Carbodiimide modification of superhelical PM2 DNA: considerations regarding reaction at unpaired bases and the unwinding of superhelical DNA with chemical probes

J. Lebowitz*, A. K. Chaudhuri†, A. Gonenne† and G. Kites†


Received 15 December 1976

ABSTRACT

Superhelical PM2 DNA I can be modified with N-cyclohexyl-N'-B-(4-methylmorpholinium)ethyl carbodiimide (CMC). The transition of the sedimentation coefficient uncorrected for buoyant density change \( S_{20,*} \) vs. \( \% \) reactivity in terms of base pairs shows the following characteristics. The \( S_{20,*} \) increases by 4.5 S units upon 1% modification. There is a plateau in \( S_{20,*} \) between 1 and 4% reactivity. The extent of reactivity was determined by buoyant density and \(^3^C\) radioactive CMC binding measurements. Further reactivity was not explored since Pulleyblank and Morgan's (22) data of \( S_{20,*} \) vs. \( \% \) reactivity from 6 to 34% was previously published. The initial results obtained in this study are complementary to the cited results of the above authors. Consequently, both sets of data taken together represent a complete description of \( S_{20,*} \) vs. \( \% \) reactivity with CMC. It is shown that the model in which superhelical DNA is proposed to contain small intrastrand hairpin regions can be extended to account for the observed transitions in \( S_{20,*} \) vs. reactivity.

INTRODUCTION

Examination of the secondary structure of superhelical DNA has revealed sensitivity to chemical probes for unpaired bases, single strand specific nucleases, and DNA binding proteins. These studies have been reviewed in considerable detail recently and a model has been presented to interpret the results of chemical, nuclease and DNA protein binding probes of single strand character in superhelical DNA. In brief, it was proposed that supercoiling produces interrupted secondary structure at a critical superhelix density. If sufficient intrastrand complementarity exists due to regions of high AT content or regions of two fold symmetry then small intrastrand hairpin regions would be stabilized. This would produce localized sites for the action of single strand nucleases or chemical probes. It is envisioned, in the latter case, that reagents specific or preferential for unpaired bases react at the hairpin sites producing localized helix-coil transitions which increase the flexibility...
of the DNA substantially. This could account for a variety of reagents, of widely differing molecular weights, producing similar changes in sedimentation velocity with very limited reactivity.

In order to extend our abilities to explore superhelical DNA it is necessary that a variety of different experimental approaches be utilized. In particular, we would like to extend our capacity to study structure-function relationships of supercoiled DNA. A major question that arises, if the hairpin model is correct, is the role unpaired bases play in biological function. Two areas of considerable interest regarding superhelical DNA are transcription and recombination. In the latter case, it is striking that a large number of circular molecules have the capacity to integrate their host cell chromosome. A recent study of the binding of homologous ϕX-DNA fragments to superhelical ϕX-RF suggests that supercoiling may promote a recombination event. Do unpaired regions act to initiate this process? In regard to transcription, considerable evidence has been amassed showing enhanced template activity for negative superhelical DNA (see subsequent paper for review). Again, it is not difficult to envision a role for unpaired bases in the transcription process.

An extremely useful approach to structure-function relationships is the chemical modification of superhelical DNA at unpaired sites; removal of excess reagent, followed by an analysis of the modified DNA in a biological assay. In order to pursue this approach we have explored the reactivity of N-cyclohexyl-N'-β-(4 methylmorpholinium)ethyl carbodiimide (CMC) with superhelical DNA. This reagent reacts with unpaired guanine and thymine (uracil) residues at the imino site. Figure 1 shows the product of the reaction with uracil. It is clear that this bulky reagent requires unpaired bases since it can not be accommodated without either transient distortion of the Watson-Crick structure or preexisting interrupted secondary structure.
Studies with t-RNA have revealed reaction only at sites which are unpaired and exposed. Consequently this reagent appeared ideal for the modification of supercoiled DNA. Furthermore studies with SV40 DNA reveal that the superhelical form (FI) can be modified with CMC. In addition, the location of CMC sites was determined using the Hind restriction endonuclease. The CMC containing fragments also contain sites susceptible to S\(_1\) endonuclease.

In the case of PM2 DNA, CMC has also been utilized to measure the sense and magnitude of the superhelical content. In order to promote the CMC reaction Pulleyblank and Morgan utilized 50% ethanol to weaken the secondary structure of the DNA. These conditions did not allow these authors to detect the initial reactivity with CMC and the sedimentation velocity changes produced by this modification. In this study we will characterize this initial reactivity utilizing analytical ultracentrifugation measurements and sedimentation velocity and buoyant density. The latter can be used to measure the binding of CMC and this will be compared to the amount of radioactive \(^{14}\)C-labeled CMC bound under identical reaction conditions. It will be shown that our results can be easily extended with the addition of Pulleyblank and Morgan's data, allowing for a complete description of the transition of sedimentation velocity vs. percent reactivity of base pairs with CMC.

**MATERIALS AND METHODS**

**Preparation of PM2 DNA (Form I).** PM2 DNA form I was prepared as described by Espejo, and Dean and Lebowitz. Both form I and form II DNA were dialyzed against and stored in 0.1 M sodium borate (pH 8.0) at -20°.

**Chemicals and Isotopes.** All chemicals were reagent grade and buffers were prepared in deionized water. N-cyclohexyl-N'-e-(4-methylmorpholinium) ethylcarbodiimide p-toulene sulfonate (mol. wt. 423.5) was purchased from Aldrich Chemical Co. \(^{14}\)C-labeled CMC (specific activity 2.07 mC/mM) was prepared by the New England Nuclear Corp. (Boston, Mass.) custom synthesis laboratory, using 1-cyclohexyl-3-[2-morpholino(4)-ethyl]carbodiimide that was prepared by Dr. N. P. Salzman (NIH) following published procedures. This intermediate carbodiimide was converted to CMC by reaction with \(^{14}\)C-labeled methyl toluene sulphonate in the New England Nuclear Corp. laboratory.

**Analytical Ultracentrifugation.** Band sedimentation velocity experiments were performed in a Beckman Model E ultracentrifuge as previously
described. The sedimentation solvent was 2.8 M CsCl, 0.01 M sodium borate, pH 8.0. Sedimentation coefficients ($S_{20,w}$), assuming constant buoyant density, were corrected to sodium DNA according to the method of Bruner and Vinograd. A rigorous application of this empirical method for evaluating $S_{20,w}$ values requires a knowledge of the buoyant density and a linear decrease of the uncorrected $S$ value times the relative viscosity versus the density of the sedimentation solvent. Although we have obtained buoyant density values for CMC modified DNA an examination for the linear dependence of sedimentation velocity vs. density would have required a vast amount of data. Hence, for comparative purposes with previous data in the literature it was decided to utilize the Bruner-Vinograd procedure assuming a constant buoyant density since sufficient data was not available for a rigorous test of the correction procedure.

Reaction of CMC with PM2 DNA. Metz and Brown employed extremely high concentrations of DNA to drive the reaction of $^{14}$C-labeled CMC to completion. For example, denatured calf thymus DNA at a DNA molar phosphate or nucleotide concentration of $5.30 \times 10^{-3}$ ($N_T$) was reacted with 0.05 Molar $^{14}$C-CMC. The rate did not reach a plateau until 8 hours. Consequently this second order reaction is extremely slow. In order to achieve a detectable reaction at lower concentrations of PM2 DNA we utilized the following procedure. The modification of PM2 DNA FI or FII was carried out at 30°, unless otherwise indicated, using a 500-fold excess molar ratio of CMC to DNA molar phosphate or nucleotide concentration ($N_T$). The latter was always between $0.75 \times 10^{-4}$ and $2.27 \times 10^{-4}$ $N_T$. Aliquots were withdrawn at indicated times and the modified DNA was purified from any excess CMC by either chromatography using Bio-Rad G-25 Sephadex or by exhaustive dialysis against 1M NaCl, 0.01 M phosphate buffer, pH 5.9.

Radioactive $^{14}$C-CMC binding was analysed with a scintillation fluid mixture prepared as follows: 600 ml toluene, 300 ml Triton-X, 100 ml water and 42 ml Spectrofluor. The amount of $^{14}$C-labeled CMC bound to PM2 DNA, after background subtraction, was converted into disintegrations per minute after correcting for the counting efficiency. This data was converted into moles of CMC per mole of DNA nucleotide for comparison with the buoyant density results.

Buoyant Density Experiments. Buoyant densities of the chemically modified DNAs were determined at 20°C and 44,000 rpm. The solvent for buoyant density experiments was prepared by dissolving appropriate amounts of CsCl in 0.01M phosphate buffer, pH 5.9. This buffer was chosen because
carbodiimide-modified DNA is more stable at this pH during the long time of equilibrium sedimentation. M. lysodeikticus DNA was used as a marker and its buoyant density was taken as 1.7245. Buoyant densities of CMC-modified DNAs with reference to marker DNA was calculated following the procedure described by Szybalski which corrects all buoyant density values to the center of the cell. The appropriate correction factors were taken directly from the standard curve for CsCl at 44,000 rpm as determined by Szybalski.

Partial Specific Volume Measurement of CMC. The partial specific volume of CMC was determined using a Mettler-Paar DMA02D precision density meter. The details of the use of this mechanical oscillation technique for partial specific volume measurements have been described by Kratky et al. Ten measurements were made at 20°; the average partial specific volume of CMC is 0.81387 ± 0.007 in 0.04 M Tris buffer, pH 7.9. Since the thermodynamically correct neutral component is CMC-C1 for the buoyant density analysis we attempted to evaluate the partial specific volume of CMC in the appropriate CsCl solutions. Unfortunately, we could not handle the extremely high density solutions without appreciable errors. However, the partial specific volume of .814 was utilized previously to evaluate CMC binding to SV40 DNA and this was compared to 14C-labeled CMC binding with excellent results (5,21). In addition, % reaction of CMC has been obtained using Δρ vs. ΔA290. A comparison of this approach with our data from the equations below reveals excellent agreement. For example, a Δρ of .0029 and .0027 (this study) was found to produce 1% modification of the basepairs. A Δρ of .013 and .012 (this study) was found to produce 4.5% modification. The ability to determine CMC binding by three independent methods with excellent agreement strongly supports the use of the measured partial specific volume of CMC in the analysis below as well as the capacity to make a composite of the hydrodynamic data (Figure 6).

Binding Analysis from Buoyant Density Data. The extent of reaction between a small molecular species and a buoyant macromolecule can be calculated from buoyant density shifts according to the procedure described by Bauer and Vinograd. The first step in the calculation is the evaluation of the change in hydration Δr and the net hydration r upon binding CMC. This is done by using equations (101a) and (101b) of the above study and these are repeated below:

Δr (θ) = 0.04092-0.7282 + 4.592n^2-16.08n^3 + 20.56n^4  \quad (1)

and

r = r_0 + Δr (θ) - Δr (θ_0)  \quad (2)
where $n = 0-1.5$ and $\theta$ is the buoyant density. In our study $\Delta r^-(\theta)$ and $\Delta r^-(\theta_0)$ are the hydration changes of PM2 DNA with bound carbodiimide and the unbound DNA relative to phage T4 DNA in CsCl, respectively. $r^0$ and $r^-$ are net hydration of native DNA and carbodiimide-reacted DNA respectively. In order to obtain $r^-$ equation (1) is used twice, once to calculate $\Delta r^-(\theta_0)$ for unreacted PM2 DNA and finally with the measured $\theta$ value for DNA containing CMC. The value of $r^0$ is calculated by the relationship of Vinograd and Hearst:

$$\theta = \frac{1 + r^0}{(\tilde{v}_3 + r^0 \tilde{v}_1)}$$

where $\tilde{v}_3 = 0.479$, the partial specific volume of anhydrous DNA and $\tilde{v}_3 = 1.0$, the partial specific volume of water. This completes the evaluation of $r^-$ to be used below. $\nu^-$, the net weight binding ratio for CMC to PM2 DNA for a given measured buoyant density, is calculated using equation (19) of the Bauer and Vinograd analysis:

$$\nu^- = \frac{1 + r^- + \nu^-}{(\tilde{v}_3 + r^- \tilde{v}_1 + \nu^- \tilde{v}_4)}$$

where $\tilde{v}_4 = 0.814$, the partial specific volume of CMC determined above.

Finally, $\nu^-$ is multiplied by the molecular weight ratio of an average cesium-bound nucleotide and CMC to obtain the number of moles carbodiimide per mole nucleotide.

**RESULTS**

**Sedimentation Velocity Analysis.** Studies with probes of the structure of superhelical DNA have shown that the initial reactivity produces an increase in sedimentation velocity. This is also true for CMC reactivity with SV40 DNA. Consequently, changes in $S_{20,*}$ were used to monitor the reaction of CMC with PM2 DNA. When PM2 DNA is incubated at 30° with a 500-fold excess of CMC and aliquots examined as a function of time we observed the changes in $S_{20,*}$ for FI shown in Figure 2. The superhelical form increases its $S_{20,*}$ value from 28.5 to 33 S. However there is no change in the $S_{20,*}$ value of PM2 DNA F II during the time in which FI undergoes a large increase in sedimentation velocity. The data do suggest a possible increase in $S_{20,*}$ for FII after 20 hours. The results show that the superhelical form appears to be far more hydrodynamically sensitive to chemical modification than the nicked circular form.

This increased sensitivity of FI as judged by $S_{20,*}$ criteria could be misleading since we have no measure of the relative structural sensitivities of DNA I and DNA II to CMC modification. Consequently, it is essential that we have a direct analysis of the amount of CMC covalently bound to each form of PM2 DNA. This was done by two different experiments, buoyant...
density measurements and a binding analysis using $^{14}$C-labeled CMC.

Carbodiimide binding analysis using buoyant density and radioactivity measurements. Separated DNA I and DNA II were reacted with CMC under the conditions described in the legend to Figure 3. It is clear from the buoyant density analysis that the reaction with CMC is readily detectable, producing a shift in density from 1.6984 to 1.6868. The shift for FII is only .0003.

It is easier to interpret this data when one converts the buoyant density values to moles of CMC bound per mole of nucleotide. This is done as described in Materials and Methods using the measured $\bar{v}_4$ of CMC. The data are presented in Figure 4 along with the analysis of the reactivity of $^{14}$C-labeled CMC with form I and II of PM2 DNA under identical conditions. It is quite clear that the buoyant density analysis of Bauer and Vinograd and the measurement of bound CMC radioactivity give identical results. Furthermore the substantial $S_{20,\text{w}}$ change of FI occurs upon 1.0% of the nucleotides undergoing a reaction with CMC (8 hour point). It is seen in Figure 4 that .2% of the nucleotides are reactive in FII with the possibility of a slow increase with time.

Figure 2. Changes in sedimentation velocity ($S_{20,\text{w}}$) of superhelical (O) and nicked (•) PM2 DNA as a function of reaction time with CMC. Reaction carried out at 30° in .1M Borate, pH 8.0 [N] = $1.5 \times 10^{-4}$M and [CMC] = $7.5 \times 10^{-2}$M.
Figure 3. Buoyant density changes of closed supercoiled (O) and open nicked (●) PM2 DNA as a function of reaction time with Carbodiimide. Reaction carried out at 30°C in 0.1 M Borate, pH 8.0 \([N_T] = 2.27 \times 10^{-4} M\) and \([CMC] = 1.13 \times 10^{-4} M\).

Figure 4. Moles of CMC bound per mole of nucleotide as a function of reaction time. The symbols for the respective analysis, buoyant density or \(^{14}C\) labeled CMC binding are shown at the top of the Figure.

Sedimentation velocity changes of PM2 DNA in the presence of CMC as a function of temperature. The \(S_{20,\text{w}}\) transition shown in Figure 2 was performed at 30°. It was of interest to examine the reactivity of CMC at a range of temperatures from 25° to 50°, using \(S_{20,\text{w}}\) as a criteria for modification. PM2 FI and FII were incubated at selected temperatures for 24 hours, the sample was cooled and the \(S_{20,\text{w}}\) determined. Appropriate
Figure 5. Changes of sedimentation velocity of closed supercoiled (○) and nicked (•) PM2 DNA as a function of reaction temperature. Each experiment was carried out for a 24 hour period in 0.1 M Borate, pH 8.0. \([N_T] = 1.5 \times 10^{-4}\) and \([\text{CMC}] = 7.5 \times 10^{-2}\text{M}\). Control experiments for supercoiled (○) and nicked (■) DNA without any CMC.

controls of DNA incubated at the same temperature without CMC were also performed. The results are shown in Figure 5.

Form II appears to react at 25°, after 24 hours, but further increases in \(S_{20,*}\) are very gradual as a function of temperature. Form I shows a 3 unit \(S\) increase at 25° and further increases as reactivity is promoted by incubation at higher temperatures. Note, that heating in the presence of CMC produces a curve for the \(S_{20,*}\) vs. reaction temperature whereas incubation at 30° produces a plateau in \(S_{20,*}\) vs. time.

DISCUSSION

In the introduction we cited that CMC reactivity with superhelical DNA was performed by another laboratory to measure both the sense and magnitude of the superhelical turns in this DNA. Pulleyblank and Morgan\(^{22}\) used a large excess of CMC (10% W/V) in the presence of 50% ethanol at room temperature. They measured % reaction from a linear calibration curve of spectral change (\(\% \Delta A_{290}\)) vs. the buoyant density change. The spectral analysis requires exchanging p-toluene sulphonate for the bromide anion which does not contribute significantly to the optical density. A comparison of the buoyant density change vs. % reactivity between their results and ours shows excellent agreement (see Materials & Methods). However, since a denaturing solvent was present it enhanced the rate of the CMC reaction with the inability to detect the initial 6% binding and
the $S_{20,*}$ changes produced by this modification. Consequently, they reported $S_{20,*}$ vs. % reactivity starting with a value of 6.5% modification in terms of base pairs. Since we have been able to measure the initial 4.0% reactivity in terms of base pairs, we can plot both sets of results for $S_{20,*}$ vs. CMC reactivity to obtain a complete analysis of the sedimentation velocity behavior of PM2 FI upon CMC modification. This is shown in Figure 6.

It is apparent that both studies are complementary and do present a complete picture of the $S_{20,*}$ behavior vs. % reaction of CMC. Hence, the major observations of CMC modification of PM2 DNA FI are as follows:

i. CMC produces a large increase in the $S_{20,*}$ of superhelical PM2 DNA with a reactivity of 1% of the base pairs.

ii. Further reactivity from 2 to 4% of the base pairs occurs which is not accompanied by any $S_{20,*}$ change (plateau region Figure 6), but can be clearly detected by buoyant density or radiolabel measurements.

iii. The agreement is excellent between the CMC buoyant density binding data and the $^{14}$C-labeled CMC binding measurements.

iv. The plateau region is followed by a sharp decrease in $S_{20,*}$ which undoubtedly represents the major loss of negative supercoils followed by

![Figure 6. Changes in $S_{20,*}$ for superhelical PM2 DNA vs. % reaction with CMC in terms of base pairs. (•) are the results of this study (Figures 2 & 4) and (■) are the published results of Pulleyblank and Morgan, ref. 22.](image)
the introduction of positive superhelical turns. Reactivity above 20% produces a fast sedimentating denatured structure. Alkali, HCHO and \( \text{CH}_3\text{HgOH} \) all produce similar effects.

In order to analyze the \( S_{20,*} \) vs. % reaction produced by CMC we must review some previous considerations regarding superhelical DNA. It was proposed by Upholt, Gray and Vinograd\(^{29} \) that superhelical DNA behaves like an extended tightly wound linear molecule hydrodynamically at or near the native superhelix density. They viewed the removal of superhelical turns as first producing a decrease in rigidity which is accompanied by an increase in \( S_{20,w}^* \). This can be viewed as a transition from the rigid extended form (rod) to a spherically coiled form (wormlike coil). Additional unwinding continues to remove superhelical turns and opens the DNA into an open circular coil with a large change in hydrodynamic volume. This change from a wormlike coil to an open circular form appears to be a characteristic transition for the loss of the majority of the superhelical turns. The above interpretations were developed to account for the behavior of superhelical DNA assuming no interruptions in secondary structure. In order to account for the reactivity of \( \text{CH}_3\text{HgOH} \), Woodworth-Gutai and Lebowitz\(^4 \) have extended the hydrodynamic considerations of Upholt et al. by proposing the introduction of small hairpin regions in superhelical DNA. Any disruption of these sites by chemical modification leads to large changes in \( S_{20,*} \) due to increased flexibility as defects are generated by the localized melting of the hairpins. It was shown that this model could account for a variety of observations regarding the reactivity of superhelical DNA. We now propose to extend the hairpin model by an examination of the possible reaction paths available for chemical modification of superhelical DNA and the hydrodynamic changes that can be anticipated as reactivity proceeds.

The reaction of any chemical probe will be a competition between pre-existing hairpin regions and sites that become available due to a local transient unwinding of the intact duplex, i.e. breathing of the secondary structure. Consequently two reaction paths exist. Path A is the reaction with any hairpin site. Path B can be described by the reaction scheme shown for the opening and closing reactions of the cooperative "breathing" unit (one or more base-pairs), \( k_3 \) represents the second order rate constant of the reaction of the probe with the transiently open form, and \( k_4 \) represents the first order rate constant of the back reaction if it occurs. If \( k_2 > k_3 \) and \( k_4 \) then it can be shown\(^{30} \) that the observed rate constant
equals the following expression

$$k_{\text{obs.}} = \frac{k_1 \cdot k_3}{k_2} = k_{\text{conf.}} \cdot k_3$$  \hspace{1cm} (5)

Equation (5) simply states that the observed rate of reaction of a chemical probe is equal to the probability or fraction of open conformations (open/closed) times the rate of reaction of the unpaired state with the probe, i.e. the rate of the reaction with single stranded DNA. Therefore a separate measurement of $k_{\text{obs.}}$ and $k_3$ for duplex and denatured DNA respectively allows one to measure $k_{\text{conf.}}$, the extent of transiently open conformations. Obviously, if a DNA molecule contains a fraction of unpaired bases than the initial observed rate $k_{\text{obs.}}$ will be comparable to single stranded DNA and this will lead to an abnormally high $k_{\text{conf.}}$. This is true for PM2 F1 DNA\(^3\) where $k_{\text{conf.}}$ is .55-.60 for the initial HCHO reactivity at 30°. The $k_{\text{conf.}}$ for duplex DNA without any defect has been estimated from theoretical considerations\(^3\) to be $10^{-4}$. Quite clearly it can be concluded that if 55-60\% of the base pairs were transiently open at 30° reactivity would rapidly denature the DNA in a very cooperative melting transition. Furthermore, the hydrogen exchange of F1 PM2 DNA is similar to FII showing that most of the slowly exchanging hydrogens are not affected by supercoiling.\(^3\) This supports the existences of two types of structure and readily supports the reaction scheme proposed in Figure 7. Reaction path A is highly favored over reaction path B and reagents specific or

![Figure 7](image_url)

**Figure 7.** A diagram of the reaction paths of a chemical probe with superhelical DNA which leads to the unwinding of superhelical turns and finally to the denatured or condensed closed form I DNA. Path A is self explanatory and Path B is described in terms of respective rate constants for the reaction of transiently open duplex sites.
preferential for unpaired bases will react with preexisting hairpin sites. Modification of these sites leads to localized melted regions. If the rigidity of the DNA is decreased by the creation of new flexibility points in the molecules we would anticipate a significant decrease in frictional coefficient, i.e. a decrease in the persistence length of the DNA. This would account for the sharp transition in $S_{20,w}$. We now attempt to visualize the next stage or stages of reactivity and the effects produced on the hydrodynamic behavior of FI.

The mechanism of DNA unwinding under the action of a slowly reacting agent has been studied theoretically and experimentally. In brief, the reaction is first characterized by path B, modification of transiently exposed bases preventing reformation of base pairing. These defective sites can now act as nucleation sites for further unwinding. Naturally, if defects preexist in the DNA propagation of unwinding will occur from these sites and the initial rate of unwinding will be enhanced. It is our view that the shape of the $S_{20,w}$ vs. % reactivity curve reflects the relative rates involved in the respective paths for unwinding superhelical DNA. If the number of defects in the DNA (all reacted sites) is the rate limiting step then it would be anticipated that path A, reaction at hairpin sites, would occur rapidly and a lag period would occur which requires the accumulation of additional reaction sites via path B. The cooperative melting occurs with defects acting as the centers of unwinding. This would explain the plateau region which we propose to be the accumulation of small defects which are apparently not sufficient to change the sedimentation velocity of the flexible wormlike coil until sufficient supercoiling is lost and there is a conversion to the open circular form.

It is possible that the plateau region is a balance of different effects as the DNA undergoes a complex set of events which can result in a loss of supercoiling and changes in chain flexibility, hydration, partial specific volume and hydrodynamic volume. Although we can not exclude the possibility of compensating effects a number of observations strongly suggest that the plateau does represent the accumulation of defects without causing any significant changes in $S_{20,w}$.

The rate of reaction of HCHO can be monitored spectrophotometrically. When the initial reaction of PM2 FI reached equilibrium we determined an $S_{20,w}$ values of 32S. If this represents the saturation of hairpin sites further reactivity would occur at duplex regions either by propagation from the disrupted hairpins or via path B. Since equilibrium was reached
we believe propagation to be very slow from a limited number of sites. Consequently heating of the DNA was performed in the presence of 4% HCHO in order to promote additional reactivity. The DNA sample was held for 10 minutes at a particular temperature, e.g. 35°, cooled and an aliquot taken for spectral and $S_{20,*}$ measurements. The process was repeated by short temperature intervals. Since this published data is important for our model and for comparative purposes we present it again in Figure 8.

The examination of the $S_{20,*}$ behavior of heated (equilibrium reacted) HCHO DNA I showed a flat plateau until the dip region in two different sedimentation solvents, whereas the O.D. 270nm value continued to increase (not shown) indicating continued reactivity. Consequently, HCHO reactivity appears to separate into two stages, first hairpins or single strand regions are saturated (path A) and then duplex structure reacts via path B as the temperature is increased. The hydrodynamic transitions observed; $S_{20,*}$ increase, plateau and dip are clearly comparable to the CMC-induced transitions.

![Figure 8. Changes in sedimentation velocity of superhelical PM2 DNA in the presence of HCHO as a function of the mode of heating. PM2 DNA I was treated in two different ways. (1) The sample was brought to a specified temperature for 10 minutes in 8% HCHO, cooled in ice, and the AOD read at 20°C (data not shown). An aliquot was removed for sedimentation velocity analysis. The transition obtained is that of the open (O) circles. (2) The sample was reacted in 4% HCHO at 30° until equilibrium was achieved by optical density criteria at 270 nm. Melting was continued by the heating procedure described above and the transition followed in two solvents: 1.5 M CsCl, .03 M Tris, pH 7.9 (■); and .01 M Na$_3$B$_4$O$_7$ • 10 H$_2$O and 0.1 M NaCl, pH 9.0 containing 50% D$_2$O v/v (□). For complete details see reference 1.](image-url)
However, if PM2 DNA is just heated in the presence of HCHO, cooled and examined for changes in $S_{20}^*$, we observe a bell shaped curve of $S_{20}^*$ vs. temperature. The maximum $S_{20}^*$ is 37S and this is followed by the dip regions. This is shown in Figure 8 (dashed curve) and was also published previously. Unfortunately, we did not realize the importance of the HCHO observations until we examined CMC reactivity. We believe that incremental heating promotes reactivity by paths A and B (Figure 7) and consequently generates considerably more nucleation sites for unwinding, thus producing a more flexible wormlike coil and a more cooperative unwinding to the open form. Hence we do not see a plateau in $S_{20}^*$ vs. temperature but a bell shaped curve. The heating of CMC also produces a broad bell shaped curve (Figure 5) and we again have an analogous situation to HCHO reactivity. For the case of $\text{CH}_3\text{HgOH}$ reactivity with PM2 FI we did not observe a plateau but a sharp bell shape curve followed by the characteristic dip region. Since $\text{CH}_3\text{HgOH}$ is known to cooperatively denature duplex DNA at a critical concentration we simply interpret the $\text{CH}_3\text{HgOH}$ reaction as proceeding via paths A and B simultaneously so that cooperative melting occurs very sharply when sufficient $\text{CH}_3\text{HgOH}$ is present. It is important to note that when single strand character is missing from the DNA, i.e. superhelical PM2 DNA molecules of low superhelical density we do not observe a bell shape curve but simply a plateau in $S_{20}^*$ followed by a dip in the loss of supercoiling.

Consequently, the proposal for the reaction of chemical probes with superhelical DNA (Figure 7) qualitatively accounts for the $S_{20}^*$ behavior produced by a variety of reagents and extends the model proposed by Woodworth-Gutai and Lebowitz. It has been demonstrated that the essential features of the model, intrastrand hairpin regions which are easily modified and the accumulation of many additional sites of reactivity by transient unwinding of duplex structure can account for the differences in transitions in $S_{20}^*$ vs. reactivity observed thus far for superhelical DNA. It should be emphasized that considerable structural details remain to be resolved before a full understanding of the behavior of superhelical DNA. Although we have focused exclusively on the hairpin as our major working hypothesis, we are aware that other structural features such as branching may play a role in reactivity and hydrodynamic behavior and require further consideration.

With this background regarding the structural features of superhelical DNA we now focus our attention on the effects produced by CMC on transcription.
ACKNOWLEDGEMENTS

This research was supported by Public Health Service Grant from the National Cancer Institute CA 17077-03 and one of us (J.L.) is currently a recipient of Public Health Service Career Development Award CA-00141-05 from the National Cancer Institute. We thank Dr. Tom Tice, Richard Woodward and Pat Hale for helpful discussions. It is a great pleasure to thank Bonnie McLay for help in the preparation of this manuscript.

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* Address all reprint requests to J. Lebowitz.