to 300 μg/ml, but then remained constant to 500 μg/ml. At these high dye levels, however, the sensitivity for detection by fluorescence is somewhat reduced because of the fluorescence of free dye.

The optimum choice of an initial dye concentration also depends on the initial density. Low dye concentrations can be used if the density is sufficiently high that the bands form near the top of the cell where the dye is concentrated. The final choice was 300–500 μg/ml of dye, either EB or PI, and density of 1.58 gm/ml. Centrifugation for 24 hours at 43 krpm in a 3.0-ml volume in an SW50 rotor is sufficient for preparative purposes, although 48 hours was routinely used in this study. The details of the centrifugation are unimportant and a wide variety of rotors (including angle rotors) has been used in this laboratory. A tube containing a reference DNA in the open and closed form was always included in the same rotor for purposes of quantitative comparison.

The effect of molecular weight and superhelix density on the separation enhancement of PI over EB: The increased separation of SV40 DNA's in PI as compared with EB is shown in Figure 1*a*, *b*. The same result (Table 2) was obtained in a similar experiment with HeLa cell mitochondrial DNA (M-DNA), which has a molecular weight approximately three times higher than SV40 DNA. The ratio of separations with PI over EB, χ, is approximately 1.8 in each case. This is the expected result, since all of the properties that determine the buoyant density of a DNA-dye complex are intensive, and there should be no effect of molecular weight.

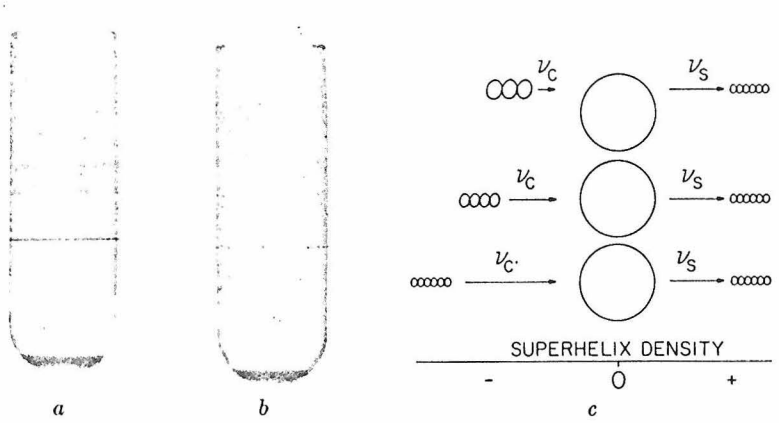


FIG. 1.—Fluorescent bands of open (*upper*) and closed (*lower*) SV40 DNA in a buoyant CsCl density gradient containing (*a*) ethidium bromide and (*b*) propidium iodide. The initial densities were 1.58 gm/ml. The tubes were centrifuged in the same rotor under standard conditions (Table 2). (*c*) Diagrammatic representation of the binding of an intercalating dye to three closed circular DNA's with differing initial superhelix densities. The dye-free molecule is shown at the left, the relaxed molecule in the center, and a highly twisted one at the right.

We have prepared two artificial forms of closed SV40 DNA to test the effect of the superhelix density on χ. A preparation of SV40 I DNA was treated with pancreatic DNase at very low levels to produce some nicked SV40 II with 3′-hydroxyl groups at the hydrolyzed bond. Species II was then purified and closed with the enzyme polynucleotide ligase.[8–10] The first product, SV40 L1 DNA, was obtained by enzymatic closure in moderately low salt. The second product, SV40 L2 DNA, was obtained similarly except for the presence of ethidium bromide during the action of the ligase.

SV40 L1 DNA has a low superhelix density. Viral λ DNA closed under the incubation conditions and used to prepare SV40 L1 DNA has been calculated to

TABLE 2. *The buoyant separation Δr between the closed and open forms of four DNA's in cesium chloride containing ethidium iodide (EI) and propidium bromide (PB).*

DNA	Rotor	Δr_{PI} (cm)	Δr_{EB} (cm)	$\chi = \Delta r_{PI}/\Delta r_{EB}$	$\bar{\chi}$
SV40 L1	SW50.1†	0.822	0.429	1.91	1.91
	SW50.1*	0.895	0.470	1.90	
SV40 I	SW50	0.532	0.300	1.77	1.80
	SW41	0.570	0.310	1.84	
	SW50.1†	0.619	0.345	1.79	
	SW50.1*	0.669	0.370	1.81	
SV40 L2	SW50.1†	0.347	0.201	1.72	1.72
	SW50.1*	0.389	0.228	1.71	
HeLa M-DNA	SW50	0.608	0.345	1.76	1.76

The dye concentration for all experiments was 330 μg/ml and the initial density was 1.58 gm/cm.[3] Three-ml volumes were used in all experiments except in the SW41 rotor, where 6 ml were used. All runs were 20°C for 48 hr except for the SW41, which was run for 72 hr. The nominal rotor speed for the three rotors used was 43 krpm for the SW50, 35 krpm for the SW41, and 40 krpm for the SW50.1. The measured speed in the experiment marked with an asterisk was 39 krpm as compared with 40.5 krpm in the experiment marked with a dagger. This difference accounts for the variation of Δr in the two experiments.

have about 15 per cent of the superhelix density of SV40 I.[13] SV40 L1 DNA should have a similarly low superhelix density. SV40 L2 DNA, on the other hand, has a high superhelix density because it was closed in an underwound condition. The extent of this underwinding, and the final superhelix density, depends on the amount of dye bound to the DNA at the instant of closure.[14] Rough calculations demonstrate that the superhelix density of SV40 L2 DNA should be substantially higher than that of SV40 I DNA.

The separations of these forms of SV40 DNA have been determined in EB and PI in order to see if there was any effect of the initial superhelix density on χ (Table 2). There seems to be no large effect, although a trend may be indicated.

Fluorescence: EB and PI were compared for fluorescence enhancement[15] at 365 mμ and found to be the same. This implies that the detection sensitivity of PI and EB should also be equal. However, in practice it seems that PI is somewhat less sensitive than EI for detecting small amounts of DNA.

Removal of dye: Several methods are available for removal of EB and PI. Removal is greatly facilitated by high salt because of the reduced binding affinity of DNA for dye. Dialysis against low ionic strength medium is inefficient. The dye is easily removed from the DNA in the 4.5 *M* CsCl by passage through a Dowex 50 column,[1] or by extraction with isopropanol[16] or isoamyl alcohol followed by ether extraction. The extraction methods have the advantage that they are quick and can be used on small samples with little dilution.

Variation of the buoyant separation with superhelix density: Consider the three forms of SV40 DNA which have been made as described above. SV40 L1 has a low superhelix density, SV40 I has an intermediate superhelix density, and SV40 L2 has a high superhelix density. The binding of an intercalative dye to these molecules is shown schematically (Fig. 1*c*), in which the horizontal coordinate represents the superhelix density. The figures at the left represent the molecules before addition of dye. A certain amount of dye, ν_c, binds to the molecule and converts it to the open conformation. This amount depends on the initial superhelix density, since $\nu_c = (\pi/10\phi)\sigma_0$,[2, 17] where ϕ is the unwinding angle of the base pairs upon binding one molecule of the intercalating dye. The relaxed closed circular molecules bind further dye, ν_s, and are eventually "saturated" in that the free energy of the dye binding is effectively counterbalanced by the free energy of superhelix formation.[2] If ν_s is a constant with respect to σ_0, the amount of dye bound at saturation, $\nu = \nu_c + \nu_s$, should depend on σ_0, and closed circular molecules with high (negative) superhelix densities would exhibit small separations in a dye–buoyant density system. A high initial superhelix density results in increased binding at saturation and a decreased buoyant density. There is, however, a limit for the sum $\nu_c + \nu_s$ which cannot exceed $\nu_{II,\,max}$, the maximum number of binding sites, expressed in moles dye per phosphate, on the nicked circular form. As the limit is approached for molecules with high superhelix density, the assumption that ν_s is independent of σ_0 may no longer be valid.

The effect of variations of the superhelix density is demonstrated by the results obtained with closed SV40 DNA's. Figure 2*a*, *b* presents the experiment in which the three closed forms and the open form were banded in dye–cesium chloride gradients containing either EB or PI. The experiments have also been con-

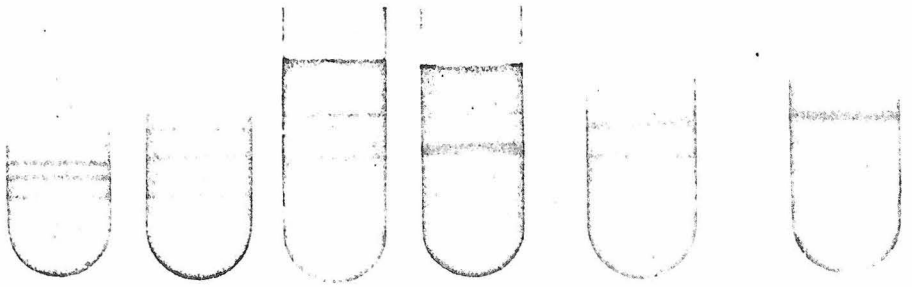


FIG. 2.—The effect of superhelix density on the buoyant density in dye-CsCl gradients. Fluorescent bands of SV40 DNA in buoyant CsCl containing (*a*) ethidium bromide and (*b*) propidium iodide. In order of increasing densities, the bands are SV40 II DNA, SV40 L2 DNA, SV40 I DNA, and SV40 L1 DNA. A comparison of the buoyant separations of (*c*) HeLa cell M-DNA and (*d*) SV40 I DNA in propidium iodide–CsCl density gradients. A comparison of the buoyant separations of (*e*) HeLa cell M-DNA and (*f*) chicken liver M-DNA in ethidium bromide–CsCl density gradients.

ducted with each of the DNA's separately to be sure of the assignment of the bands. The result is that SV40 L2 DNA, the high superhelix density DNA, shows a very small buoyant separation from the open form. The native DNA, SV40 I DNA, shows an intermediate separation and SV40 L1 DNA, the low superhelix density DNA, shows a large buoyant separation. The trend of these data is in the direction expected; Δr varies inversely with σ_0. The sensitivity of the separation to a given change in the superhelix density is about 1.8 times as large for PI as for EB.

The buoyant separation between SV40 I and II in an EB-CsCl gradient was measured six times under standard conditions (Table 3) and found to be 0.297 $\pm$ 0.004 cm (SE). The separation is thus very reproducible. In three experiments with HeLa M-DNA in EB, it was found that the separation 0.340 $\pm$ 0.005 cm was slightly but significantly larger than the SV40 separation. This was also the case in PI, where there was no doubt about the larger separation for HeLa cell M-DNA, 0.608 cm, as compared with 0.545 cm for SV40 DNA (Fig. 2*c*, *d*). An experiment with a mixture of SV40 I, SV40 II, HeLa M I, and HeLa M II DNA's in EB showed a slight splitting of the bottom bands, but not of the top bands. The obvious interpretation of these results is that HeLa cell M-DNA has a lower superhelix density than SV40 I DNA.

The separation between closed and open DNA's in a dye-CsCl density gradient should also depend on the buoyant density of the DNA in the absence of dye, and hence on the G + C content of the DNA. This effect has been estimated to result in about a $^1/_2$ per cent increase in the closed to open separation of DNA per 1 per cent increase in G + C content[3] for the case in which the binding constant is independent of G + C and $\bar{v}_4 \approx 1$.

We have measured the buoyant separations of rat and rabbit liver M-DNA in EC and have found them to be about the same as or slightly less than SV40 DNA (Table 3). The separation for chicken liver M-DNA is smaller than that for HeLa M-DNA (Fig. 2*e*, *f*). The separation measured for sea urchin (*L. pictus*)

M-DNA is considerably larger than for rat and rabbit liver M-DNA's, but less than for HeLa M-DNA (Table 3). This implies that the superhelix densities of HeLa cell and sea urchin M-DNA are lower than that of the liver M-DNA's. The results are in agreement with those of Smit and Ruttenberg[18] in that there appears to be little difference in superhelix density between SV40 DNA and rat liver DNA. Furthermore, these results confirm the expectation that if two DNA's have equal superhelix densities, they will have an equal separation in these systems.

TABLE 3. *The buoyant separation between the open and closed forms of six DNA's relative to the comparable separation for SV40 viral DNA measured in each experiment.*

		$\Omega = \Delta r/\Delta r_{SV40\ I}$	
Expt.	Material	Ethidium bromide[1]	Propidium iodide
1.	SV40 L1 DNA	1.26 ± 0.01 (2)	1.33 ± 0.01 (2)
2.	HeLa M-DNA	1.12 ± 0.005 (2)	1.12 (1)
3.	*L. pictus* M-DNA		1.07 (1)
4.	SV40 I	1.00	1.00
5.	Rabbit liver M-DNA	0.97 (1)	
6.	Rat liver M-DNA	0.96 (1)	
7.	SV40 L2 DNA	0.60 ± 0.02 (2)	0.57 ± 0.01 (2)

Typical values of Δr_{SV40} are given in Table 1. The average value of Δr_{SV40} was 0.297 ± 0.004 (SE) in six experiments in an SW50 rotor at 43 krpm. The number of determinations of each ratio is given in parentheses. For details of composition and centrifugation see text.

The superhelix density of the *in vitro* closed SV40 L1 DNA should be about the same as that measured for *in vitro* closed λ DNA made under the same conditions. The latter value is 15 per cent of the value of viral SV40 DNA. The superhelix density of HeLa cell M-DNA can be estimated to be 61 per cent or 69 per cent of that of SV40 viral DNA if a linear interpolation is made with the data for EB or PI, respectively. A similar estimate with the data for PI gives a value of 82 per cent for sea urchin egg DNA. These numbers indicate the magnitude of the variations in superhelix densities and should not be considered as accurate determinations. The linearity of superhelix density with separation has not yet been established, nor has the effect of base composition been examined. It has been shown in this laboratory that the minimum in an EB-sedimentation velocity titration of HeLa cell M-DNA occurs at a much lower free-dye concentration than in a titration with SV40 I DNA,[19] confirming the above interpretation.

All closed circular DNA molecules isolated to date contain superhelical turns. Chicken[20] and rat[18] liver mitochondrial DNA's, papilloma viral DNA's,[21,22] SV40 viral DNA,[2] and polyoma DNA[5, 17] have superhelix densities which are approximately equal, although there are small but definite differences between SV40 and polyoma DNA's.[19] The superhelix densities of HeLa cell and sea urchin egg mitochondrial DNA's are considerably lower. The biological problem of the origin of superhelical turns[23] has not been solved. Wang[13, 24] has shown that about 15 per cent of the superhelix density of SV40 DNA can be accounted for by changes in the average angle between base pairs due to changes in ionic strength, and that temperature has a small effect on superhelix density. Since the internal physical-chemical environment of cells is unknown, the relative con-

tribution of the former effect cannot be assessed. Any proposed mechanism for the origin of superhelical turns must include the potential for a variability.

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PROPOSITION I

Experiments combining the effects of denaturation and ethidium bromide binding on closed circular DNA's are proposed to determine both the direction of superhelical turns in naturally occurring closed circular DNA's and the direction of the angle of unwinding of ethidium bromide intercalation.

The intercalating dye, ethidium bromide, has been used extensively in the study of the number of superhelical turns in closed circular DNA (1). At a critical dye concentration, all superhelical turns are removed and the properties of the DNA are identical with those of nicked circular DNA at the same dye concentration. As more dye is bound, superhelical turns of the opposite sign are introduced. The binding of ethidium bromide has been estimated from model building studies of Fuller and Waring (2) to cause an unwinding of the Watson-Crick double helix decreasing the angle between base pairs by 12°. Based on this assumption, native closed circular DNA must be closed while in an underwound or partially denatured state and thus has negative superhelical turns to compensate for the extra positive turns formed after closure. These extra positive Watson-Crick helical turns arise either from the formation of hydrogen-bonds and Watson-Crick helical turns in a previously denatured region or by an increase in the angle between base pairs under the conditions of observation compared to those of closure.

The superhelix density of polyoma DNA has been determined by both a sedimentation velocity-dye titration (3) and by an alkaline buoyant density titration (4) which is independent of bound dye. This second method assumes that the early fractional shift in the buoyant density of the closed circular DNA at a lower pH than the density shift corresponding to the denaturation of the nicked circular form is the result of the denaturation (and coincident unwinding) of sufficient base pairs to

remove the superhelical turns. This is expected to occur before the strand separation for nicked circular DNA due to the positive free energy of superhelix formation (5). The data obtained by the alkaline titration gave the same number of superhelical turns as was obtained by the EB titration, supporting the assumed unwinding angle and direction. However, neither the model building studies nor the agreement between the dye and alkali titrations give conclusive proof of the direction of unwinding of EB intercalation.

Alternatively the buoyant density titration may be interpreted as the denaturation of sufficient base pairs to allow the strands to wind more tightly about themselves and release positive superhelical turns. This could occur if the free energy required to wind the denatured region more tightly were less than the free energy released by the removal of superhelical turns. This possibility, although energetically less favorable than the mechanism for releasing negative superhelical turns, cannot be discarded for that reason.

This alternative explanation of the alkaline titration requires that the intercalation of EB increase the angle between base pairs and unwind positive superhelical turns. The DNA must then be in an overwound state when closed.

To further clarify the direction of unwinding and the direction of superhelical turns in naturally occurring closed circular DNA's, experiments combining denaturation and EB binding are proposed. A key assumption in these proposals is that there is a positive free energy change in going from an open denatured loop region to a denatured

region which maintains or increases the number of turns previously present in the Watson-Crick helical structure. The twisted denatured region is thus energetically less stable than a region where loops may form.

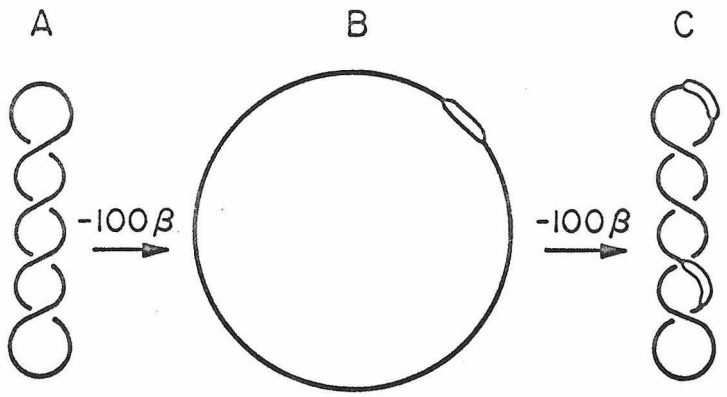
Follett and Crawford (6) have observed the heat denaturation of human papilloma viral DNA (a closed circular DNA of molecular weight 5×10^6 daltons) in 12% formaldehyde in the electron microscope. They observed a decrease in the number of superhelical turns to zero followed by an increase as temperature was increased. Alkaline sedimentation studies on polyoma (4) and papilloma (7) DNA's confirm this behavior. The sedimentation coefficient of the closed circular DNA initially decreases to the value for the nicked circular form and then increases to a value approximately 2.5 times its initial value.

Figure 1 shows the proposed effects of denaturation on closed circular DNA's with both positive and negative superhelical turns. Case 1 shows the previously proposed conformations (4, 7) for a closed circular DNA with negative superhelical turns as it undergoes denaturation. Case 2 shows the corresponding forms for a closed circular DNA with positive superhelical turns if it is to undergo the conformational changes seen in the electron microscope and in sedimentation studies.

In this discussion, I use a modified form of Bauer and Vinograd's (8) equation relating the numbers of various types of turns in the molecule

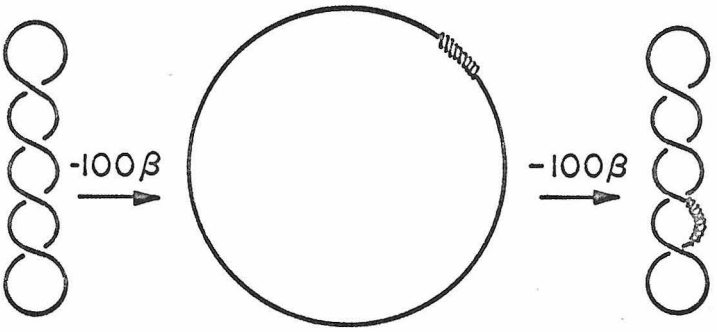
$$\alpha = \tau + \beta + \delta \qquad (1)$$

Case 1
α = 900



A	B	C
β = 1000	β = 900	β = 800
τ = -100	τ = 0	τ = +100
δ = 0	δ = 0	δ = 0

Case 2
α = 1100



β = 1000	β = 900	β = 800	~~800~~
τ = +100	τ = 0	τ = +100	-100
δ = 0	δ = +200	δ = 200	+400

where α is the winding number or topological linking number which is invariant for any particular closed circular DNA, β is the number of Watson-Crick turns in the DNA, τ is the number of superhelical turns and δ is the number of turns in denatured regions. β for the undenatured molecule is arbitrarily assigned the value 1000 for both cases. In case 1, τ for the undenatured molecule is assigned a value of -100 superhelical turns and in case 2, +100. Values of α for these two cases are thus 900 and 1100, respectively, In case 1, upon the denaturation of 100 Watson-Crick turns ($\Delta\beta$ = -100), form B is obtained with no superhelical turns and a single stranded loop region of length equivalent to 100 Watson-Crick turns. Upon further denaturation of an additional 100 Watson-Crick turns, τ increases to +100 (form C) to maintain the equality of equation (1).

For case 2, upon the denaturation of 100 Watson-Crick helical turns (form B), τ goes to zero and δ to 200 to compensate for the loss of superhelical turns. As further denaturation to form C occurs, positive superhelical turns would be reformed. The assignment of positive superhelical turns to this form is based on the following argument. Follett and Crawford (6) have demonstrated that superhelical turns are formed as further denaturation occurs beyond form B. The direction of the turns may be considered to be either positive or negative. If 100 positive turns are formed δ will be equal to 200. If 100 negative turns are formed the corresponding value for δ is 400. The free energy of formation of positive and negative superhelical turns has been shown by Bauer and Vinograd (5) to be approximately equal.

The formation of negative superhelical turns requires more δ turns in the same sized denatured region than the formation of positive superhelical turns required involving additional free energy. Thus the superhelical turns in form C are assumed to be positive. The exact assignment of the distribution of turns between τ and δ in case 2 and the assignment of a value of zero to δ for case 1 have been made for purposes of discussion and does not imply that there would be no variation from these assumed numbers.

The addition of ethidium bromide to form C would give different results for these two cases. In case 1, the addition of EB to form A removes negative superhelical turns and is equivalent to a positive change in τ. The addition of EB to form C in case 1 would thus increase the number of positive superhelical turns and cause the sedimentation coefficient to increase. In case 2, the addition of EB to form A is equivalent to a negative change in τ. When added to form C, it would decrease the number of positive superhelical turns. As increasing amounts of EB are added the sedimentation coefficient would decrease until a "relaxed" form is reached and then increase as negative superhelical turns are added. Thus the difference in the sedimentation behavior of the two forms would provide a means of determining the direction of turns initially present, as well as the direction of unwinding of EB.

Partially denatured forms 1-C or 2-C may be formed by a combination of a variety of denaturation conditions including low ionic strength (9, 10), elevated temperatures, presence of formamide (11),

formaldehyde (12) or DMSO (13), and high concentrations of certain anions such as Cl_3CCOO^- and ClO_4^- (14).

It is proposed that conditions be chosen such that the effect of addition of EB to the DNA in the appropriate conformation may be observed by analytical centrifugation. Extremes of pH, high temperatures, and the presence of formaldehyde are undesirable for a number of reasons. A set of conditions which could be used is 36% formamide in the presence of 0.01 M salt which would give a melting temperature (T_m) for a DNA with a G-C content of 42% (that of SV40 DNA) of 40°C. This value was calculated from the dependence of T_m on ionic strength (9) and formamide concentration (11). Since the phenomena of interest occurs in the early stages of denaturation this would probably give the desired effect as a temperature of 30-40°C. By varying conditions of temperature and formamide concentration, it should be possible to locate appropriate conditions to study the effects of adding increasing amounts of EB.

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PROPOSITION II

Experiments to determine the effect of partial denaturation on the relative sedimentation coefficient of native closed circular DNA at low ionic strength are proposed to determine if any portion of the sedimentation behavior observed with native SV40 closed circular DNA at low ionic strength at 20°C is due to denaturation.

As ionic strength is lowered, the relative sedimentation coefficient of native closed circular DNA (sedimentation coefficient of closed circular DNA divided by sedimentation coefficient of nicked circular DNA measured under the same conditions) has been shown to pass through a maximum at an ionic strength of approximately 0.01 M (1, 2, 3). The hydrodynamic conformational changes causing this maximum are not well understood. A portion of the increase in the relative sedimentation coefficient as ionic strength is decreased from 1.0 M to 0.01 M may be accounted for by a decrease in the negative superhelix density with decreasing ionic strength (4). A decrease in negative superhelix density gives an increase in sedimentation coefficient for DNA's with superhelix densities in the range -0.039 to -0.025 as a result of the local minimum in this region of the sedimentation coefficient versus superhelix density curve (5). The remainder of the increase may be due to electrostatic effects causing a compacting of molecules with this superhelix density at low ionic strengths. As ionic strength is lowered below 0.01 molar, the relative sedimentation coefficient begins to decrease and falls to a value of 1.2 at an ionic strength of 10^{-3}. The relative sedimentation coefficient of a DNA with much larger negative superhelix density ($-\sigma_0 = 0.086$) also decreases rapidly as ionic strength is lowered in this range (3). This rapid decrease in the relative sedimentation coefficient may be due to partial early denaturation which removes the strain of superhelical turns.

Studies by Dove and Davidson (6), and Colvill and Jordan (7) have shown that denaturation of DNA begins at temperatures between

20° and 40°C at ionic strengths of 10^{-3} and lower. At these ionic strengths the transition breadth of melting is significantly widened compared to that at high ionic strength. The temperature of the beginning of denaturation is thus lowered more than the lowering of the actual melting temperature. Since closed circular polyoma DNA undergoes early melting in alkali (8), it is reasonable to expect that partial denaturation of closed circular DNA's may occur at 20°C at ionic strengths of 10^{-3} M.

It is proposed that the contribution of partial denaturation to the sedimentation behavior at this ionic strength be checked by measuring the relative sedimentation coefficient as a function of temperature. These measurements should be made on DNA's with different superhelix densities at various ionic strengths in the region of the maximum of the relative sedimentation coefficient of native closed circular DNA's. Appropriate ionic strengths for such measurements would be 0.01, 0.003, 0.001 and 0.0003. If the phenomenon is related to denaturation, variations in temperature should show significant variations in the relative sedimentation coefficient of DNA's with high values of $-\sigma_0$. As denaturation occurs, the sedimentation coefficient should show a change similar to that seen in sedimentation velocity-dye titrations (4).

The Watson-Crick winding angle of DNA and the superhelix density are dependent on temperature at high ionic strengths (4, 9). However, this dependence is weak and would be expected to give smaller changes in the sedimentation coefficient than would be observed with a melting phenomenon. The sedimentation behavior of a closed

circular DNA with a superhelix density near zero would distinguish between denaturation and changes in σ with temperature as it would not be sensitive to early melting but would be sensitive to changes in σ.

An alternative procedure to varying temperature would be to sediment in the presence of formamide which lowers the melting temperature by 0.7°C per percent formamide (10). Any possible effect of formamide on the superhelix density could be checked by testing the effect of low concentrations of formamide on the relative sedimentation coefficient in high salt of a DNA with a value of σ_0 approximate -0.01 whose sedimentation coefficient is sensitive to changes in σ.

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PROPOSITION III

Experiments are proposed to determine the fate of a naturally occurring cross-link in vaccinia viral DNA during DNA replication.

Vaccinia viral DNA has been shown by Berns and Silverman (1) to be a naturally cross-linked double stranded linear DNA with a molecular weight of approximately 1.5×10^8 daltons. More than 90% of the DNA molecules appear to be crosslinked. When the DNA is broken by shear into five equal sized fragments only 20% of the fragments show cross-linking when sedimented through alkaline sucrose. When the DNA is sonicated into fragments of molecular weight 10^6 daltons, there is no detectable renatured material when neutralized alkaline denatured molecules are banded in CsCl. Berns and Silverman (1) estimate their limits of detection at 5 out of 150 fragments. Thus there is probably only one cross-link per molecule or a number of cross-links in a given localized region. They also report that the cross-links are resistant to deoxycholate, pronase, phenol, ether and high pH.

From a comparison of the relative sedimentation rates of T4 and vaccinia DNA's in alkaline sucrose gradients, it appears that the cross-link is internal rather than at the end of the molecule, as the DNA sediments faster than would be expected for a linear single stranded DNA of twice the molecular weight of vaccinia single strands. Internally cross-linked molecules are more compact when denatured and would be expected to sediment more rapidly (2).

The existence of a cross-link creates problems for the process of DNA replication. During semiconservative replication, the cross-links must be broken and reformed at some later stage.

Experiments are proposed to determine the fate of the cross-links

in replicating molecules. Vaccinia DNA replication offers a system which is well suited for replication studies. Although the buoyant density of vaccinia DNA does not differ significantly from that of host cell (HeLa) DNA, vaccinia DNA replicates in the cytoplasm and may be easily separated from nuclear DNA. Nuclei may be removed from a cell homogenate by low speed centrifugation, leaving vaccinia DNA in the supernatant. Furthermore, it is possible to get large numbers of viral particles (100-200/cell) absorbed by the cells (3). 5000 to 10,000 virus particles are produced per cell and three to four times that amount of intracellular viral DNA may be recovered (4).

Dahl and Kates (5) have developed an isopycnic sucrose density gradient technique to partially purify intracellular structures containing vaccinia DNA associated with protein. They obtain four distinct components, two of which contain parental DNA and two containing primarily progeny DNA. The complexes are reasonably stable and retain the ability to synthesize viral messenger RNA in vitro with an endogenous RNA polymerase (6). From a knowledge of the density, type of DNA (parental or progeny), DNAse sensitivity and type and amount of RNA synthesized, they have assigned probable structures and functions to the four components. Parental DNA is recovered in two light bands at density 1.157 and 1.175 gm/ml. The upper band is insensitive to DNAse and is considered to be viral cores bound to membrane. The band at density 1.175 is 30-50% sensitive to DNAse and is considered to be cores and uncoated DNA which are membrane bound. At two hours post-infection, two bands of progeny DNA are

seen, one at a density of 1.18 and the other on an 80% sucrose cushion (density 1.285 gm/ml.). At three hours post-infection, most of the progeny DNA is in the dense band, which also contains the structure on which most of the transcription of late viral genes occurs. Pulse labeled DNA at 1.75 hours post-infection appears in the dense band, but previously labeled progeny DNA occurs in the band at density 1.180 gm/ml.

Experiments using differential labeling techniques are proposed to follow the status of the cross-link in parental and progeny DNA in these various structures. Virus labeled with 5-Bromuracil and H^3 thymidine is to be prepared by the technique of Joklik (7). HeLa cells are to be infected with this virus and grown in the presence of P^{32}. The intracellular DNA-protein structures are to be isolated as a function of time by the procedure of Dahl and Kates (5). The DNA may then be freed from the protein by treatment with sodium dodecyl sulphate. Neutral sucrose sedimentation with a known marker DNA would be done to obtain the molecular weight distribution of the double stranded DNA's. Alkaline sucrose velocities would give an indication of the number of single stranded breaks in the DNA and the molecular weight distribution of the single stranded DNA. Banding reneutralized alkali denatured DNA in CsCl would then give the proportion of the single stranded fragments which are crosslinked and band as double stranded DNA. Further alkaline and neutral velocity studies of the isolated cross-linked and noncross-linked fractions would provide further information about the molecular weight distributions of these two populations.

The parental DNA may be followed by both the tritium marker and the BU density label. The density label may be used to determine if semiconservative replication of the parental DNA occurs and if the cross-link in parental DNA is broken and reformed in heavy-light progeny DNA. Progeny DNA can be followed by the P^{32} marker.

Initially, samples should be made at one hour intervals for five hours after infection. Shorter time intervals should be considered on the basis of the data obtained with this sampling. Pulse labeling at various time points after infection may also prove to be useful.

Experiments to further define the number of cross-link locations and to determine if the cross-links occur at random or at regular positions may be done by electron microscopy of molecules which have been denatured and spread in formamide. Formamide both denatures DNA and prevents random base-base interactions from occurring (8). This makes it possible to measure the lengths of denatured DNA strands.

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PROPOSITION IV

Experiments are proposed to determine the locations of DNA and DNA polymerase within mitochondria by the stepwise disassembly of mitochondria and subsequent examination of fractions for DNA content and DNA polymerase activity. This would provide further information about the function and replication of mitochondrial DNA and mitochondria.

The existence of a distinct DNA component in mitochondria has been well-known for several years (1). The incorporation of labeled nucleotide triphosphates into isolated mitochondria has been studied (2) and mitochondrial DNA polymerases have been isolated from yeast (3) and rat liver (4, 5). Good experimental data to determine the locations of mitochondrial DNA and mitochondrial DNA polymerase in the mitochondria have not been obtained. Electron microscopy indicates that the DNA is probably located in the matrix of the mitochondria and bound to membranes (6). Kroon (7) studying the synthesis of protein in mitochondria, has found DNA associated with mitochondrial subparticles formed by digitonin treatment of mitochondria and Neubert, Oberdisse, Schmieder and Reinsch (8) report that DNA remains attached to membranes during their procedure for isolating DNA dependent DNA polymerase.

The DNA polymerase has been assumed to be located in the matrix region of the mitochondria along with the mitochondrial DNA. Various reports indicate that it is either a soluble enzyme in the matrix or weakly bound to the inner membrane. The enzyme has been isolated as a soluble enzyme in the supernatant after spinning down the disrupted mitochondrial membrane system by Neubert et al. (8), Iwashima and Rabinowitz (9) and Kalf and Ch'ih (4). Schulz and Nass (10) have reported that they have found a mitochondrial membrane associated DNA polymerase activity. Meyer and Simpson (5) solubilized only about 10% of their activity when they disrupted their mitochondria by grinding frozen mitochondria with alumina if they did not include

1.0 M-NaCl. The remainder of the activity sedimented with the mitochondrial membranes.

The existence of membrane bound DNA and DNA polymerase in mitochondria would be consistent with recent work showing membrane bound DNA (11, 12) and DNA polymerase (13, 14) in bacteria. This would add to the growing number of similarities seen between bacteria and mitochondria (15).

A procedure for the localization of DNA and DNA polymerase within the mitochondria is proposed. Tritiated thymidine is to be injected intravenously into rats. Mitochondria are to be isolated from the livers by the method of Clayton, Smith, Jordan, Teplitz and Vinograd (16). DNA from a portion of the mitochondria is to be extracted with sodium dodecyl sulfate and banded by the EB-CsCl method of Radloff, Bauer and Vinograd (17) to determine the amount of label and DNA. A portion of the isolated mitochondria are to be incubated with C^{14} dCTP and the amount of label incorporated measured to demonstrate DNA polymerase activity.

The mitochondria are then to be treated with 1% digitonin to remove the outer membrane according to the method of Schnaitman and Greenawalt (18). Fractions of outer membrane (OM), intermembrane space (IS) and inner membrane (IM) plus matrix (M) are isolated by differential centrifugation after digitonin treatment. Each fraction is to be checked for the amount of labeled DNA present and for the amount of labeled nucleotide incorporated into DNA using the DNA polymerase assay system of Meyer and Simpson (5). The fractions should

also be checked for activities of adenylate-kinase, monoamine oxidase, cytochrome oxidase and malate dehydrogenase to determine the effectiveness of the digitonin treatment and subsequent isolation of mitochondrial fractions. The enzymes are known to occur respectively in the intermembrane space, outer membrane, inner membrane and matrix (19).

Both DNA and DNA polymerase activity are expected to be found associated with the IM plus M fraction. Upon obtaining a good intact IM+M fraction, it is to be treated with phospholipase A which apparently makes small holes in the inner membrane allowing the soluble matrix material to escape while maintaining structurally intact IM (20). The IM is to be spun down and the supernatant and pellet are to be checked for the previously mentioned activities. A further more drastic treatment of the IM with the non-ionic detergent Brij 58 (14) should fragment the IM into smaller pieces releasing all of the matrix material to solution. The IM fragments could be collected on a cushion in a sucrose gradient as done by Knippers and Stratling (14) in the work with bacterial DNA polymerase.

If these procedures are carefully worked out, unambiguous assignments of the location of DNA and DNA polymerase in the mitochondria should be possible. In the event that the proposed method is not entirely satisfactory, there are a number of other procedures for disassembling mitochondria. Brdicakz, Pette, Brunner, and Miller (20) have reported an alternative method for the isolation of an intact IM+M fraction which give IM with slightly less leakage of M than is

obtained by the digitonin method. The OM is disrupted by rapidly freezing mitochondria in slightly hypertonic solutions.

A somewhat different and perhaps less drastic method for looking for the location of DNA polymerase would be to embed the mitochondria in a matrix of agar according to the method of Smith, Schaller, and Bonhoeffer (13) before or after treatment with digitonin and to wash out soluble material before assaying for DNA polymerase.

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PROPOSITION V

It is proposed that a combination of radioactive and density labeling of mitochondrial membranes in yeast be used to further define the nature of mitochondrial biosynthesis. Yeast auxotrophs for unsaturated fatty acids and choline are to be labeled by growing in bromostearate and C^{14} labeled choline. Isolated intact mitochondria and inner and outer membrane fragments are to be studied to determine the nature of mitochondrial biosynthesis.

The mechanism of the formation of mitochondria has been a subject with many different theories, none of which have had any strong experimental support until recently (1). Schatz (1) has grouped these theories into three differing concepts: (a) synthesis from existing cellular organelles, (b) de novo synthesis from precursor molecules and (c) growth and division of existing mitochondria. This last mechanism has received strong experimental support from the work of Luck (2, 3, 4). Choline requiring yeast mutants were grown in the presence of labeled choline (3, 4). Unlabeled choline was then substituted for the labeled material and the mitochondria were observed by autoradiography as a function of time. The number of disintegrations was found to be distributed randomly among the mitochondria and was diluted at the same rate as the increase in the mass mitochondria.

Luck (2) also found that the density of mitochondria from choline requiring mutants varied depending upon the amount of choline present in the growth media. When mitochondria grown in low concentrations of choline were switched to media with high concentrations of choline, the buoyant density of the mitochondria was found to shift as a homogeneous band as a function of time. Both of these experiments indicate that existing mitochondria continuously incorporate material and grow gradually. The synthesis of new mitochondria de novo would give a separate distinct class of mitochondria rather than a gradual transition.

Fox, Law, Tsukagoshi, and Wilson (5) have developed a new density method for labeling membranes by growing E. Coli essential

fatty acid auxotrophs in the presence of bromostearate. Membranes of these bacteria band 0.06 gm/ml denser than membranes from bacteria grown on oleic acid. This technique should also be useful in density labeling mitochondrial membranes.

Auxotrophs for choline and unsaturated fatty acids are available in Neurospora (2, 8). A strain carrying both mutants may be obtained by standard yeast genetic techniques (9, 11).

The effect of growth in bromostearate on the density of mitochondria and mitochondrial fragments is to be determined. If sufficient density labeling occurs, an auxotroph for both unsaturated fatty acids and choline is to be made. The yeast are to be grown in the presence of labeled choline until maximum labeling is obtained. The labeled choline is then to be replaced with non-labeled choline and bromostearate added at the same time. The mitochondria are to be isolated as a function of time by the technique of Luck (4). The change in labeling and density of the mitochondria are subsequently to be followed and compared with the increase in mass of the total mitochondria. To obtain further information the outer and inner membranes may be fractionated (10) to determine if there is any difference between the growth of these two membranes. The expected results for the growth and division model for mitochondrial synthesis would be a gradual shift in density with a corresponding decrease in the radioactive labeling. The specific activity of the mitochondria should be constant independent of density at any particular time point.

A matter of further interest is whether the growth of the mitochondria is random throughout the entire mitochondrion or whether growth occurs in specific regions of the mitochondrion. This may be determined by fragmenting the membranes into small pieces (12) and determining if the labeling remains random throughout all particles.

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PROPOSITION VI

It is proposed that the properties of a genetically fused bi-functional enzyme containing histidinol dehydrogenase and imidazoleacetol phosphate: L-glutamate amino transferase be compared with the properties of the two individual wild type enzymes.

A genetically fused bifunctional enzyme from Salmonella typhimurium containing histidinol dehydrogenase and amino transferase has been obtained by Yourno, Kohno, and Roth (1) by two sequential mutational events near the intercistronic region. This fusion is believed to be caused by frameshift mutations causing the message punctuation signals to be misread allowing one polypeptide containing both activities to be formed. The single polypeptide folds and associates to retain a substantial part of both enzymic activities and also aggregates to form multimers.

The two enzyme activities which are fused are in the histidine operon and catalyze two steps near the end of histidine synthesis. The two reactions are the transaminase reaction which transfers an amino group from aspartate to imidazoleacetol phosphate by the reaction:

imidazoleacetol phosphate + L-glutamate $\rightleftharpoons$
L-histidinol phosphate + α-keto glutarate

The second enzyme converts L-histodinol to L-histidine by the reaction:

$$\text{L-histidinol} + 2\text{DPN}^+ + H_2O \rightarrow \text{L-histidine} + 2\text{DPNH} + 2H^+$$

Histidinal has been shown to be a non-isolable intermediate in this reaction. Loper and Adams (2) have purified and studied the properties of the wild type enzyme. Wolf and Loper (5) have further extended these studies. The exact mechanism of the reaction is not known.

The kinetics of the amino transferase have been studied by Albritto and Levine (3) and Martin (4) showing it to follow a bisubstrate ping-pong type mechanism.

The bifunctional enzyme is to be prepared from strain TR1024 by the method of Martin, et al. (7) as modified by Yourno, Kohno, and Roth (1). Wild type histidinol dehydrogenase is to be prepared by the method of Loper and Adams (2) and amino transferase by the method of Martin and Goldberger (6). The kinetics of the bifunctional enzyme are to be compared with those of the two native enzymes using the assays of Martin and Goldberger (6) and Albritton and Levine (3) for the forward and reverse reactions of the amino transferase and the assay of Loper and Adams (2) for histidinol dehydrogenase.

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