NON-MONOTONIC DEPENDENCE OF SEDIMENTATION COEFFICIENT AND INTRINSIC VISCOSITY ON SUPERHELIX DENSITY AT POSITIVE SUPERHELIX DENSITIES

A Thesis

Presented to the Department of Biophysical Sciences

College of Natural Science and Mathematics

University of Houston

In partial fulfillment

of the requirements for the degree

Master of Science

By

Paul P. Lau

December 1974

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ABSTRACT

The sedimentation coefficient (s°) and viscosity of closed circular bacteriophage PM2 DNA, artificially produced so as to possess a low absolute superhelix density, has been measured as a function of ethidium bromide (EB) concentration in a solvent of approximately 0.1 M ionic strength. Under these conditions, which allow the introduction of a substantial number of positive superhelical turns into the molecule with increasing ethidium bromide concentration, the hydrodynamic behavior of the closed circle is not a monotonic function of the superhelix density, and clearly displays a local minimum in the case of the s° titration curve. As such non-monotonic behavior has previously been demonstrated in the case of negative superhelix densities, it is now clear that the curve of s° vs superhelix density is non-monotonic and contains local minima on both sides of zero superhelix density.

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INTRODUCTION

I. INTRODUCTION

Circular DNA's have been well characterized in recent years. They are classified as single-stranded rings, covalently closed duplex rings, and nicked circles, which are duplex rings containing one or more interruptions in one or both strands (Vinograd and Lebowitz, 1966; Bauer and Vinograd, 1971). Closed circles exist in a twisted or supercoiled configuration in neutral salt solutions at room temperature (Vinograd et al., 1965). This may be envisioned as arising from a deficiency in the number of Watson-Crick turns in the molecules at the time the last phosphodiester linkage is made (this could be due to a small local region of unstacked bases, or to a slight reduction in the rotation angle of the Watson-Crick helix over the whole molecule). When the constraint causing the deficiency in the number of the Watson-Crick turns is then removed, the covalently intact double stranded circle must necessarily acquire superhelical turns as the helix returns to, or nearly to, its normal Watson-Crick structure. This is due to the topological constraints imposed on the system, which require that the total number of twists of one strand about the other must be constant as long as both strands are covalently intact. This has been shown diagrammatically by Vinograd, Lebowitz, and Watson (1968), and a proof based upon topological considerations has been given by Glaubiger and Hearst

(1967). In solutions near neutral pH, the introduction of a single strand scission into a closed circular duplex generates a swivel in the complementary strand at a site in the helix opposite to the scission. The twists are then released and a nicked circular duplex, which sediments more slowly than the superhelical closed circle, is formed. These circular duplexes dissociate in denaturing solvents, such as strongly alkaline solutions, to form circular and linear single strands which sediment at slightly different velocities (Vinograd et al., 1965). The superhelix density, which is the number of superhelical turns per ten base pairs, has been defined by Bauer and Vinograd (1968) as an intensive measure of supercoiling in closed circular DNA's. All naturally occurring and artificially produced superhelical DNA's known to date have superhelical turns, and hence superhelix densities, of the sense defined as negative by Bauer and Vinograd (1968).

Natural sources of closed circular supercoiled DNA are widespread. Some oncogenic animal virus DNA's, the intracellular forms of many bacteriophage DNA's, the mature form of at least one bacteriophage, and bacterial episomal and plasmid DNA's are found to be closed circles. They are also found in subcellular organelles, such as kinetoplasts in some protozoa and mitochondria in higher organisms (see Bauer and Vinograd (1971) for a list of references). In this work, we have chosen Pseudomonas page PM2 (Espejo and Canelo, 1968) as the source of DNA;

PM2 is the only phage so far known to contain closed circular duplex DNA in its mature form (Espejo, Canelo, and Sinsheimer, 1969). This system is chosen mainly due to the ease of preparing large quantities of closed circular DNA without going through tedious procedures; for instance, the difficult tissue culture techniques employed in the preparation of closed circular DNA from animal viruses.

An understanding of the biological role and significance of supercoiling of these circular DNA's requires a detailed knowledge of the physical structure and chemical properties of these molecules. Much work concerned with the effect of intercalating dyes upon the properties of closed circles has been done in a number of laboratories (Crawford and Waring, 1967; Bauer and Vinograd, 1968; Wang, 1969a,b; Gray, Upholt, and Vinograd, 1971; Waring, 1971; Revet, Schmir, and Vinograd, 1971). The intercalation mechanism was first proposed by Lerman (1961) in connection with duplex DNA in which planar portions of the dye molecules are inserted between the base pairs of the DNA duplex, unwinding the Watson-Crick helix. This has the effect of removing the superhelical turns in a supercoiled closed circular duplex DNA (Crawford and Waring, 1967; Bauer and Vinograd, 1968). Although the unwinding of the duplex by the intercalating dye, ethidium bromide, has been disputed by Paoletti and Lepecq (1971), who have asserted that the effect of ethidium bromide is

instead to increase the angle between base pairs, it has been demonstrated in recent work from Vinograd's laboratory (Schmir, Revet, and Vinograd, 1974) that the dye does indeed unwind the DNA helix.

There are various means to monitor the dye titration of a closed circular DNA. The methods employed have been sedimentation velocity titrations (Crawford and Waring, 1967; Bauer and Vinograd, 1968; Wang, 1969a,b; Gray et al., 1971), viscometric-dye titrations (Revet et al., 1971), and buoyant density-dye titrations (Bauer and Vinograd, 1968). Wang (1969a,b), Gray et al. (1971), and Upholt, Gray and Vinograd (1971) have demonstrated a non-monotonic dependence of sedimentation coefficient at infinite dilution ($s°$) on the (negative) superhelix density of closed circular DNA. The value of $s°$ increases, starting from a superhelix density (σ) of zero, until a leveling off is observed near $\sigma = -0.020$, followed by a decrease to a local minimum near $\sigma = -0.039$, and a monotonic increase in $s°$ at all the more negative superhelix densities experimentally accessible to date. Upholt et al. (1971) gave an explanation of this behavior based on their electron microscopy data, which showed the various configurations of in vitro closed circular DNA's of various superhelix densities: The molecules appear to undergo a decrease in radius of gyration as superhelical turns are introduced into the completely relaxed closed

circle, accompanied by an increase of the s° value in the s° vs σ curve. In a critical region, the molecules appear to stiffen into an extended interwound superhelical form, giving rise to the local minimum, before they display superhelical branches at higher absolute superhelix densities, causing a monotonic increase in the s° value thereafter. The viscosity monitored as a function of superhelix density would be expected to be the inverse of the sedimentation behavior, displaying maxima where minima are observed in the sedimentation titration curves and vice versa. This behavior has been demonstrated by Revet et al. (1971) in ethidium bromide titrations of PM2 I DNA monitored by capillary viscometry.

This phenomenon, if its explanation is as given above, is expected to have a symmetrical relationship; that is, a tightly negatively supercoiled circle would pass through a local minimum as it is unwound, through the absolute minimum where the superhelix density is zero, and then through another local minimum as a large number of turns of the opposite sense (positive) are introduced. Ostrander and Gray (1973) did not observe a "leveling off" in the s° vs pH curve in PM2 I DNA in their alkaline titration, whereas suggestions of such a leveling off region were observed in alkaline titrations of molecules of lower superhelix densities (Dawid and Wolstenholme, 1967; Rush and Warner,

1970). Here Ostrander and Gray (1973) reasoned that the existence of a larger alkaline denatured region in the more highly negatively supercoiled DNA's, required for the DNA to reach the minimum in the curve where the original superhelical turns are relaxed, is available to take up the positive superhelical turns of further denaturation. Thus, the positive superturns cause crossing over and entanglement of the two strands in the denatured region; the denatured region is large enough that the molecule will never assume the extended interwound superhelical form and a local minimum will not be observed. In this view, the higher the superhelix density of the native closed circle, the less likely the leveling off region will be observed. Native PM2 I is the most highly supercoiled naturally occurring DNA found to date (Gray et al., 1971). It is believed that the leveling off in the s° vs pH curve, where observed, is actually the local minimum at the positive superhelix densities. The most pronounced leveling off found so far is for mitochondrial DNA (Dawid and Wolstenholme, 1967) which has a low superhelix density (Kasamatsu, Robberson, and Vinograd, 1971). If the symmetry in the s° vs pH curves is real, a curve of s° vs pH for the in vitro closed PM2 circles containing low absolute numbers of superhelical turns is expected to display a leveling off region at a pH slightly above that at which the original superturns are unwound.

In this work, titrations of PM2 closed circular DNA of low absolute superhelix density were carried out with ethidium bromide, employing solutions of low ionic strength in order to increase the intrinsic binding constant of the dye (Lepecq and Paoletti, 1967). These titrations were monitored hydrodynamically both by capillary viscometry and by band velocity sedimentation. The results indicate that the non-monotonic behavior of hydrodynamic properties as a function of superhelix density is symmetric; that is, local maxima and minima exist on both sides of $\sigma = 0$.

II.

MATERIALS AND METHODS

II. MATERIALS AND METHODS

Chemicals

Ethidium bromide (EB) and propidium diiodide (PI) were purchased from Calbiochem. Optical grade and biological grade CsCl were purchased from Schwarz Bioresearch. Pancreatic DNase (DN-C), bovine serum albumin (BSA) (Fraction V) and β-DPN were supplied by Sigma Chemical Co. Deuterium oxide (D_2O, 99.87 mole percent) was purchased from Bio-Rad. All other chemicals were reagent grade.

Preparation of PM2 Phage DNA's

PM2 phage was grown, harvested, and purified as described by Espejo and Canelo (1968). Phage DNA was extracted according to the procedure of Espejo et al. (1969). PM2 phage and its Pseudomonas BAL 31 host were the gifts of Dr. R. T. Espejo. PM2 I DNA (native closed circles) was isolated by the PI-CsCl buoyant density method (Radloff et al., 1967; Hudson, Upholt, Devinny, and Vinograd, 1969) and collected after tube puncture while monitoring under long-wave UV light. The lower bands were pooled and dialysed into BE buffer (0.1 M NaCl, 0.02 M tris, 0.001 M EDTA, pH 8.1) (Espejo et al., 1969) in dialysis tubing pretreated by boiling in the presence of EDTA, after the PI had been removed by the isopropanol extraction method of Cozzarelli, Kelly, and Kornberg (1968) as described by Ostrander and Gray (1973).

Nicking of PM2 I DNA by DNase

Nicking of PM2 I DNA by limited pancreatic DNase action was used to produce PM2 II DNA (nicked circles) with an average of less than one scission per molecule. Incubation was performed in the medium used by Hudson et al. (1969), as modified by Ostrander and Gray (1973), containing 1/10 volume 0.07% BSA, 1/10 volume 0.12 M $MgCl_2$, 5/10 volume pretreated PM2 I DNA (heated at 60°C, about 10°C below its T_m, for 5 to 10 minutes), 1/10 volume DNase I stock (approximate concentration 2 x 10^{-3} µg/ml) and 2/10 volume DNase I buffer (0.015 M NaCl, 0.01 M tris, 0.001 M EDTA, pH 8.1, in which buffer all components above were dissolved), at 30°C for four hours. BSA and $MgCl_2$ solutions were placed in boiling water for 5 to 10 minutes before such solutions were used; this was to prevent adventitious nicking, as was the heating of the DNA (Ostrander and Gray, 1973). The reaction was stopped by adding at least 1/10 volume of EDTA (concentration 0.2 M) and placing the mixture on ice. This reaction yielded 30% nicked circles and 70% form I DNA's upon band sedimentation analysis in standard alkaline CsCl solution (2.83 M CsCl, 0.1 M KOH, 0.001 M EDTA, pH 13) (Vinograd et al., 1965). About 0.5 M NaCl was added to the lamellar solutions in order to inhibit the nicking sometimes observed in the wells of the Epon band-forming centerpieces when the DNA was in dilute salt solution (Ostrander and Gray, 1973).

Nicked DNA was isolated by PI-CsCl buoyant density gradients, followed by isopropanol dye extraction and two dialyses into DNase I buffer.

Closure of PM2 II by Polynucleotide Ligase

Enzymatic closure of about 25 μg/ml of PM2 II DNA was carried out in a DNase I buffer solution of a total volume of 21 ml, containing 0.02 volume of polynucleotide ligase in 50% glycerol solution which contained 0.05 M potassium phosphate, pH 6.5 (samples were the gifts of Dr. J. C. Wang), 1/10 volume DPN (2×10^{-3} M), 1/10 volume heat-treated BSA (0.07%), and 0.025 volume heat-treated $MgCl_2$ (0.12 M) at 15°C for 24 hours (Mertz and Davis, 1972). The reaction was stopped by adding 0.003 volume of 1.0 M EDTA to the mixture and placing on ice. Fifty percent closed circles were produced as monitored by alkaline CsCl band sedimentation. Closed circular DNA was isolated from PI-CsCl gradients, dialysed into BE buffer, and concentrated by dialysis against dry sucrose, followed by a second dialysis into BE buffer.

Viscometric Dye Titrations

Viscometric dye titrations of the closed PM2 DNA were carried out in a Cannon-Ubbelohde semi-micro dilution viscometer (Cannon Instrument Co.) mounted in a water bath thermostatted at 20 ± 0.01°C, in BE buffer.

The viscometer was cleaned with $K_2Cr_2O_7$-H_2SO_4 cleaning solution, rinsed with deionized water and acetone, and dried at 40°C. The viscometer contained 92.9 µg/ml of DNA in BE buffer. The titrant solution contained the same concentration of DNA as in the viscometer and 50 µg/ml of EB in the same solvent. The titrant was added with a digital pipette (Greiner Scientific Products) affixed to a piece of intramedic tubing which was inserted into the dilution bulb of the viscometer. The solutions were well-mixed with a flow of air through the dilution bulb before each measurement of flow time. At least four determinations of the flow time were made for each titration point. The titrations were completed in approximately eight hours. An aliquot of the titrated solution was withdrawn and assayed for percent nicking by band sedimentation in standard alkaline CsCl solution. The solutions of BE buffer, DNA and EB in this solvent were all passed through fine (4 to 5.5 micron pore size) sintered glass filters before they were used for preparations of the titrant and the DNA solution. Pipets and all glassware were cleaned and blown out with streams of filtered nitrogen gas, in order to avoid dust particle contamination. The absorbance of the filtered DNA and EB solutions were measured in a Cary 14 spectrophotometer. The concentrations of the titrant and the DNA solution made from these solutions were calculated as described below.

Determinations of the critical binding ratio, ν', and the critical free EB concentration, C', for PM2 I DNA in the 50% D_2O solvent (below) at 20°C were performed by the capillary viscometric dye titration procedure described by Révet et al. (1971). ν' and C' are the ratio of bound EB per DNA nucleotide and free dye concentration, respectively, at the point where $\sigma = 0$. DNA solutions were approximately 50, 100, 150, 200 µg/ml in the 50% D_2O solvent. The titrant solution contained 74.9 µg/ml of EB in the 50% D_2O solvent. All solutions were filtered through sintered glass filters as above before their concentrations were measured spectrophotometrically. ν' and C' were obtained from the least-squares analysis of the slope of a plot of C_T' against N_T' (where C_T' is the total molar concentration of EB at the maximum of the flow time vs ethidium bromide concentration curve and N_T' is the total molar nucleotide concentration) with the aid of an Olivetti 101 Programma computer. The determination of C' under these conditions was unsatisfactory and was done by sedimentation velocity measurements as will be described.

Analytical Band Velocity Dye Titrations

The band sedimentation-dye titration experiments were performed in a Beckman Model E ultracentrifuge equipped with electronic speed control unit, photoelectronic scanning-multiplexer system and monochromator. The monochromator was

set at 265.4 nm (Bauer and Vinograd, 1968). A four-hole rotor and type I 12 mm Epon band-forming centerpieces (Vinograd, Radloff, and Bruner, 1965) were used. A 50% (v/v) D_2O solution in BE buffer was used as sedimentation solvent. This 0.1 M NaCl-containing buffer was used in order to increase the binding affinity of the DNA for EB, which reduces the maximal free dye concentration required for the DNA to become sufficiently positively supercoiled to reach a local minimum (as compared to the higher dye concentrations which would be required at much higher salt concentrations) (Lepecq and Paoletti, 1967). This was necessary so that the analytical centrifuge absorption optical system could be employed to monitor the sedimentation process without the background ultraviolet absorption due to the dye becoming prohibitively large; the buffer itself will not support band sedimentation and the use of D_2O is indicated in order to produce density gradients sufficient to stabilize the bands (Vinograd, Bruner, Kent, and Weigle, 1963). The maximal photomultiplier slit width required for the highest dye concentration was 0.18, compared to the 0.12 mm width employed in the absence of high background absorption.

A stock solution of ethidium bromide at about 100 µg/ml was made in the 50% D_2O solvent, an aliquot of which was assayed for its concentration with a Cary 14 spectrophotometer. The sedimentation solvent at each EB concentration,

volume of 1 ml, was made up in a small stoppered tube, containing the 50% D_2O solvent accurately added with a Manostat digital pipette, and an appropriate amount of EB in this solvent (estimated with the modified Scatchard relation; discussed in RESULTS) introduced with a Hamilton microsyringe. A stock solution of EB near 100 µg/ml in BE buffer was also made, the concentration of which was also assayed spectrophotometrically. The lamellar solution, total volume of 20 µl, contained 0.1 µg of DNA in BE buffer. For the higher dye concentrations employed (above 0.5 µg/ml), an amount of EB required to give a free dye concentration equal to that in the sedimentation solvent was added to the sample well in order to minimize concentration gradients of EB in the ultracentrifuge cell. Hamilton microsyringes were used for all additions and mixings. All sedimentation experiments were performed at 20°C and 26 Krpm. Temperature was regulated by the standard RTIC system. The uncorrected sedimentation coefficients were evaluated from the least squares analysis of the slopes of ln $\bar{r}$ vs t plots (where $\bar{r}$ is the band maximum position at time t (Vinograd and Bruner, 1966)) with the aid of an Olivetti 101 computer or the UNIVAC 1108 computer. Twelve to sixteen ln $\bar{r}$ vs t points were obtained for each s° value determination; all points more than two standard deviations away from the least squares line were discarded. All s° values were obtained at least in

duplicate. The standard deviation (three or more determinations) or ± one-half the range (two determinations) of all the determinations obtained at a single dye concentration are shown on the graphs where these exceed the size of the plot symbol.

Sedimentation-dye titration (Gray et al., 1971) of the PM2 I DNA (native closed circles) was necessary to obtain C' for PM2 I DNA in the 50% D_2O solvent system. The experiments were carried out in the same manner as described above. The same titrant solution was employed.

Sedimentation dye titrations of the PM2 ligase closed circular DNA was used to obtain ν', the critical molar binding ratio of this ligase closed circle in standard neutral CsCl solution (2.83 M CsCl, 0.01 M tris, 0.001 M EDTA, pH 8.1). The titrant contained 110 µg/ml of EB in the standard neutral CsCl solvent. The lamellar solution of 20 µl contained 0.1 µg of DNA in BE buffer. The experiments were carried out at 20°C and 32 Krpm.

Preparative Ultracentrifugation for Estimation of Superhelix Density σ_o

A solution of 0.2 µg each of PM2 ligase closed circles and PM2 II DNA in 3 ml of CsCl in BE buffer, density 1.55 g/ml, containing 330 µg/ml of EB, was centrifuged in the Spinco Model L ultracentrifuge for 36 hr at 20°C and 40 Krpm

in a SW 50.1 rotor, with a reference tube containing PM2 I and II DNA's in a gradient of the same constituents.

Photography and Measurement of Band Separations in EB-CsCl Gradients

The DNA gradients were photographed under ultraviolet light and the band separations were measured as described by Hudson et al. (1969) and Gray et al. (1971), using the apparatus described by Watson, Bauer, and Vinograd (1971), which in this instance incorporated a used Graflex Graphic camera with 2 1/4" x 3 1/4" sheet film. Tubes with reference and unknown DNA's were photographed in fluorescence on a single sheet of film and the separations of closed and nicked circular bands measured using a Nikon projector at 10 x magnification. The equations and calculations involved are given under RESULTS.

Spectrophotometry

Spectrophotometric measurements were carried out with a Cary 14 spectrophotometer. The reciprocal extinction coefficient of PM2 DNA was assumed to be 50 μg/ml/$OD^{1\ cm}_{260\ nm}$ (Hershey, 1968), and that of 50 μg/ml of EB in DNase I buffer or 100 μg/ml of EB in BE or in the 50% D_2O solvent, to be the value of 79.15 μg/ml/$OD^{1\ cm}_{485\ nm}$ obtained by Ostrander and Gray (1974). For EB at approximately 100 μg/ml in 2.83 M neutral CsCl, a reciprocal extinction coefficient of 81.4 μg/ml/$OD^{1\ cm}_{486\ nm}$ (Gray et al., 1971) was used for calculation.

III.

RESULTS

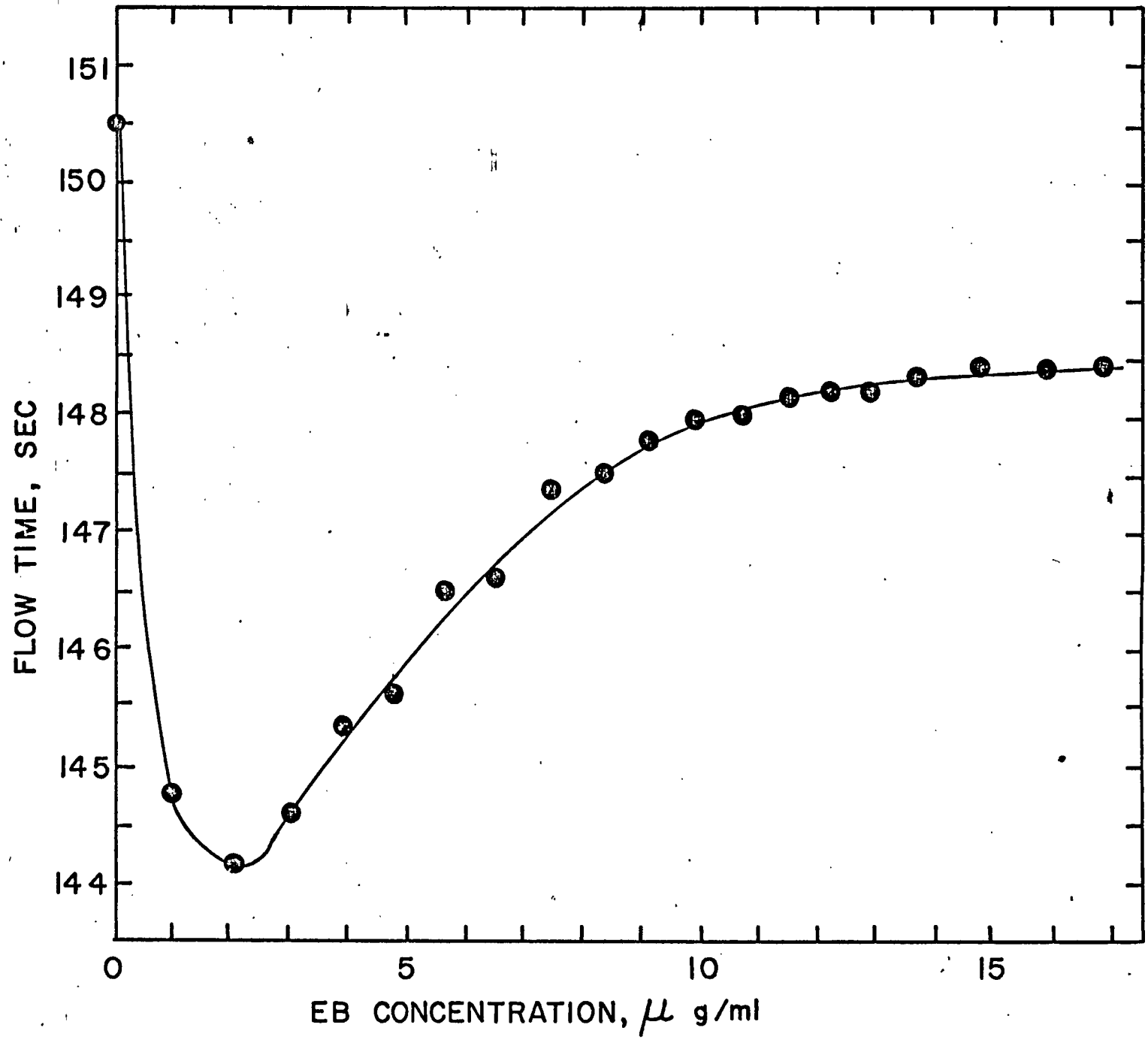
III. RESULTS

Viscometric Dye Titrations

The curve (Fig. 1) obtained for the viscometric dye titrations of the PM2 ligase closed circle has a local minimum at a dye concentration of approximately 2 μg/ml and displays a leveling off region between dye concentrations of 12 and 17 μg/ml. Failure to obtain a decrease in viscosity for higher dye concentrations is believed due to the presence of increasing percentages of nicked circles arising during the prolonged titration time required, which could readily obscure any decrease in viscosity for the closed circles (see DISCUSSION). That such nicking did occur was demonstrated by alkaline band sedimentation experiments performed upon the samples before and after the eight-hour viscometric titration experiment. An increase of approximately 6% in the percentage of nicked circles was found between the two determinations above from cutting and weighing of the bands from photoelectric scanner tracings (Douthart and Bloomfield, 1968; Ostrander and Gray, 1973). Samples withdrawn at one hour intervals from the titrant solution (50 μg/ml EB) standing on ice for a total of eight hours and from an aliquot of the DNA solution (no EB) in the viscometer mounted in the thermostatted bath for the same amount of time at 20°C, were likewise assayed for percentage of nicking with respect to time. The nicking rates of the closed

FIGURE 1

Viscometric ethidium bromide titration of the PM2 ligase closed circle at 20°C in BE buffer performed as described in MATERIALS AND METHODS. The standard deviations of the four determinations of each flow time do not exceed the size of the plot symbol for any of the data. The titration was completed in approximately eight hours.


151
150
149
148
147
146
145
144
FLOW TIME, SEC
0
5
10
15
EB CONCENTRATION, μ g/ml

circles in the titrant and in the DNA solution in the viscometer are roughly constant and proceed at rates of about 2.0 ± 0.3% and 9.0 ± 0.1% in eight hours, respectively (Fig. 2). The enhanced rate of nicking at low ionic strengths in the presence of ethidium bromide is consistent with previous observations (Ostrander, D.A., and Gray, H.B., Jr., unpublished results). The necessity for large binding constants, hence low ionic strengths, in these studies precluded the use of more concentrated salt solutions, in which the nicking rate would be negligible. The rates of nicking, which change as the ethidium concentration is increased in the viscometric titrations (Fig. 2), as well as the unknown effects of hydrodynamic shear on the nicked and closed circular forms of DNA, prohibit any reliable point-by-point correction for these effects.

Determination of ν' and C' for PM2 I DNA by Capillary Viscometry

ν' and C' can be calculated from the amounts of DNA and dye present at the maxima of viscosity dye titration curves (Fig. 3), where the molecule assumes the fully relaxed configuration, with the relationship (Révet et al., 1971):

$$C'_T = \nu' N'_T + C' \qquad (1)$$

where C'_T is the total dye concentration and N'_T is the total nucleotide concentration, both in moles/liter; the primed

FIGURE 2

Nicking rates of ligase closed circles. ●, titrant solution (50 μg/ml EB) on ice, O, DNA solution (no EB) in the viscometer at 20°C. All data were obtained from analysis of at least two scanner tracings from each experiment. The straight lines were obtained by linear least squares analysis.

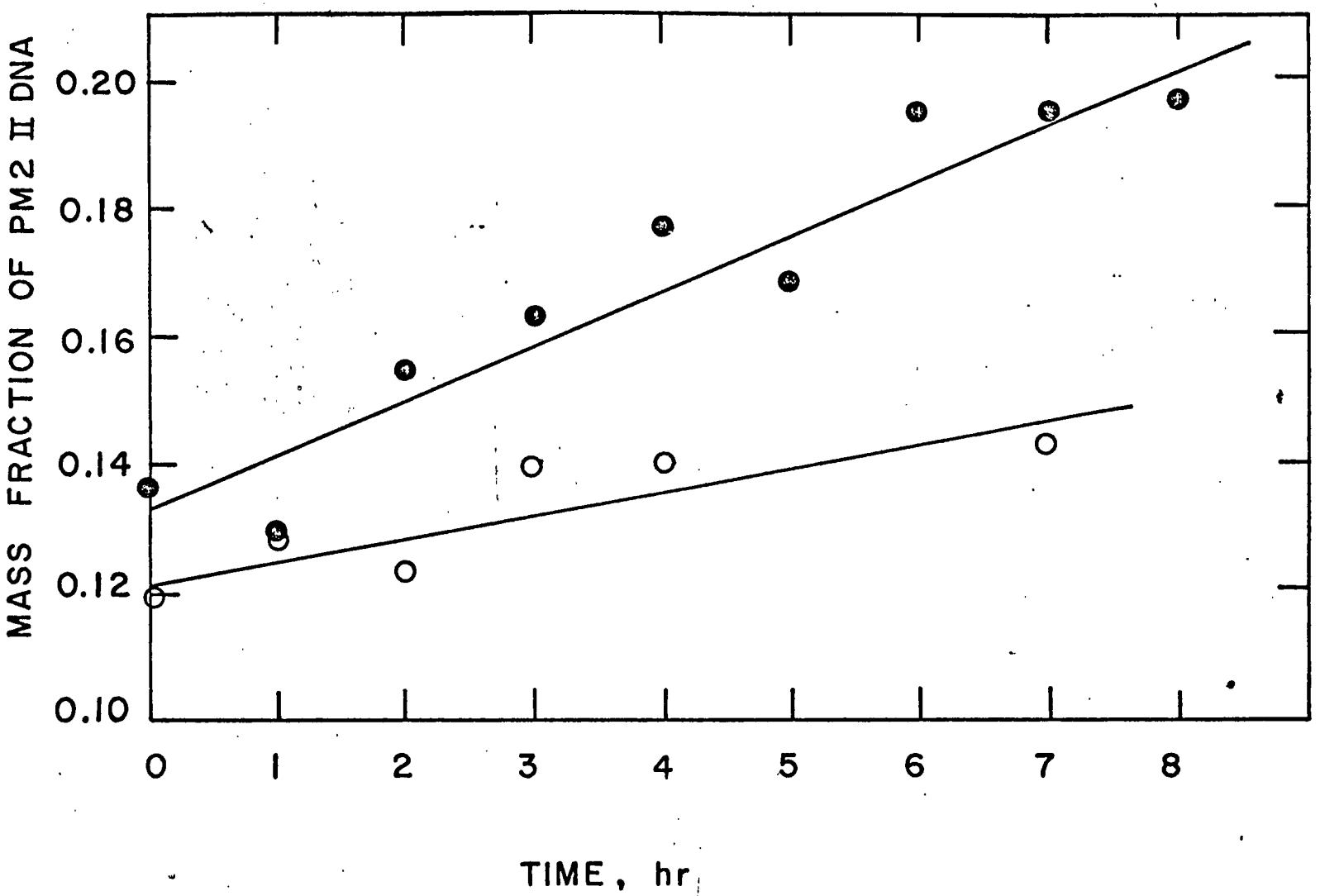
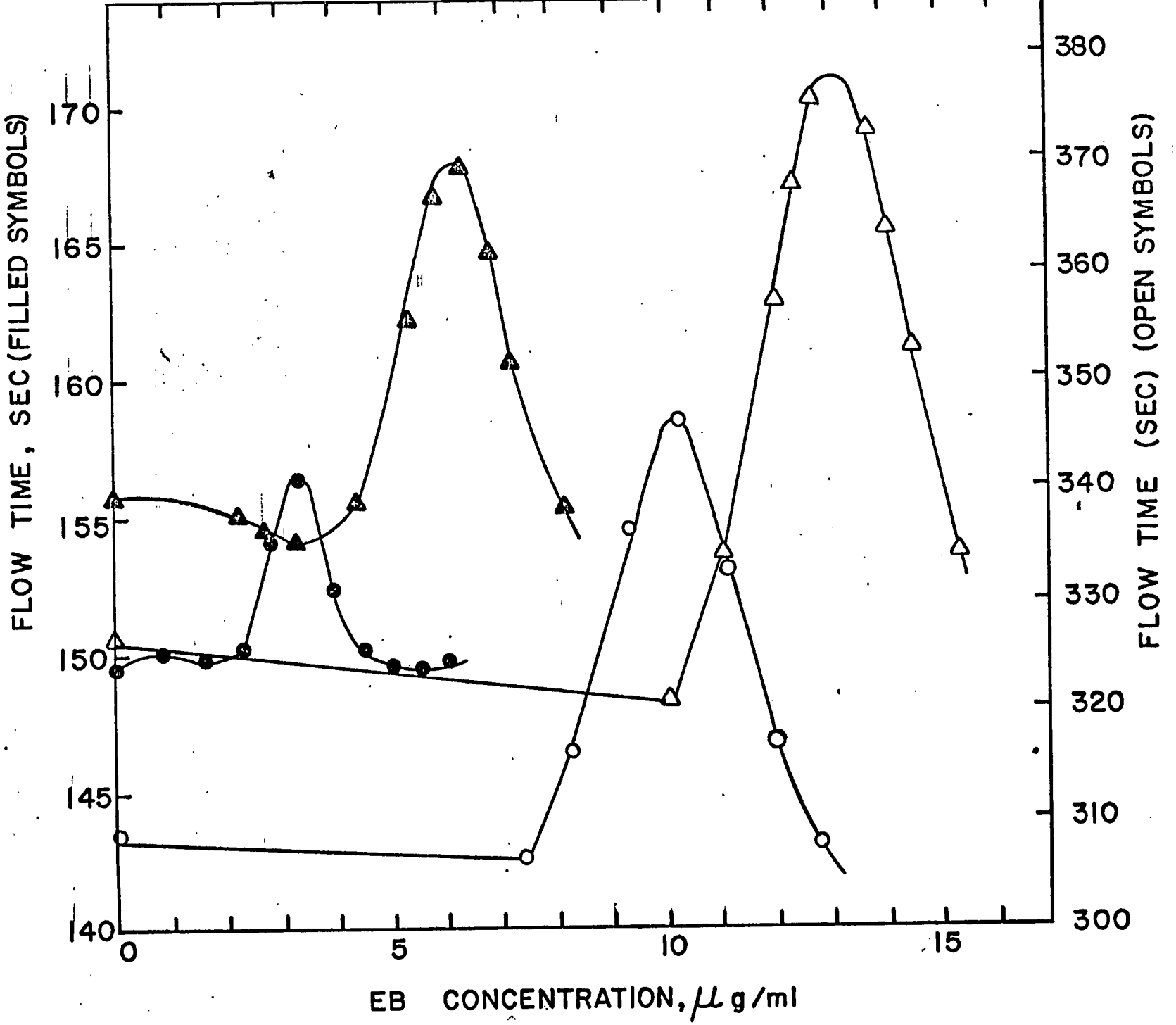

MASS FRACTION OF PM2 II DNA
0.20
0.18
0.16
0.14
0.12
0.10
0
1
2
3
4
5
6
7
8
TIME, hr

FIGURE 3

Viscometric dye titrations of PM2 I DNA at various concentrations in 50% D_2O-BE buffer. ●, ▲, O, Δ represent DNA concentrations of 46.4, 88.9, 125.5 and 165.9 μg/ml, respectively. The two series at the higher DNA concentrations, for which the right-hand ordinate scale applies, were obtained with the use of a viscometer of smaller capillary diameter than that used in the other experiments. Flow times were measured at least in triplicate; standard deviations do not exceed the size of the plot symbols.


FLOW TIME, SEC (FILLED SYMBOLS)
FLOW TIME (SEC) (OPEN SYMBOLS)
EB CONCENTRATION, μg/ml
170
165
160
155
150
145
140
380
370
360
350
340
330
320
310
300
0
5
10
15

symbols refer to the relaxed ($\tau = 0$) conformation of the closed circle. A C_T' vs N_T' curve (Fig. 4) was obtained from the data of Fig. 3, giving a slope which yields the critical molar binding ratio ν' of 0.084 ± 0.005 directly and a Y-intercept, C', of -0.805 ± 0.601 μg/ml, which nominally corresponds to the free dye concentration in equilibrium with the fully relaxed circular DNA-dye complex. The negative value of C' obtained above is of course meaningless per se, although it implies a very small positive number could be the correct value when its standard deviation is taken into account. A reasonable value for C' (0.375 ± 0.010 μg/ml) was thus obtained by sedimentation velocity-dye titration as described below. ν' was then obtained from least squares analyses of a new curve containing the original four points from the viscometric titrations (Fig. 3) and the new value for C' obtained from the sedimentation velocity-dye titration experiments (point at $N_T' = 0$ in Fig. 4), and has the value of 0.078 ± 0.004. This value was retained for subsequent calculations.

Sedimentation Velocity-Dye Titrations

The value of C' needed to calculate the intrinsic binding constant K (below) for PM2 DNA at 20°C in the 50% D_2O solvent was obtained from an s° vs EB concentration curve for native PM2 I DNA (Fig. 5). The EB concentration, which

FIGURE 4

Plot of C_T' *vs* N_T'. ●, viscometric titration data obtained from Fig. 3; ▲, sedimentation titration datum obtained from Fig. 5.

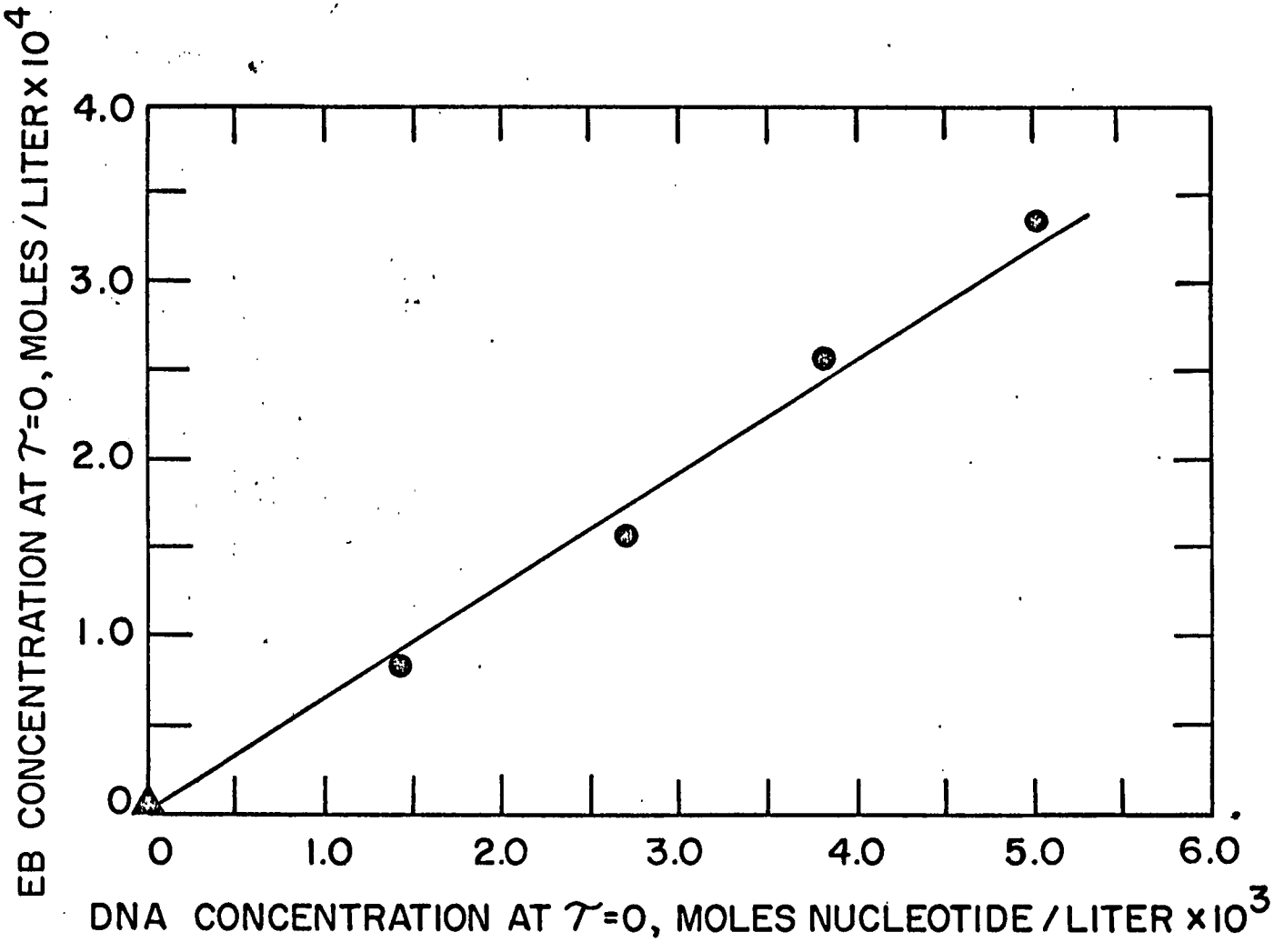
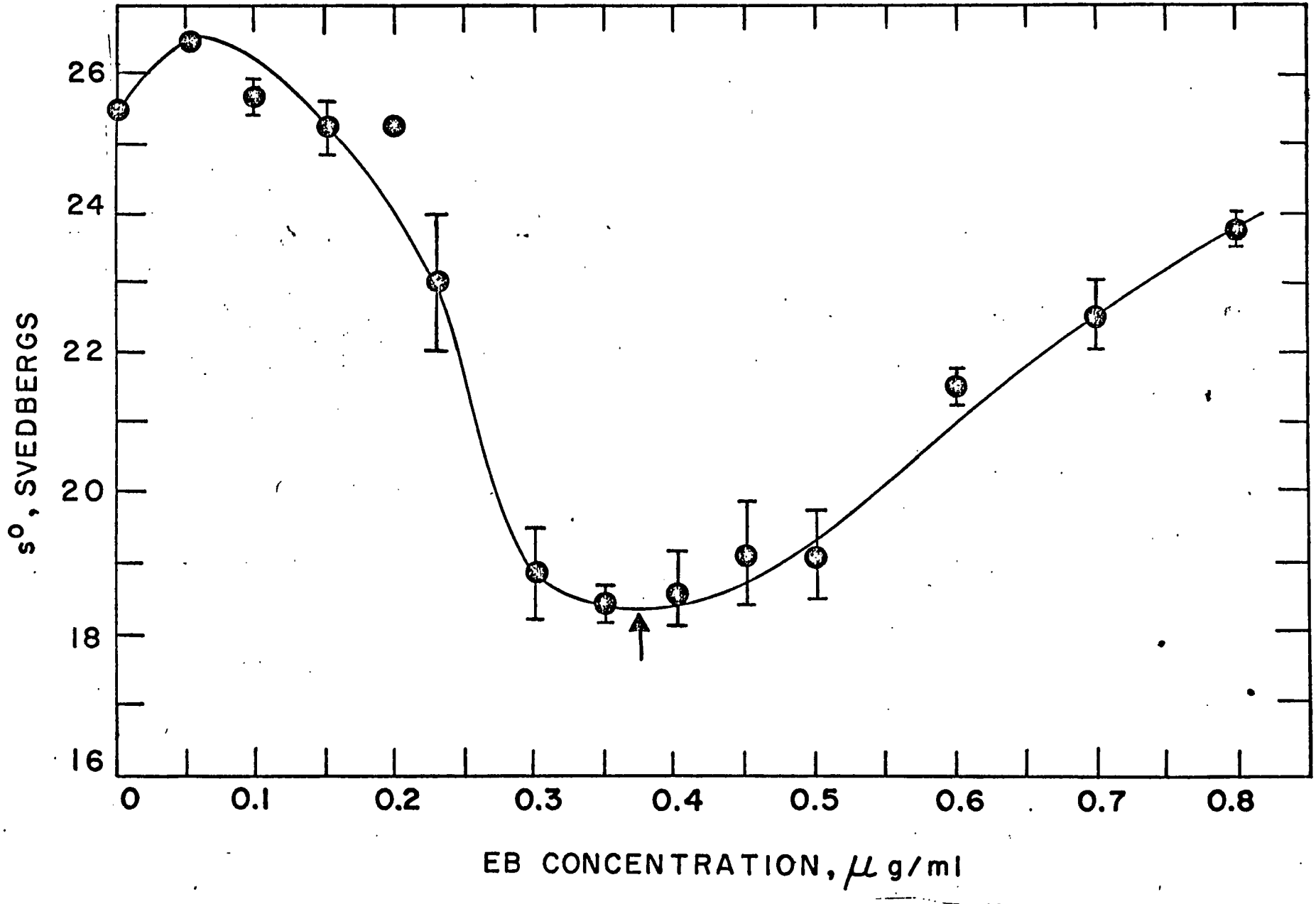

EB CONCENTRATION AT τ=0, MOLES/LITER x 10^4
4.0
3.0
2.0
1.0
0
0
1.0
2.0
3.0
4.0
5.0
6.0
DNA CONCENTRATION AT τ=0, MOLES NUCLEOTIDE/LITER x 10^3

FIGURE 5

Sedimentation dye titration of PM2 I DNA in 50% D_2O solvent at 20°C. Error bars represent the ranges (two determinations) or standard deviations (three or more determinations) where these errors exceed the size of the plot symbol. All points are the averages of at least two determinations. Arrow indicates position of minimum.


26
24
22
20
18
16
s^o, SVEDBERGS
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
EB CONCENTRATION, μg/ml

represents the free EB concentration in such experiments (below), is taken to be 0.38 ± 0.01 μg/ml at the minimum of the titration curve of Fig. 5. The data of Fig. 5 are not corrected for the effects of bound ethidium on the buoyant density, stiffness, or contour length of the DNA.

The s° vs c curve for the enzymatically closed circle (Fig. 6) contains, in a distorted form, information as to the behavior of the s° value as a function of free dye concentration at positive superhelix densities. The lower curve is for PM2 II DNA. The free dye concentration is the same as the initial dye concentration, inasmuch as the amount of dye bound by the small amount of DNA in the band is negligibly small, and dye was also added to the lamellar solutions as described in MATERIALS AND METHODS. The sedimentation coefficients of Fig. 6 are not corrected for the dye binding effects.

We have converted these curves to the more meaningful s° vs ν curves (Fig. 7) following the procedure of Upholt et al. (1971). Assuming ν_m (the maximum number of ethidium binding sites per nucleotide) is 0.241 (Gray et al., 1971), the Scatchard equation,

$$\nu' = K\, C'\, (\nu_m - \nu') \qquad (2)$$

where the primes have the meaning given earlier, may be used to calculate the intrinsic binding constant, K, with ν' and C' determined experimentally as above. The value of K, for PM2 DNA in the 50% D_2O-H_2O solvent at 20°C, is thus obtained

FIGURE 6

Sedimentation velocity dye titrations of PM2 ligase closed circle (●) and nicked circle (O) in 50% D_2O solvent at 20°C. The sedimentation coefficients are not corrected for dye binding effects. Error bars have the same meaning as in Fig. 5.

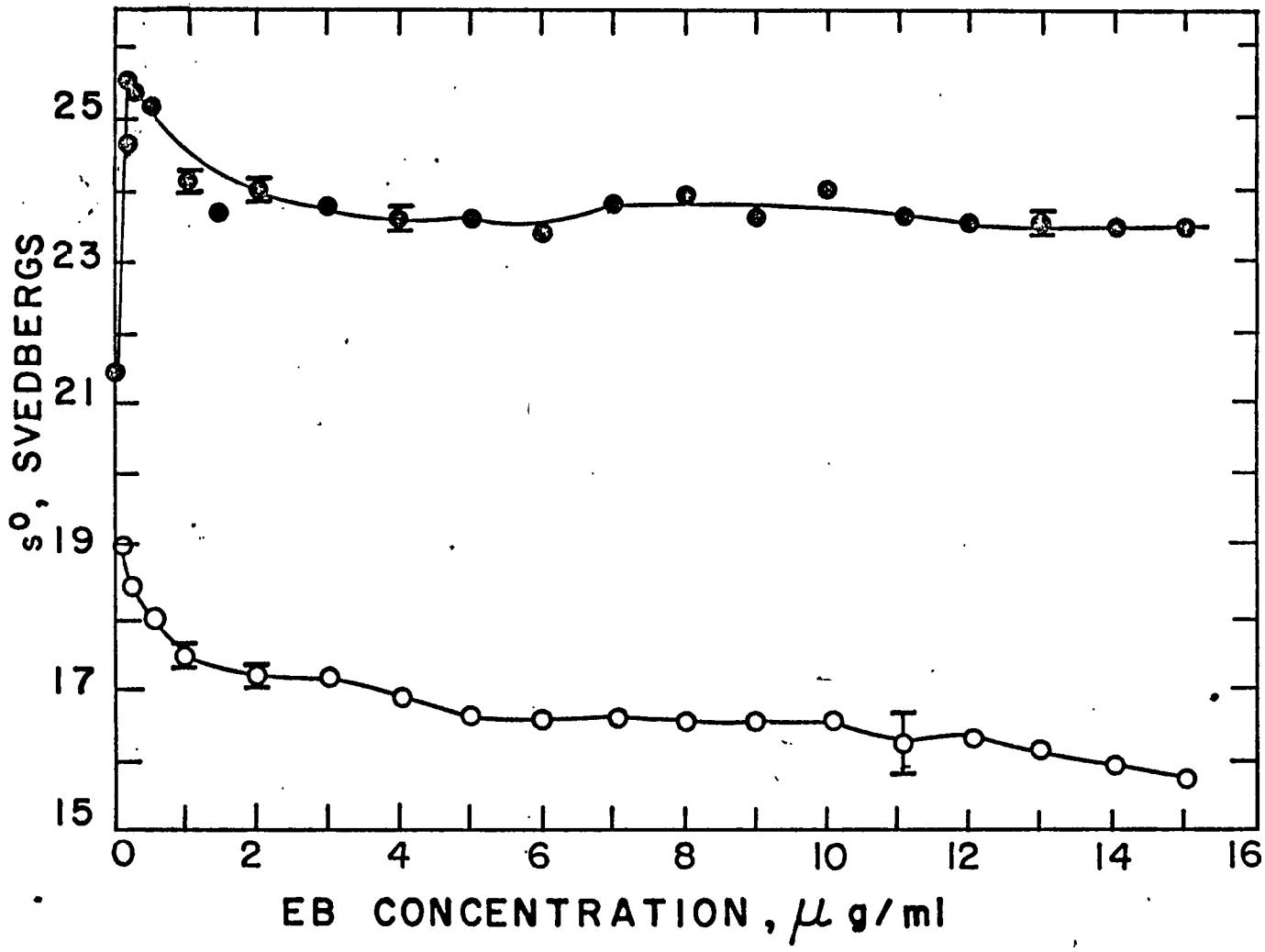
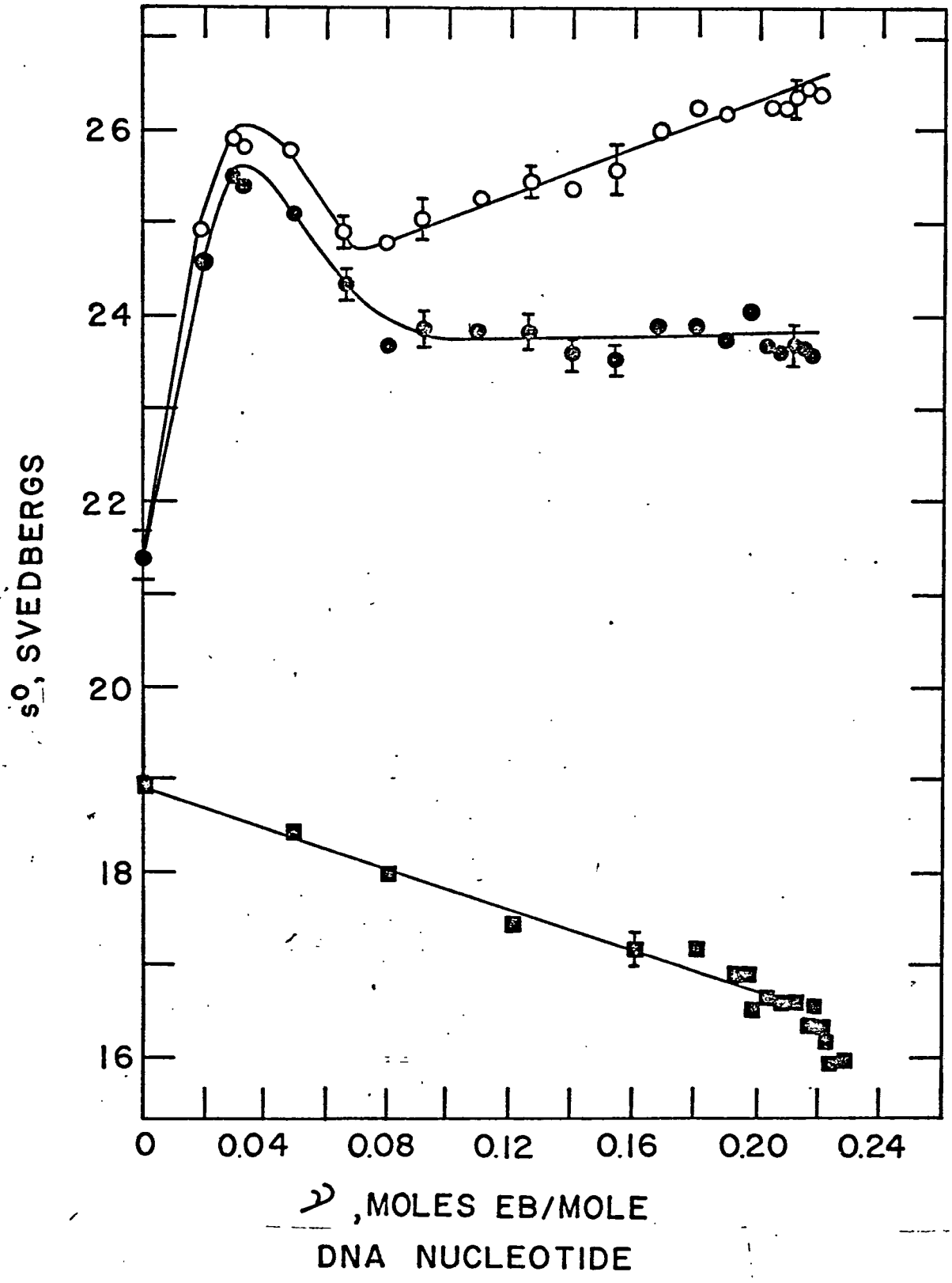

25
23
21
19
17
15
s^o, SVEDBERGS
0
2
4
6
8
10
12
14
16
EB CONCENTRATION, μ g/ml

FIGURE 7

s° vs ν for PM2 ligase closed circle and nicked circle. ●, ligase closed circle with s° values not corrected for dye binding effects; O, ligase closed circle with s° values corrected for dye binding effects (see text); ■, nicked circle. The error bars have the same meaning as in Fig. 5.


26
24
22
20
18
16
s^0, SVEDBERGS
0
0.04
0.08
0.12
0.16
0.20
0.24
ν, MOLES EB/MOLE
DNA NUCLEOTIDE

as $4.075 \pm 0.004 \times 10^5$ 1/mole. The value of ν corresponding to each value of C for the PM2 II DNA may then be calculated from eq. (2) to convert the lower curve of Fig. 6 to that of Fig. 7, as there is no dye binding restriction due to supercoiling for the form II DNA, and eq. (2) is thus applicable at all dye concentrations (Gray et al., 1971).

The value of ν', the number of moles of EB bound per mole nucleotide at the minimum in the sedimentation velocity dye titration curve, is related to the superhelix density of the dye-free molecule, σ_o, by the equation (Bauer and Vinograd, 1968):

$$\sigma_o = -0.67\ \nu' \qquad (3)$$

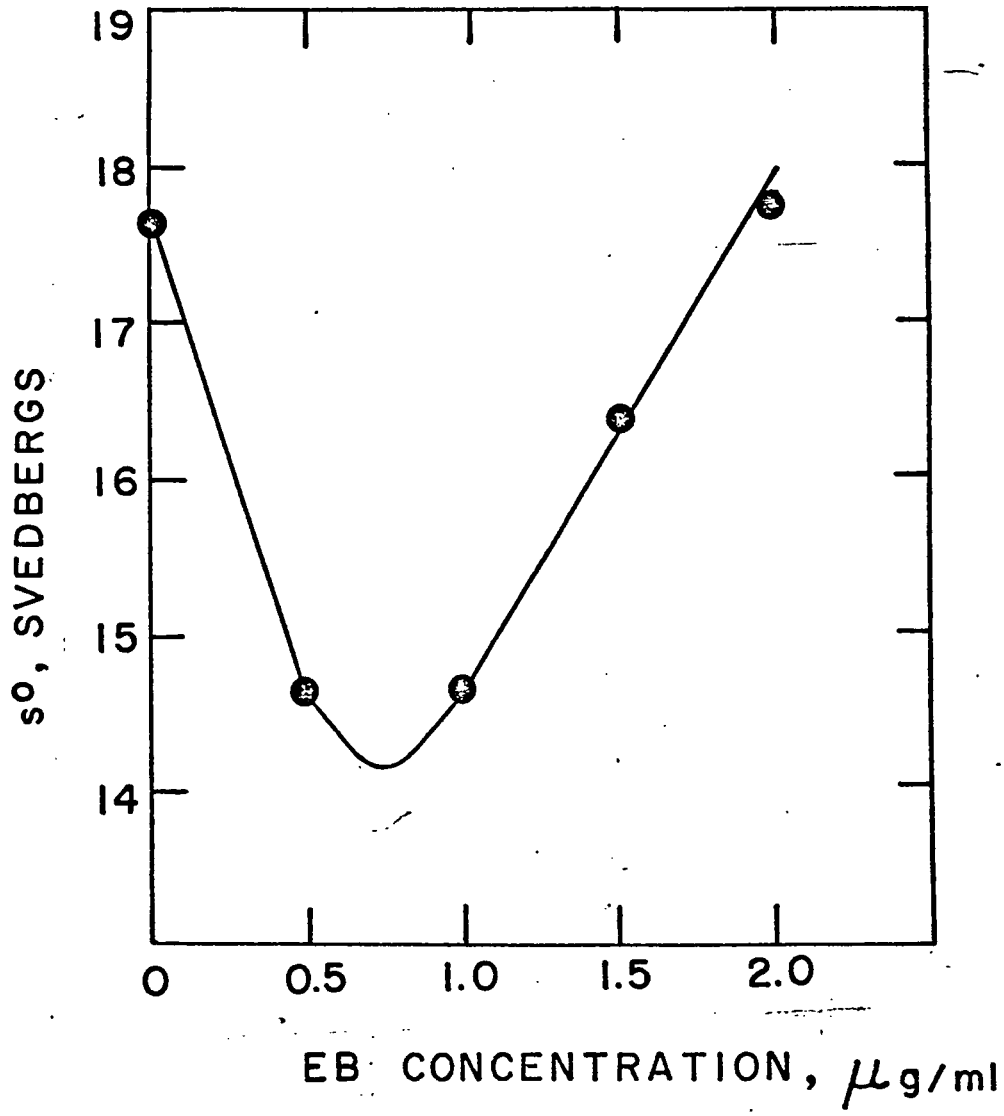
ν' was determined for the ligase closed circle by the sedimentation velocity-dye titration method (Fig. 8) under the conditions used by Gray et al. (1971) and calculated as 0.0107, assuming their values of 2.45×10^4 1/mole and 0.241 for K and ν_m, respectively, for PM2 DNA in neutral 2.83 M CsCl solution at 20°C. This value is required in the calculation of ν for the closed circle at various free dye concentrations (below). The modified Scatchard relation of Bauer and Vinograd (1970):

$$\nu = KC(\nu_m - \nu)\exp\{-[a(\nu-\nu') + b(\nu-\nu')^2]\} \qquad (4)$$

which takes into account the effect of the free energy of superhelix formation on the binding affinity of EB by a closed circular DNA, was used to obtain values of ν from

FIGURE 8

Sedimentation dye titration of the ligase closed circle in standard neutral CsCl solvent (see text) at 20°C. The s° values are uncorrected for the dye binding effects. Only one determination was made for each point.


19
18
17
16
15
14
s°, SVEDBERGS
0
0.5
1.0
1.5
2.0
EB CONCENTRATION, μg/ml

the experimental values of C, assuming a = 23 and b = 100 (Bauer and Vinograd, 1970; Upholt et al., 1971), with the aid of a plot of ν vs C calculated at various values of ν. The value of ν' here refers to that of the ligase closed circle. The s° vs c curve for the ligase closed PM2 circles of Fig. 6 is thus converted to the middle curve of Fig. 7.

The correction of s° values was made to exclude effects other than those due to the changes in supercoiling of the DNA's (mainly the EB binding effects). These would include the buoyant effect (Upholt et al., 1971), which is not expected to be very large in the solvent employed here, and the stiffening and elongation of the DNA molecule (Reinert, 1973) caused by the binding of ethidium bromide. This correction, which results in the upper curve of Fig. 7, was done simply by assuming that these effects are identical in both nicked and closed circular DNA's, and attributing all the observed decrease in s° for PM2 II to those effects. Thus, if a given level of dye binding (given value of ν) results in a decrease in s° for PM2 II from its value at $\nu = 0$ of a certain percentage, the value for the closed circle is increased, at that same value of ν, by the same percentage. The non-monotonic behavior of the sedimentation coefficient for positive superhelix densities is thus clearly established (Fig. 7, uppermost curve).

Determination of the Superhelix Density of the Ligase Closed Circular DNA

The superhelix density of the ligase closed circles was initially measured by the buoyant method of Gray et al. (1971). The relative buoyant separation between closed and nicked circular forms, Ω_c, which characterized the relative differences in the amount of EB bound, is

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

where the correction factor, f^{-1}, is unity in this case for which the reference DNA and the unknown DNA are of the same DNA species (same G-C content), $\frac{\bar{r}}{\bar{r}^*}$ is the ratio of the average distances from the axis of rotation for the unknown and reference DNA's, and $\frac{\Delta r}{\Delta r^*}$ is the value of the buoyant separation of the unknown closed circular DNA from the nicked circle divided by that for the reference closed circular DNA and nicked circle (The asterisk refers to the reference DNA.). PM2 I and II DNA's comprised the reference species, with the superhelix density for PM2 I DNA taken as -0.053 (Gray et al., 1971). With $\frac{\Delta r}{\Delta r^*}$ measured as 0.985, the value of $\Omega_c = 1.567 \pm 0.008$ was obtained, with the standard deviation resulting from numerous redeterminations of the separation between bands from several different photographs of the same unknown and reference gradients.

The corrected relative buoyant separation in CsCl density gradients containing 330 μg/ml of EB is empirically related to the difference in superhelix density as

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

σ_o, obtained from eq. (5) and eq. (6) is 0.006 ± 0.005. A positive value of σ_o is not expected. The same parameter was clearly shown in the sedimentation-dye titration of Fig. 8 to have a negative value; if the superhelix density in the absence of dye is actually positive, no minimum will occur in such titrations. With the aid of the Scatchard relation (eq. (2)), and the value for C' from Fig. 8, (0.76 μg/ml), the value of σ_o is approximated to be -0.0072 which is more reliable and is not significantly different from that obtained by the buoyant method when its standard deviation is taken into account. This value was used to calculate ν' in eq. (4) as described above.

IV.

DISCUSSION

IV. DISCUSSION

Viscometric Dye Titration of Ligase Closed Circles

Ostrander and Gray (1973) have demonstrated that the Huggins constants for nicked and closed circular PM2 DNA's are not significantly different from zero at moderate to high salt concentrations in a series of pH titrations performed with a Zimm-Crothers low-shear rotating cylinder viscometer. Révet et al. (1971) have shown the effects of DNA concentration on the reduced viscosity for PM2 I and II DNA's, obtained by viscometric dye titrations in a capillary viscometer of average shear gradient $\sim$1000 sec^{-1}, to be rather small. It is known that high shear gradients will decrease the Huggins constant (Crothers and Zimm, 1965). Inasmuch as the reduced viscosity is weakly dependent upon DNA concentration and only qualitative results (presence or absence of non-monotonic behavior at positive superhelix densities) were desired, we have reported flow times directly as a function of dye concentration. The correct relative reduced viscosities between various data points in the curve of Fig. 1, however, are not obtained due to the high shear gradient in the capillary viscometer, which has a greater effect on circles of low superhelix density than on more highly supercoiled circles, giving rise to shear dependence of the relative intrinsic viscosities as well as of the intrinsic

viscosities themselves. The increasing and unknown concentrations of nicked circles in the titration mixture (below) further preclude interpretation of the data of Fig. 1 as relative reduced viscosities as a function of dye concentration.

The behavior of the viscosity of closed circular PM2 DNA at positive superhelix densities is seen to be non-monotonic in Fig. 1. The initial decrease represents the initial positive supercoiling of this DNA; the viscosity is decreasing from that of the relaxed closed circle in this region. A closed circular DNA of higher absolute superhelix density (more negatively supercoiled) will of course display a maximum viscosity as the molecule passes through the fully relaxed conformation in going from negative to positive superhelix densities, as clearly displayed for PM2 I DNA in Fig. 3. That the ligase closed circular DNA of these studies fails to display a maximum in viscosity (Fig. 1) or minimum in sedimentation coefficient (Figs. 6, 7) in the 50% D_2O solvent must be attributed to the difference in solvent conditions from those of Fig. 8, in which the molecule is clearly negatively supercoiled. This point is discussed more fully later.

The increase in viscosity observed in the curve of Fig. 1, beginning at an EB concentration of about 3 μg/ml, comprises strong evidence that the hydrodynamic behavior of

closed circular DNA is non-monotonic with increasing positive superhelix density. It is difficult to attribute this increase to increasing percentages of nicked circles (below), as the curve clearly levels off above an EB concentration of about 14 μg/ml.

The failure to obtain a decrease in viscosity after a local maximum is reached is presumed due to the presence of increasing amounts of nicked circular DNA arising during the course of the titration. Assuming the viscous increments due to the two forms to be additive, corrections of the observed flow times for percent of nicked circles could in principle be made so that a decrease in viscosity should be observed in the region of higher dye concentrations. However, due to the difficulty of obtaining the intrinsic viscosity of the pure closed circle at various dye concentrations in a solvent system of such low salt concentration and high dye concentrations, in which nicking is appreciable during the time course of an experiment, as well as the difficulty in obtaining the percent of nicked circles at any given point in the titration, such corrections were not attempted. Sedimentation velocity dye titrations, which are not affected by the presence of nicked circles, were thus required to further investigate the non-monotonic behavior of the conformational change of the macromolecule as a function of positive superhelix density.

Sedimentation Velocity-Dye Titration of Ligase Closed Circles

The data of Fig. 6, when corrected to the upper curve of Fig. 7 as previously described, clearly display a non-monotonic dependence of s° upon increasing positive superhelix density. The initial increase in s°, up to $\nu = 0.015$, represents the initial introduction of positive superhelical turns into the molecule, as is also seen for native PM2 I DNA in Fig. 5 in the same solvent; the absence of the point corresponding to $\sigma = 0$ in Figs. 6 and 7 is due to the effects of changing solvents as already mentioned. The decrease to the local minimum at $\nu = 0.042$ in the upper curve of Fig. 7 is followed by a monotonic increase at all higher values of the molar binding ratio.

It is recognized that the increase in s° after the local minimum in Fig. 7 (but not the presence of the non-monotonic behavior) depends upon the correction for the effects of bound ethidium upon factors other than supercoiling. This, in turn, depends upon the assumptions that such effects can be separated from those upon supercoiling, and are the same, at a given value of ν, as the effects upon the nicked circle. In addition, the numerical values of the various parameters of eq. (4), both measured in this work and taken from the literature, are in most instances subject to substantial experimental uncertainty. It may further be pointed out that the value for ν' used in eq. (4) refers to the DNA in

the 2.83 M CsCl solvent; and not in the 50% D_2O solvent; a zero or negative value of ν', although the latter would be difficult to interpret physically, is actually required (see below). Accordingly, the corrections employed in the upper curve of Fig. 7 have been repeated employing somewhat different values of the intrinsic binding constant K, which substantially changes the values of ν calculated in eqs. (2) and (4). A value of 7.0×10^5 1/mole may be very crudely extrapolated for the conditions of these experiments (ignoring any possible effects of the D_2O) from the data of Lepecq and Paoletti (1967); although seven times larger than the value of K from these studies, the qualitative behavior of the upper curve of Fig. 7 is still observed. Finally, ν' has been set equal to zero in eq. (4) and the calculation repeated with the same qualitative result. Likewise, the arbitrary use of K set equal to 0.2×10^5 liters/mole does not abolish the non-monotonic dependence of s° upon ν. Thus, the result here obtained does not depend upon precise measurement of the experimental variables involved nor upon the exact validity of the assumptions above.

The effect of the change in solvent from that of Fig. 8 (2.83 M CsCl) to the 50% D_2O solvent (0.1 M in NaCl) may be roughly estimated. Wang (1969a) and Upholt *et al.* (1971) have made use of the dependence of s° of supercoiled closed circular DNA upon the number of superhelical turns to estimate

the effects upon σ_o of changes in temperature, counterion, and ionic strength. Both groups obtain an increase of the superhelix density (this corresponds to the unwinding of a negatively supercoiled molecule) of 0.002 in going from 2 M CsCl to 2 M NaCl; this will be taken as the difference which would be observed in going from 2.8 M CsCl to 2.8 M NaCl. Upholt et al. (1971) give data in Figs. 5 and 7 of their paper which may be crudely calculated to yield an increase in superhelix density of 0.003 in going from 2.8 M NaCl to 1.0 M NaCl; this is based on their measurement of the ratio s°_I/s°_{II} (sedimentation coefficient of the closed circle relative to that of the nicked circle) for artificially closed circles of SV40 DNA as a function of superhelix density in 1.0 M NaCl, and the measurement of the same ratio for a closed circle of few superhelical turns as a function of NaCl concentration. In going from 1.0 to 0.1 M NaCl, the low superhelix density closed circle yields no further information, as it is too near the principal minimum of the s° vs σ_o curve; highly supercoiled DNA, however, does display a dependence of s° upon NaCl concentration in the 0.1 to 1.0 M NaCl concentration range which allows a further increase in σ_o of 0.003 in going from 1.0 to 0.1 M NaCl to be estimated. With the temperature unchanged at 20°C and the effects of D_2O upon supercoiling unknown and thus necessarily neglected (it seems reasonable that this would not produce a very

large effect), a total increase in σ_o of 0.011 is estimated in changing between the solvent of Fig. 8 and that of Figs. 6 and 7. With σ_o estimated at -0.007 from Fig. 8, it stands to reason that the ligase closed PM2 molecule of these studies might have a small positive superhelix density (estimated at 0.004), in the absence of EB, in the 50% D_2O solvent. This is therefore offered as the explanation as to why the viscosity and sedimentation titration curves do not pass through a principal maximum and minimum, respectively. The value of employing a closed circular DNA of low superhelix density, especially with respect to the sedimentation studies, lies in the fact that a fraction of the available dye binding sites do not have to be occupied before the molecule acquires a positive superhelix density, as would be the case had native PM2 I DNA been employed (e.g., had the titration of Fig. 5 been extended to higher EB concentrations). The advantage, as for the use of the low ionic strength solvent as discussed in MATERIALS AND METHODS, is that dye concentrations at which a local minimum might be observed can be reached before the background absorption due to the dye becomes unworkably large for analytical centrifugation using absorption optics. It is likely that attempting to extend the titration of Fig. 5 would have led to marginal and possible even negative results.

In the s° vs σ_o curves demonstrated by Upholt et al. (1971) and Wang (1969a), the s° values correspond to those

of closed circular DNA's with different superhelix densities in the absence of dye. It is, however, impossible to perform this type of experiment at positive superhelix densities, since no method for producing dye-free closed circles containing substantial numbers of positive superturns has been discovered to date. When $s°$ *vs* σ or ν curves converted from $s°$ *vs* c curves obtained from a series of sedimentation dye titrations of closed circles of different (negative) superhelix densities, and involving different DNA species, were superimposed on a single plot of $s°$ *vs* σ_o, very similar shapes and closely overlapping local minima and maxima of the curves were observed (Upholt *et al.*, 1971). Thus, we have converted the $s°$ *vs* c curves to the more meaningful $s°$ *vs* ν curves in order to better envision the non-monotonic behavior at positive superhelix densities, as well as to carry out the correction for the effects of bound dye. This conversion substantially expands the "hump" of the curve, and contracts the region of higher dye concentrations (*e.g.*, Figs. 6 and 7). The final curve of $s°$ *vs* ν (Fig. 7) is qualitatively similar to the plots of $s°$ *vs* ν obtained by Upholt *et al.* (1971) in their EB titration of native PM2 I DNA in 2.83 M CsCl. The absolute values of σ for the local maxima and minima for negatively and positively supercoiled DNA's may be estimated from the foregoing and from Fig. 3 of Upholt *et al.* (1971). The plot of $s°_{20,w}$ *vs* ν in the

latter figure may be converted to a plot of $s^{\circ}_{20,w}$ *vs* σ_o simply by using the formula

$$\sigma - \sigma_o = 0.67\nu \qquad (7)$$

where σ and ν are the superhelix density and dye binding ratio, respectively, at any EB concentration. With σ_o = -0.053 as before, the above figure gives σ = -0.017 and -0.035 for the local maximum and minimum, respectively. Taking σ_o = +0.004 for the ligase closed circle of these studies in the 50% D_2O solvent (above) and the values of ν from Fig. 7, the local maximum and minimum for positively supercoiled PM2 DNA occur at σ = +0.028 and +0.052, respectively. Considering the experimental errors involved, the agreement may be considered excellent, and gives credence to the idea that the curve of s° *vs* σ_o is symmetric about σ_o = 0.

The value for ν' for native PM2 I DNA, obtained from the data of Figs. 3 and 5, is 0.078 ± 0.004, compared with 0.080 ± 0.005 obtained in the 2.83 M CsCl solvent by Gray *et al.* (1971). Although these values themselves are in agreement, the increase in σ_o predicted above for the solvent change would yield a value of 0.063 for PM2 I DNA in the D_2O solvent. Even though little confidence can be placed in the numerical value obtained for the effect of the solvent change, the assessment of the direction of the effect is undoubtedly correct.

It is seen from these studies that the use of the method of Révet et al. (1971) for determination of ν' and C' requires the additional step of a sedimentation titration to obtain C' if low ionic strength solvents are employed; this is simply because the higher binding constant results in a value of C' which is too near zero to be determined accurately by the viscometric titration method itself.

J. C. Wang (personal communication) has obtained evidence that the true angle of intercalation of EB in duplex DNA is approximately 26°; all superhelix densities to date have been obtained assuming an intercalation angle of 12° (e.g., eq. (3) is obtained using this value). If the above is true, then all superhelix densities calculated to date are low in absolute value by a factor of approximately two. It should be pointed out that none of the salient conclusions drawn from these studies would be altered by changing the value of the intercalation angle, as this does not affect the correction of the data of Fig. 6 to that of Fig. 7, and positive and negative superhelix densities (e.g., in the comparison above for minima and maxima) would be changed by the same factor.

The explanation for the non-monotonic dependence of s° upon σ_o proposed by Upholt et al. (1971) was reviewed in the INTRODUCTION. The data presented here, which firmly support the existence of a non-monotonic dependence of

hydrodynamic properties of closed circular DNA upon σ_o at positive superhelix densities, are amenable to the exact same explanation. It will probably not be possible to demonstrate conclusively whether the curves are indeed symmetric about $\sigma = 0$, with local minima and maxima at the same absolute superhelix densities, unless a method is devised for the production of closed circular DNA's which are highly positively supercoiled in the absence of intercalating agents. The good correlation between plots of s° vs σ at negative superhelix densities observed independently of whether a family of DNA's of different σ_o's or a dye titration of a single closed circular species is involved (Upholt et al., 1971), is a further reason for confidence in the qualitative validity of the conclusions drawn from these studies.

V.

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V. REFERENCES

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