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NAME OF AUTHOR/*NOM DE L'AUTEUR* DAVID EDWIN PULLEYBLANK

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NAME OF SUPERVISOR/*NOM DU DIRECTEUR DE THÈSE* Dr. A.R. Morgan

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PERMANENT ADDRESS/*RÉSIDENCE FIXE* 6216 Mackenzie St.,
Vancouver B BC

NL-91 (11-73)

THE UNIVERSITY OF ALBERTA

STUDIES ON SUPERHELICAL DNA

by

DAVID E. PULLEYBLANK

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "STUDIES ON SUPERHELICAL DNA" submitted by DAVID E. PULLEYBLANK in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

A. Richard Morgan
.....
Supervisor

Lloyd M. Kay
.....

Peter C. Dalton
.....

M. N. G. James
.....

Daniel C. Wemmer
.....
External Examiner

Date 2/1/[illegible]
.....

W. J. Rattray
.....

ABSTRACT

Superhelical closed circular DNA is not only of widespread occurrence but has been extensively utilized to study the conformation of DNA. Superhelix densities of these DNAs have routinely been calculated using the assumption that ethidium unwinds the primary helix by 12°. This assumption has been questioned by Paoletti and LePecq (1971b) who proposed that ethidium winds the duplex by 13°. This thesis unequivocally demonstrates by a combination of chemical and physical means that ethidium unwinds the duplex but that the unwinding angle and consequently superhelix densities are probably between two and three times the previously assumed values. The consequences of this finding in terms of models of chromatin structure, and other naturally occurring structures for DNA are discussed. Two additional studies related to superhelical coiling of DNA have been carried out. The free energy of supercoiling under conditions close to physiological has been measured by a fluorimetric technique. The values measured for PM2 DNA and M13 RFI are close to those calculated for DNA of similar superhelix densities using the results of Bauer and Vinograd (1970) obtained for SV40 DNA at buoyant equilibrium in 5.8 M CsCl.

A simple method is proposed for the measurement of superhelix density, using a fluorimetric technique.

A protein capable of releasing strain in closed circular DNA molecules due to supercoiling has been extensively purified from calf thymus. This enzyme had been previously reported in E. Coli by Wang (1971), and in a nuclear extract from secondary mouse embryo cells by Dulbecco and Champoux (1972).

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1. INTRODUCTION

DNA, unlike other biological macromolecules, has long been thought to have a monotonous secondary structure. This view has in large part been a consequence of the success of the Watson-Crick (1953) model of the DNA duplex. It appeared that it would be necessary to look elsewhere for an understanding of the highly specific interactions that occur between regulatory proteins and the native helix. Recently evidence from a variety of sources has suggested that the situation could be more complex than had generally been supposed. For example it has been shown in prokaryotes that many of the RNA polymerase promoter sites contain sequences within a single strand that are capable of specific self interaction (Allet et al. 1974), so called palindromic sequences. Examples of such sequences have recently been discovered in eukaryotic DNA (Wilson and Thomas 1973), although their function is not yet understood. A more general problem of DNA conformation is the question of how exceedingly long molecules are manipulated during the cell cycle, especially at cell division.

A primary concern of this thesis has been the determination of the change in winding angle of the double helix that accompanies intercalative binding of the drug ethidium to DNA. This parameter is important in its own right since numerous compounds, including antibiotics and carcinogens bind to DNA by intercalation (Waring 1970). The

value is also important to our understanding of other problems of DNA conformation, since it permits measurement of a parameter called the superhelix density in covalently closed circular DNA. Superhelical coiling of these DNA molecules is a consequence of distortion of their *in vivo* structure away from the B form of the helix.

This introduction will emphasize the variability of DNA conformation and the importance of superhelical coiling both *in vivo* and *in vitro*. It will illustrate the way in which knowledge of the superhelix density can be used to restrict the range of acceptable models for the conformations observed.

Crystalline Structures

Historically, X-ray crystallography has been the leading technique in the study of DNA conformation. Its application, in combination with the known chemical properties of the molecule led to the highly sucessful Watson-Crick (1953) model for the structure of DNA. Early in the structural studies it was recognized that more than one form of the crystalline structure existed (Franklin and Gosling, 1953). Over the past twenty years versions of the model have been refined to the point where very close agreement between observed and calculated X-ray diffraction has been obtained for the two major crystalline modifications, the A and B forms. A third partially crystalline modification, the C form has been studied in considerable detail (see Arnott 1969 for review).

The A form is normally found in fibres of sodium DNA when there is little or no excess salt present, or when the relative humidity of salt containing fibres is reduced below 92% (Cooper and Hamilton, 1966). It is characterized by a right handed helical pitch of 28.15 A, with 11 base pairs per turn of helix. In the latest model (Arnott et al., 1969) the bases are tilted at an angle of 69.8° to the helix axis, with the centre of the base pair displaced 4.72 A from this axis. In an earlier model (Fuller et al., 1965) the bases were twisted 8° with respect to each other. This is stereochemically undesirable since it reduces the strength of hydrogen bonding between the strands of the helix. In the more recent model the twist has been reduced to 1.2°. Further characteristics of the A form are the conformation of the sugar residue which is C3' endo, and the rotational angle X between the O4'-C1' bond and the base plane along the C1'-N1 bond which is 0°, lying within the range observed in mononucleotides exhibiting the C3' endo sugar conformation (Sundaralingam, 1969).

The B crystalline form is generally found at high relative humidity when excess salt is present in the crystal (Cooper and Hamilton, 1966). It is the form considered to resemble the conformation of DNA in aqueous solution, at moderate ionic strength since there is no abrupt phase transition as fibres swell and dissolve at 100% relative humidity. This form of the helix has a right handed pitch of

34 A with 10 base pairs per turn of helix, which lie perpendicular to the axis of the helix, and are centred close to it. The sugar conformation was previously thought to be C2' endo (Arnott et al. 1969), however in the latest model a C3' exo structure has been proposed which shows as good agreement with the diffraction data as the best C2' endo model, and is free from stereochemical anomalies. In this model the value of X, (142.5°) lies just outside the range for C3' exo mononucleotides (Sundaralingam, 1969).

The C form of the helix is found in fibres of lithium DNA below 66% relative humidity. It has 9.3 residues per turn of helix and a pitch of 31 A. This form has not been as well characterized as the A or B forms because the non integral helix prevents perfect crystallization. It appears to be related to the B form. In the model of Marvin et al. (1961) the base planes are 84° to the helix axis, with a twist of 5° between members of a base pair. The centre of the base pair is moved 2.13 A away from the helix axis so as to enlarge its minor groove.

Several other crystalline and semicrystalline forms of DNA have been observed, although their occurence seems to be confined to DNA of unusual base composition. A recent study of fibres of the synthetic polynucleotide dIC.dIC (Mitsui et al. 1970) is of particular interest. This DNA-like polymer has an unusual X-ray diffraction pattern which indicates a double helix with 8 residues per turn and a pitch of 25.01

On the basis of titration studies it was shown that the base pairing must be of the Watson-Crick type. At the present time models of the structure have not been highly refined, however the best of a series of models tried was a left handed helix, in contrast to the usual right handed duplex.

The X-ray diffraction pattern obtained from poly dIC.dIC resembles a previously described pattern called the D form that is obtained from ammonium and sodium salts of poly dAT.dAT (Davies and Baldwin 1963). This has a pitch of 24.5 A and a strong meridional reflection at 3.4 A, corresponding to the translation per base pair. These numbers suggest 7.5 base pairs per turn of helix, which may be 8 within experimental error. The close similarities of the two polymers with respect to base pairing and stacking reinforce the notion that the two structures are closely related. If this is so a left handed helix becomes less probable since fibres of dAT.dAT have been observed to spontaneously change from the A to the D form. Reversal of the handedness of the helix would probably be strongly inhibited by the crystal packing forces.

The polymer dG.dC has been observed to undergo a cooperative transition in 2.5 M NaCl, with a large change in its circular dichroism spectrum (Pohl and Jovin 1972). Preliminary results of Arnott's quoted in this paper suggest that the transition may be associated with a change from a C

form of helix to a D form.

The T form studied by Mokulsky et al. (1972) in fibres of T2 DNA at low relative humidity has similar helix parameters to the D form, a pitch of 24 A and 8 residues per turn of helix[1].

DNA conformation in solution:

There is no a priori reason to suppose that the conformation of a polymer in its crystalline state need resemble its conformation in solution, but in the case of DNA several lines of evidence in addition to the one mentioned point to close similarities between the crystalline B form and the conformation present in dilute aqueous solutions of moderate ionic strength. The related techniques of optical rotatory dispersion and circular dichroism are sensitive tools for monitoring small conformational changes in polynucleotides. A partially successful attempt has been made to place their use in these studies on a theoretical foundation (Johnson and Tinoco 1969). However the interpretation of the spectra obtained is

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[1] An illustration of the problems that beset work with fibre X-ray diffraction patterns has been provided by Donohue who has persistently questioned the correctness of the traditional Watson-Crick base pairing scheme (e.g. Donohue 1969). Although the weight of evidence now strongly favours traditional pairing, models can be built, using Hoogsteen base pairing (Arnott and Hukins 1973) that fit the diffraction data better than the original Crick and Watson model (1954).

still largely empirical. Tunis-Schneider and Maestre (1970) were able to obtain CD spectra in thin films of DNA under conditions where one of the crystalline forms was known to be present. The A form was found to have positive ellipticity near 260 nm, and a strong negative trough near 210 nm. In some samples of DNA in the A form a second negative ellipticity trough is found at 295 nm. This is variable in intensity suggesting that the A form is a family of structures rather than a single form (Brunner and Maestre, 1974). The spectrum of A form DNA resembles that of solutions of double stranded RNA which forms crystalline fibres with structures similar to the A form of DNA (Arnott 1969). The B form shows weaker positive ellipticity near 275 nm and a negative trough near 245 nm. The amplitudes of the positive and negative ellipticities are approximately equal. This spectrum closely resembles that of DNA in 0.1 M NaCl, providing evidence that the latter has a B like conformation. The C form lacks the positive peak at 275 nm but has a negative trough at 245 nm of the same intensity as the B form.

Further evidence that the B form of the helix is present under physiological conditions has come from X-ray scattering studies on DNA solutions. Early studies of the low angle X-ray scattering (i.e. scattering at angles corresponding to d spacings greater than 10 A) showed that the mass per unit length of DNA in aqueous solution is close

to that calculated for the B form of the molecule (Luzzati et al. 1961, Eisenberg and Cohen 1968). More recently the high angle X-ray scattering (i.e. scattering corresponding to d spacings between 3 and 30 A) of DNA solutions has been compared to the spherically averaged scattering calculated for the A, B and C forms (Bram 1971). The scattering profile obtained corresponded most closely to that predicted for the B form. By decreasing the winding angle between adjacent base pairs of the helix from 36° to 33° it was possible to improve the agreement between the calculated and observed scattering. It should be noted that the ionic strength of the solution influences the winding angle of the helix (Wang 1969). These experiments were carried out at an ionic strength of 0.05. There is evidence from IR dichroism and X-ray scattering for a B-like conformation in nucleohistone and nucleoprotamine complexes (Bradbury et al. 1962, Penghelman et al. 1955). The high angle X-ray scattering pattern of nucleohistone (Bram 1971) is suggestive of the B form of DNA. In the last case contributions to the scattering from histone obscured the fine structure. Furthermore the nucleohistone used in these experiments was solubilized in distilled water, a treatment that disrupts ordered tertiary structure (Zubay and Doty 1959).

The hydrodynamic properties of DNA show that it is an unusually stiff polymer, with very high intrinsic viscocity. An important factor in determining its rigidity is the

strong electrostatic repulsion that exists between adjacent phosphates along the helix. This effect can be overcome by the shielding effect of high ionic strength. Several workers (Scruggs and Ross 1964, Ross and Scruggs 1968, Hearst et al. 1968) have shown a dramatic decrease in the intrinsic viscosity of DNA as the ionic strength is increased up to 0.6 M. Above this salt concentration the intrinsic viscosity of DNA remains almost constant. The effect of ionic strength on the flexibility of DNA can also be observed by studying the effect of ionic strength on the sedimentation coefficient. Here the effect of decreased rigidity is partially compensated by a decrease in the partial specific volume (Rosenberg and Studier 1969, Rinehart and Hearst 1972).

There is evidence for subtle change in the conformation of DNA as the salt concentration is raised above 1 M. Tunis and Hearst (1968), Tunis-Schneider and Maestre and Studdert et al. (1972) have noted a marked decrease in the intensity of the long wavelength positive optical rotation of DNA in concentrated salt solution. When the change is observed by circular dichroism the spectrum in concentrated salt solution is like that of the C form in thin films (Tunis-Schneider and Maestre 1970).

Overwinding of the helix implicit in this interpretation has been observed in closed circular DNA, where the change in average winding angle of the helix is

accompanied by the generation of superhelical turns. The effect was first noted by Gellert (1967) and was later studied on a quantitative basis by Wang (1969). It is of interest that entirely misleading results were obtained by Bode and McHattie (1968) in an electron microscopical study of the phenomenon. These authors concluded that increased salt concentration leads to an increase in the number of superhelical turns in closed circular lambda DNA molecules. The apparent differences in superhelix density may be attributable to the greater flexibility of the DNA molecules under the high ionic strength spreading conditions.

Hydration has a strong influence on DNA conformation, for example the observed dependence of the fibre diffraction patterns on relative humidity. There is some evidence that AT base pairs have a higher affinity for water than GC pairs. Tunis and Hearst (1968 b) have interpreted the wide variation in the buoyant density of DNA with base composition in CsCl as being primarily due to stronger binding of water by AT pairs than GC pairs. Brahms and Pilet (1972), using IR dichroism to study the effect of base composition on the relative humidity at which the B to A transition took place, found that DNA with high GC content underwent the transition at higher relative humidity than AT rich DNA.

Effects such as this may have an important role in defining specificity of biological interactions. For example

a protein molecule with a DNA binding site designed to interact more strongly with DNA in the A conformation than the B form, would be expected to bind selectively to a GC rich region, since the free energy required to dehydrate such regions and promote the transition from a B to an A conformation is less than that required for an AT rich region.

The selective binding of arginine rich histones to GC rich regions of the DNA (Clark and Felsenfeld 1973) is a probable example of such a process. This hypothesis is supported by the observation that annealed complexes between DNA and the arginine rich histone GRK have CD spectra similar to the A form (Shih and Fasman 1971). In contrast polylysine and lysine rich histone KAP show preferential binding to AT rich regions (Shapiro et al. 1969), with induction of a characteristic CD spectrum (see page 12). It may be significant that the state of modification of histone KAP has a measurable effect on the CD spectrum obtained from the complex (Adler et al. 1971).

DNA conformation can be drastically affected by non-aqueous solvents. These probably exert their effects by reducing the hydrophobic stacking interactions between the bases. The most commonly used observational techniques in these studies have been CD and ORD, which permit qualitative assessment of the conformation present under the particular solvent conditions employed. For instance it has been

suggested that the CD spectrum observed by Brahms and Mommaerts (1964) for DNA in 80% ethanol solution is due to an A-like conformation of the helix. Green and Mahler (1968, 1970, 1971), and Nelson and Johnson (1970) have observed C-like spectra for DNA dissolved in ethylene glycol containing 0.05 M KF. Luzzati et al. (1964) have shown by low angle X-ray scattering that DNA in ethylene glycol is in an extended conformation. Ivanov et al. (1973) demonstrated the importance of the nature and concentration of salt in determining the appearance of the CD spectrum obtained for DNA in methanol water mixtures, adding another dimension to the problem of comparing the work of different authors.

Recently doubt has been raised about the validity of interpretations of DNA CD spectra simply in terms of the A, B or C conformations of the helix. Under various conditions DNA can adopt a conformation which gives rise to intense negative ellipticity near 275 nm. This has been called a Psi type of spectrum (Jordan et al. 1972). Studdert et al. (1969) obtained such spectra from solutions of DNA in 6 M LiCl at low temperature. Since the transition from a B-like spectrum to a C-like one with increasing salt concentration is continuous with the transition from a C-like to a Psi type of spectrum with decreasing temperature it was thought that a single mechanism was probably responsible for both transitions, and suggested that tilting of the base pairs possibly accompanied by a change in the winding of the helix

could be responsible for the spectral changes. Other situations in which a Psi type of spectrum has been observed include the complex between DNA and polylysine (Shapiro et al. 1969), the complex between T7 DNA and histone KAP (Olins and Olins 1971), T2 bacteriophage heads (Dorman and Maestre 1972), and in a condensed state of DNA resulting from excluded volume interactions with neutral polymers such as polyethyleneglycol (Lerman 1971, Jordan et al. 1972). In a recent study of this state of DNA by high angle X-ray scattering (Maniatis et al. 1974) the rather surprising conclusion was reached that the conformation of the DNA in the condensed state is very similar to the B form of the helix, although some deviation was observed from predicted scattering at very high angles. This finding was in agreement with the conclusion reached by Haynes et al. (1970) in their study of the polylysine - DNA complex.

The unusual optical rotation of DNA in the Psi state has been attributed to liquid crystalline behaviour of the condensed phase. Concentrated DNA solutions (Luzzati et al. 1961) show a peak of X-ray scattering due to intermolecular spacing, demonstrating a liquid crystalline lattice in these solutions. However such solutions do not have the optical properties of Psi DNA, although spectra of this type can be obtained at low relative humidity in films of the lithium salt of DNA and dAT.dAT (Brunner and Maestre 1974).

Unusually strong optical rotation is found in

cholesteric mesophase liquid crystals where it is associated with regular rotation of the liquid crystalline planes with respect to one another. It is possible that a regular change of DNA liquid crystalline planes could cause similar anomalous optical rotation. As will be discussed below the simplest way in which this could be achieved involves supercoiling of the DNA. Haynes et al. (1970) observed peculiar donut shaped rings 0.2 microns in diameter when examining suspensions of the DNA-polylysine complex by electron microscopy. Similar rings 0.1 microns in diameter were found by Evdokimov (1973) in solutions of DNA condensed with polyethyleneglycol, by DuPraw in frozen solutions of RFI DNA (1967), by Klimenko et al. (1967) in ruptured heads of bacteriophage T2, and by Olins and Olins (1972) in complexes of histones KAP and GRK with T7 DNA. In this last example linear aggregates were also found, which showed evidence of gentle superhelical coiling of the aggregated strands along the length of the aggregate. DNA molecules in such an aggregate would have differing orientations depending upon their position relative to the centre of the aggregate (see fig. 1). Toroidal aggregates might easily form by winding DNA strands through a circular nucleus (formed by gentle bending of a single double helix). Here again the orientation of successive layers would change as the thickness of the torus increased (Fig. 2). These arrangements of DNA helices are compatible with a slightly distorted B form helix, would be self limiting in their

Proposed Relationship Between Pitch and Radius of Superhelix for ϕ DNA

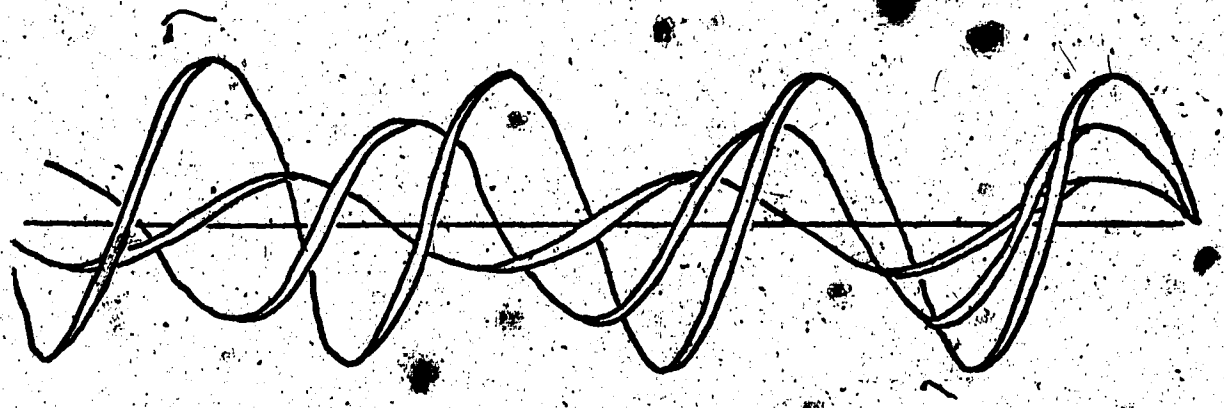


Figure 1. Layers of superhelical DNA molecules with constant superhelix density. Rotation of layers of helices with respect to each other is most apparent at the right of the diagram. Alternative arrangements can be proposed without constant superhelix density.

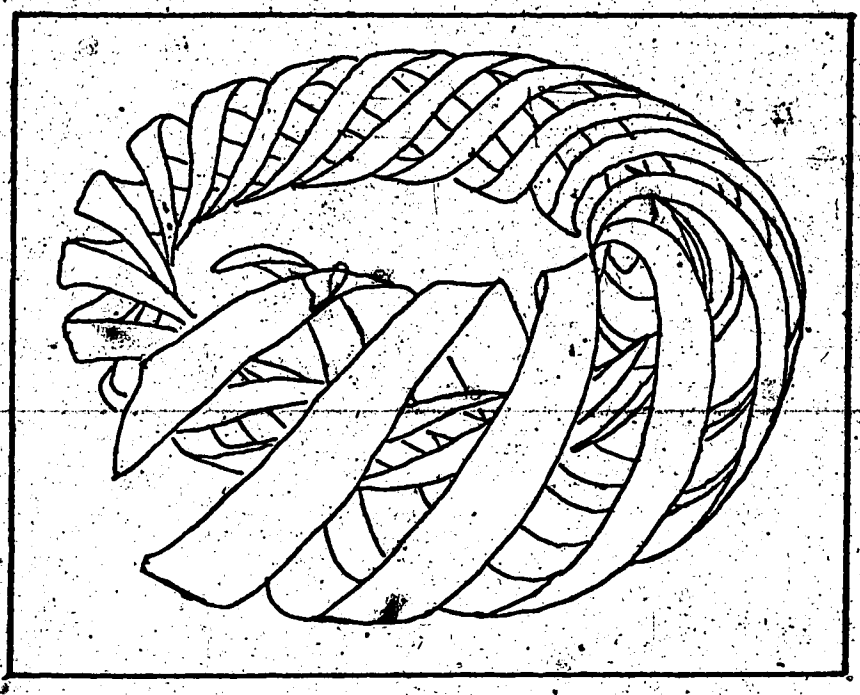


Figure 2. Proposed arrangement of DNA helices in a toroidal bundle (after M. C. Escher 1971). In this diagram the pitch of the superhelix is constant.

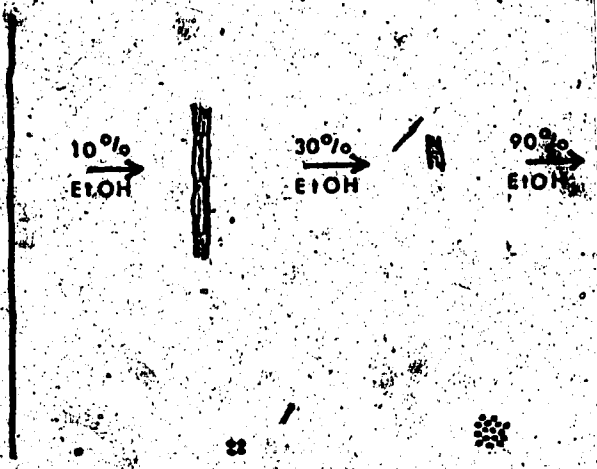
final dimensions, leading to a relatively monodisperse suspension (Shapiro et al. 1969), would not be expected to show evidence of supercoiling in the form of an X-ray scattering peak at 110 A (Maniatis et al. 1974) and avoid the need for the sharp folds in the DNA suggested by these authors.

The study of Lang (1973) on the structure of DNA in aqueous ethanol solution is of particular interest since it provides evidence that DNA can form tight supercoils in the presence of organic solvents, without the need for specific protein interactions. The distinguishing features of his study are the low concentrations of DNA employed (approximately 0.2 micrograms per ml) and the moderate ionic strength, (0.15 to 0.2 M ammonium acetate). Under these conditions intrastrand interactions compete effectively with interstrand interactions, and it is possible to observe the conformational changes that occur in a single molecule of DNA without interference by large scale aggregation (see Lang 1969). He was able to observe three successive stages in the collapse of the linear molecule. By using T7 and bacteriophage S DNA's he demonstrated that the successive stages of condensation are co-operative, and that each involves an approximately 4 fold contraction in length, accompanied by a two fold increase in diameter. These results can be explained in terms of lengthwise folding (Fig.3.1), but it is necessary to assume that the molecule

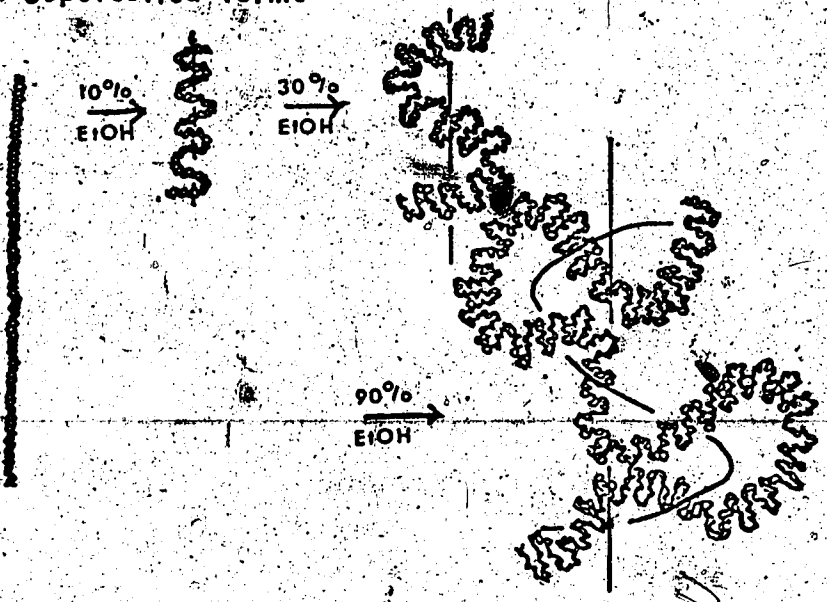
Figure 3.

Possible forms of ethanol induced superstructure

1: Folded forms



2: Supercoiled forms



is neatly folded three times at each stage of condensation. This need not be an overwhelming argument against such an arrangement if groups containing 4, 16, or 64 parallel helices have much higher stabilities than groups containing other numbers of helices. The alternative explanation is supercoiling where doubling of diameter is due to the radius of the n'th order of superhelix being one layer of n - 1'th order superhelix, and the four fold decrease in length is due to the four fold increase in cross sectional area at constant molecular volume (Fig. 3.2). This hypothesis runs into greatest difficulty when models are considered for the first order of supercoiling.

In crystals of DNA below 100% relative humidity the closest contacts between neighbouring duplexes occur at approximately 20 A. Let us assume that this is also the closest contact that adjacent turns of a superhelix can make with each other, (i.e. the minimum pitch of a superhelix is 20 A). Taking this number as the foundation for a "dry" model of a first order superhelix, 84 A of DNA initially in the B form must form a superhelical turn in order to account for the 4.2 fold axial compression observed by Lang. This in turn requires that the radius of the superhelix be around 25 A, close to value observed by Lang (20 A). However this model requires such severe distortion of the linear helix that it is unlikely to correspond to the form present under the relatively mild conditions required to promote the first

order of condensation (10% ethanol, 0.15 M ammonium acetate).

An alternative model is a "wet" one for which a reasonable pitch would be 38 A, (the distance of intermolecular spacing observed by Maniatis et al. (1974) in Psi DNA at relatively low polyethyleneglycol concentration). Since the end to end contraction observed by Lang for the first transition was 4.2 fold, 160 A or 4.7 turns of DNA initially in the B form would make one turn of first order superhelix. The diameter of the superhelix in this particular model would be 70 A, which is considerably greater than the 40 A estimated by Lang, but is probably within experimental error. In this model the distortion of the helix is less severe but is still considerable when compared to the B form of the helix.

An important characteristic of a superhelix is its handedness. In this regard a number called the superhelix density (sigma) becomes important, which can be defined as [the average right handed winding angle between neighbouring base pairs] / 360 − 1. Sigma is a measure of the distortion of the helix away from the linear B form. The distortion can be manifested either in the form of supercoiling about an axis separate from the helix axis, or by an unwinding or overwinding of the primary helix. The measurement of superhelix density is considered in detail on page 27. This definition is more general than the standard definition of

superhelix density used on page 27 in referring to closed circular DNA since it avoids the assumption of a superhelix axis separate from the helix axis. For a linear DNA duplex in the B form sigma is 0, for DNA in the A form it is -0.09 and for the C form it is +0.075. The value of sigma is independent of the choice of helix axis. In order to illustrate the way in which superhelix density in combination with axial compression ratio and the superhelical pitch can be used to generate reasonable models for supercoiling consider the superhelix densities of the models discussed above. Thus in the "wet" model if 4.7 turns of B form helix is wrapped once around an axis in a right handed direction the resulting superhelix density is 1/4.7, or +0.21, if it is wrapped in a left handed direction it would be -0.21. The value of the superhelix density could be either greater or smaller than that due to supercoiling if the primary helix were significantly distorted with respect to the B form. For example if the superhelix was left handed, and the DNA had an average of 9.3 residues per primary helical turn (i.e. the C form) the net superhelix density would be -0.1. This particular model would have 5.2 turns of primary helix in one turn of superhelix. The presence of an odd number of quarter turns (e.g. 5.25) in one turn of superhelix minimizes close contacts between the phosphates of adjacent helices, when the grooves are nearly equal in size (as in the C form). The superhelix density of the "dry" model discussed previously would be ± 0.4.

Conformational changes similar to those observed in ethanol solution occur upon the addition of salt (0.1 M) to extended chromatin fibrils prepared in 4 M urea (Varshavsky et al. 1971). The collapse of the structure depends upon the presence of the lysine rich histone KAP. Unfortunately because of the nature of the preparation accurate measurement of the axial compression ratio was not possible, although a rough estimate of 3 – 5 fold was obtained which is similar to the first condensation observed by Lang (the samples in these experiments did not contain divalent cations).

Various models of chromatin structure involving supercoiling of the DNA have been proposed (e.g. Cole 1962, Bram and Ris 1970, Pardon and Wilkins 1972). Of these the one of Pardon and Wilkins (1972) will be discussed since it is the most recent, and has the best supporting data. Several workers (Luzzati and Nicolaieff 1959, 1963, Wilkins et al. 1959, Richards and Pardon 1970) studying the low angle X-ray scattering profiles of preparations of nucleohistone have observed maxima corresponding to spacings of 110, 55, 37, 27, 22, and 18 A. Pardon and Wilkins proposed that these are orders of reflection arising from a basic period of 110 A, which they suggest represents the pitch of a supercoil with a radius of approximately 50 A. This model agrees qualitatively with the observation of fibres 100 ± 25 A in diameter in dispersed chromatin in the

absence of divalent cations, and in interphase nuclei (Ris and Kubai 1970, DuPraw and Bahr 1969). These have been called type A fibres. Fibres of larger diameter (250 ± 50 A), called type B fibres are also found in interphase nuclei, and dispersed chromatin in the presence of divalent cations. DuPraw has estimated the minimum DNA axial compression ratio of the smaller type A fibres to be 6 to 1 (DuPraw 1970), agreeing with the estimates of Bram and Ris (1971) and Luzzati and Nicolaieff (1963) proposed on the basis of X-ray scattering. These numbers are considerably greater than the axial compression ratio of 2.8 to 1 calculated on the basis of Pardon and Wilkins' model. A further difficulty with this model is that if there is no overlap of superhelices in solution, and the diameter of the DNA helix with its histone sheath is 30 A (the interhelical distance found in very concentrated nucleohistone gels) then a maximum of 17% of the available volume will be occupied by the superhelix. Pardon and Wilkins overcome this difficulty by suggesting that the nucleohistone superhelices do in fact overlap in very concentrated solution. The packing arrangements which agree most closely with the diffraction data are groups containing from one to seven superhelices sharing a common axis (a plectanemic group). This arrangement appears to be incompatible with the observation that chromatin fibrils contain only a single DNA duplex (DuPraw 1967).

The problem of low axial compression ratio in Pardon and Wilkins model does not arise if the pitch of the first order superhelix is considerably less than 110 A. The problem then becomes one of explaining the observed X-ray scattering.

A conformational change from B type to A type fibrils is associated with removal of divalent cations from the nucleohistone (Ris 1970). This can be correlated with a loss of characteristic X-ray scattering that Pardon and Wilkins associate with supercoiling, and the appearance of a new X-ray diffraction maximum at 39 A (Garret 1971). Let us suppose that this represents the pitch of a first order superhelix.

Strong evidence that the DNA in 110 A fibrils is supercoiled (or at least condensed) comes from the study of Varshavshy et al. (1970) mentioned previously which stemmed from the original observation by Georgiev et al. (1970) that 4 M urea causes chromatin to unfold reversibly into fibrils approximately 40 A in diameter. Under these conditions the histones remain associated with the DNA. Also if chromatin is dispersed in distilled water a highly viscous solution is obtained, in contrast to its condensed state in 0.15 M NaCl, in the absence of divalent cations (Zubay and Doty 1959). Histones which have been sequenced contain basic regions near their amino and carboxy termini with an intervening hydrophobic region (Hnilica 1972), suggesting that these

molecules may serve to bind adjacent turns of superhelix together through their two basic regions.

Assuming that the diameter of the helix axis in the first order chromatin supercoil is 100 A the axial compression ratio would be 9.3 to 1, close to that calculated by DuPraw (1970) for the type A fibrils. A smaller diameter of 60 A will give an axial compression ratio of 5 to 1 (cf Bram and Ris 1971). The observed change in scattering that occurs when divalent cations are added to the chromatin preparation can then be explained on the basis of second order supercoiling with a pitch approximately equal to the outside diameter of the first order supercoil of 110 A. As noted previously tightly packed nucleohistone fibrils can be spaced approximately 30 A apart. If we assume that this is the closest distance that two neighbouring supercoils can approach each other then six first order supercoils can be made to form one turn of second order supercoil with a central hole (observed by Lampert 1971), of 30 A and a diameter of 250 A, very close to the diameter found for type B chromatin fibrils, the overall packing ratio would be 56 to 1, the value calculated by DuPraw for type B chromatin fibrils.

This comparison is not entirely just since shrinkage probably accompanies drying during preparation for electron microscopy. A rough estimate of 30% is suggested by the disappearance of X-ray reflections at 110 A and 55 A when

the relative Humidity of chromatin fibrils is reduced below 98%, and their replacement by broad reflections at 76 and 38 A at humidities below 75% (Pardon and Wilkins 1972).

Secondary supercoiling of the tighter primary superhelix suggested by Bram and Ris (1971) could also have a pitch of 110 A. In this case eight turns of primary superhelix per secondary supercoil would give an axial compression ratio of 40 to 1 which, after 30% shrinkage would be 58 to 1. Unfortunately these models by no means exhaust the possibilities for secondary supercoiling with the required axial compression ratio and periodicity. The studies of Barnicott (1967) show that 110 A chromatin fibrils can aggregate in (presumably) twisted pairs under the influence of divalent cations. A second order superhelical pitch of 220 A would then be associated with a regular period of 110 A. Such a model might explain the 240 A longitudinal periodicity found in critical point dried type B chromatin fibrils by Lampert (1971). This arrangement of primary supercoils is reminiscent of the interwound form of the primary superhelix found in closed circular DNA (for examples see Bode and McHattie 1968, also plate 1, and fig 4). If in these models it is assumed that the DNA helix is neither unwound nor overwound with respect to the B form of the primary helix the superhelix density due to first order supercoiling in either Pardon and Wilkins' model or the one proposed above would be ± 0.11 depending upon the handedness

of the superhelix. In the model of Bram and Ris (1971) the superhelix density would be ± 0.17. Secondary supercoiling of the type discussed above would have a much smaller effect on the net superhelix density being only ± 0.02 because of the greater length of primary helix forming one turn of secondary superhelix.

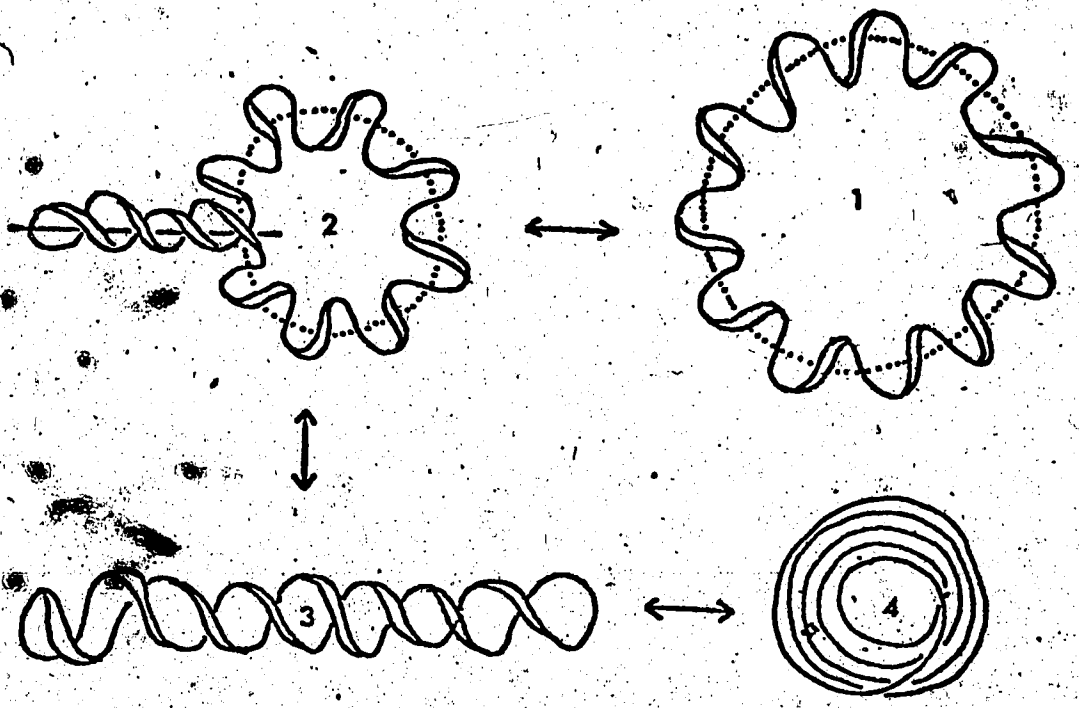
Covalently closed circular DNA

The superhelix density of a linear or nicked circular DNA molecule cannot be measured directly because the ends of the molecule are free to rotate with respect to each other. In closed circular DNA molecules which have been isolated from a wide variety of biological sources, this is not the case. Here the topological winding number (alpha) (i.e. the total number of helical turns that would be present in the molecule if it could be constrained to lie in a plane) is invariant. If this number differs from the number that would be present under the same conditions if the closed circle contained a single stranded break (beta) [used here as defined by Champoux and Dulbecco 1972] there will be strain in the molecule which can be partially accommodated by converting the difference between the two numbers into superhelical turns (tau, where tau = alpha – beta). This equation has been given analytical proof by Glaubiger and Hearst (1968). Tau does not represent the actual number of superhelical turns that are present in the molecule, since there is some distortion of the primary helix which can be

detected by circular dichroism measurements (Maestre and Wang 1971, Campbell and Lochhead 1971), but instead represents the number of superhelical turns that can be titrated with various reagents. In aqueous solution the preferred conformation of the primary helix resembles the B form as discussed previously. Under such conditions beta = number of base pairs per molecule / 10. The number of superhelical turns cannot be measured directly, and cannot be used when comparing superhelical closed circles which differ in molecular weight. The variable obtained experimentally is sigma, the superhelix density, which is the number of superhelical turns per 10 base pairs. This definition is numerically equivalent to, but narrower than the one used previously in discussing chromatin structure, since it assumes that the superhelix axis is separate from the helix axis. Numerous arrangements of the primary helix in a superhelix are possible, (see fig 4). Depending upon the superhelix density either the interwound form usually observed in electron micrographs, or a toroidal form may be the most stable conformer (Campbell and Jolly 1972, see also p 83)

The presence of superhelical turns in a covalently closed circular DNA molecule causes several differences between its physical properties and those of its nicked circular counterpart. These include increased sedimentation coefficient (Dulbecco and Vogt 1963, Weil and Vinograd 1963)

Figure 4.



Topologically Equivalent Forms of Supercoiling in Covalently Closed Circular DNA
Note the apparent reversal of handedness in the change from form 1 to 3

and decreased intrinsic viscocity (Revet et al. 1971), both of which are related to a more compact conformation in solution. There is also an altered affinity for certain intercalative drugs and dyes which is due to the distortion of the primary helix caused by the superhelical turns (Bauer and Vinograd 1968).

Several approaches have been applied to the determination of the superhelix density of these DNA's, most of which depend upon titration of the superhelical turns with an agent capable of altering the primary winding of the duplex (beta), and determination of the amount of reagent bound when no superhelical turns are present in the molecule. If one assumes a fixed known value for the change in the helical winding number per molecule of bound ligand a value for the superhelix density can be derived. The two agents that have been used most extensively are ethidium bromide, and hydroxide ion. Ethidium is an anti-trypanosomal drug which binds to DNA intercalatively, in so doing altering the angle between the base pairs at the binding site. Fuller and Waring (1964), on the basis of an X-ray diffraction study proposed a model in which this change in torsion angle is -12° (i.e. 12° unwinding). However Lerman (1961) in a similar model building study with proflavin as the intercalative dye showed that unwinding angles as high as 45° are possible. The lower of these two values has been used in all published determinations of superhelix density

using ethidium bromide. Paoletti and LePecq (1971 b) proposed an alternative model in which the distortion due to intercalation extends over a region containing 4 base pairs and the total angle change due to intercalation is +13° (i.e. 13° overwinding of the duplex). This model has an attractive feature in that it offers some explanation for the excluded site behaviour of ethidium binding to DNA (LePecq and Paoletti 1967, Bauer and Vinograd 1970). They supported their model by a study of the fluorescence depolarization due to resonance energy transfer between ethidium cations bound at nearby sites on the DNA (Paoletti and LePecq 1971 a). The stereochemical correctness of this model as well as the interpretation of the fluorescence depolarization data have recently been questioned (Pigram et al. 1973). However the question of the correct angle change due to ethidium binding remains.

Apart from these diametrically opposed models two others have been proposed which bear upon the question of sign and absolute number of superhelical turns in covalently closed circular DNA. Sobell and Jain (1972) proposed a model in which actinomycin D binds to DNA intercalatively, causing a total angle change of −34°. This is spread over 4 base pairs and so shares the excluded site feature of Paoletti and LePecq's model. Waring (1970) has shown that actinomycin D causes the same degree of unwinding as ethidium when bound to closed circular DNA. Pigram et al. (1972), have proposed

a model for the binding of daunomycin to DNA in which there is a change in torsion angle of -12°. Waring (1970) and Saucier et al. (1971), have demonstrated that daunomycin causes only one half to one third as much angle change as ethidium. Both these models therefore require that ethidium unwind the duplex by a larger angle than that proposed by Fuller and Waring (1964).

In a study of the buoyant density of polyoma DNA when measured under alkaline conditions Vinograd et al. (1968), concluded that the superhelix density must be -0.032, which appeared to be close to that measured for the closely related SV40 genome assuming 12° unwinding by ethidium. This was -0.033 by sedimentation velocity in 1 M NaCl, and -0.026 in CsCl at buoyant equilibrium (Bauer and Vinograd 1968). In a more recent study Gray et al. (1971) showed that the superhelix density of SV40 DNA is approximately 20% greater than that of polyoma DNA when measured by sedimentation velocity in 2.83 M CsCl. When the relative superhelix densities obtained by the two buoyant density methods are corrected for this difference a 50% discrepancy between the two methods is apparent. Unfortunately interpretation of the alkaline buoyant density experiment is difficult. Dean and Lebowitz (1971) have shown that the effect of formaldehyde treatment on superhelical DNA is complex, which is also true for methylmercuric hydroxide (Beerman and Lebowitz 1973). Since formaldehyde, methylmercury and alkali cause

denaturation of linear DNA some analogy may be expected between their effects on superhelical DNA. In general the greater degree of freedom available to denatured DNA causes uncertainties in the interpretation of apparent superhelix density of any partially denatured closed circle. This point has been raised by Kasamatsu et al. (1971) in their study of mitochondrial D-looped DNA.

Superhelical DNA molecules have proven to be a sensitive tool for monitoring small changes in the conformation of DNA in solution (e.g. Wang 1969, Bauer 1972, Saucier and Wang 1972). Interpretation of the results of these studies is absolutely dependent upon a knowledge of the angle change caused by the binding of ethidium to DNA. This angle change is also required for the construction of models of the in vivo conformation of the DNA molecules in question, and of chromatin as discussed above.

The proposal

The proposal for determining the sense and absolute magnitude of superhelical coiling is illustrated in Figure 5. A natural closed circular molecule is treated with a reagent that will bind to the bases and prevent hydrogen bond formation with their complimentary sequences under conditions that will lead to partial denaturation and reaction with the reagent. At this point in the procedure nothing is known about the conformation of the DNA in the derivatized sequence. The DNA is then nicked with an

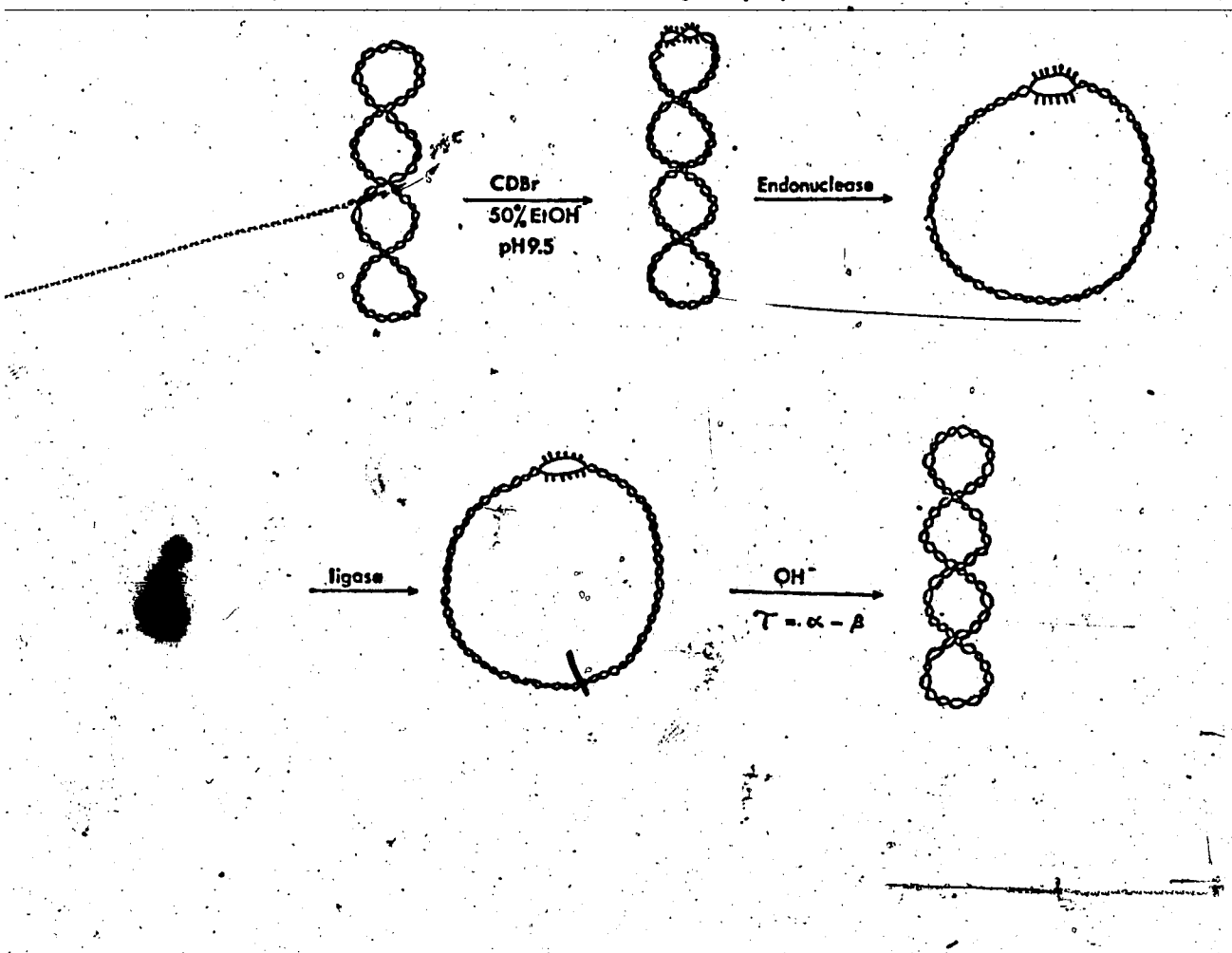


Figure 5. The synthesis of a negatively supercoiled DNA molecule. (A). Closed circular DNA is treated with a reagent under conditions that lead to partial denaturation and reaction with the reagent.
(B). The partially derivatized DNA containing superhelical turns of unknown sense is nicked to release all strain in the molecule and allow the derivatized region to adopt the entropically favourable unwound conformation.
(C). The nick is resealed with polynucleotide ligase to reform a topologically closed system.
(D). The blocking groups are removed from the derivatized region to allow the bases to reform a Watson-Crick duplex with right handed helical turns.

endonuclease to produce a 5' phosphate terminus. This has the effect of releasing all strain in the molecule and allows the derivatized region to adopt the entropically favourable unwound conformation. The nick is then resealed using polynucleotide ligase and the bound reagent is removed from the bases under suitable conditions. DNA in the previously derivatized regions will then reform a duplex with positive helical turns, the formation of which must be compensated by the creation of an equal number of negative superhelical turns. In this way a negatively supercoiled molecule can be synthesized which can be compared with natural closed circular DNA's with respect to titration of sedimentation coefficient against ethidium concentration. A deep minimum in the curve corresponding to the titration of the superhelical turns initially present followed by the creation of new superhelical turns of opposite sense would imply that natural superhelical turns are negative and thereby disprove Paoletti and LePecq's contention that ethidium winds the duplex. Furthermore accurate quantitation of the amount of reagent bound at the time of resealing would permit direct evaluation of the unwinding angle caused by ethidium.

W protein

An enzyme whose function is to release strain in DNA molecules caused by superhelical coiling was found in E. Coli by Wang (1971). Subsequently an enzyme with the same

function but rather different properties was observed in a nuclear extract from secondary mouse embryo cells by Champoux and Dulbecco (1972). The enzyme from E. Coli is capable of releasing superhelical turns of one sense (then thought and subsequently proven to be negative), and only to a limiting residual superhelix density. That from eukaryotes is capable of releasing superhelical turns of either sense and does not leave residual superhelical turns in the molecule. The enzyme has variously been called w protein (Wang 1971), swivelase (Baase and Wang 1974), unwindase and nucleoligase (possibilities considered by Champoux personal communication). For simplicity in this thesis it will be referred to as w protein. The existence of this enzyme is proof of the importance of superhelical coiling to eukaryotic cells, and has proved useful in this study of the unwinding angle due to ethidium binding since it combines the functions of the nuclease and ligase treatments required to relax strain in the derivatized supercoiled molecule.

W protein is of interest for several reasons. There are numerous possible functions for this enzyme within the cell. During DNA replication the parental molecule must be unwound ahead, or at the site of replication and the daughter molecules must subsequently be rewound (see Fig. 6). These are both operations that the eukaryotic w protein will facilitate. Unwinding followed by rewinding may also be required for the transcription of high molecular weight RNA

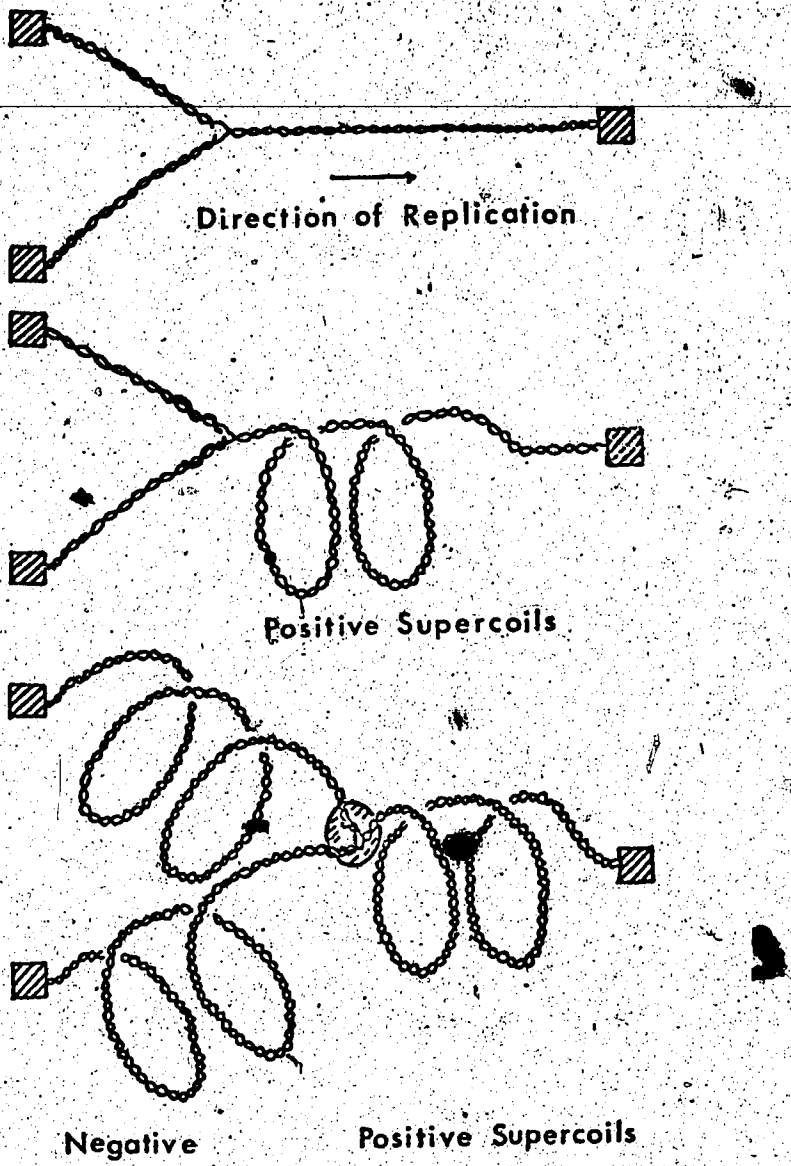


Figure 6. Possible functions of w
A. During DNA replication the duplex must be unwound ahead of the replicating fork this is equivalent to releasing positive superhelical turns.
B. The newly replicated DNA must be rewound behind the fork, this is equivalent to releasing negative superhelical turns.

from a looped chromosome, since the RNA produced must otherwise rotate once around the DNA helix for every 10 base pairs transcribed (see fig 7). This could cause entanglement, especially in mammalian cell nuclei where typical RNA molecular weights run as high a 10^7 daltons (Sherrer et al. 1966). The enzyme may also be required to permit changes in the supercoiled structure of chromatin that occur during the cell cycle and cell differentiation.

A subtle role may also exist in the control of transcription if the enzyme is actively regulated, since the torsional state of the DNA helix can affect the activity of RNA polymerase (Figure 8). The effect was first noted by Hayashi and Hayashi (1972) who showed that RNA polymerase is more active on a supertwisted closed circular DNA template than on the corresponding nicked molecule, and has more recently been extended by Botchan et al. (1973) who showed that E. Coli RNA polymerase is active on supercoiled closed circular lambda DNA but not on the relaxed molecule. When a regulatory protein dissociates from an operator sequence the alteration of transcription rate due to torsion in the template should be continuously variable. This may be of particular importance to higher organisms where fine regulation of cell growth and differentiation is required.

Apart from the direct effect on the rate at which an RNA polymerase molecule traverses its template, torsion in the template could affect the availability of regulatory

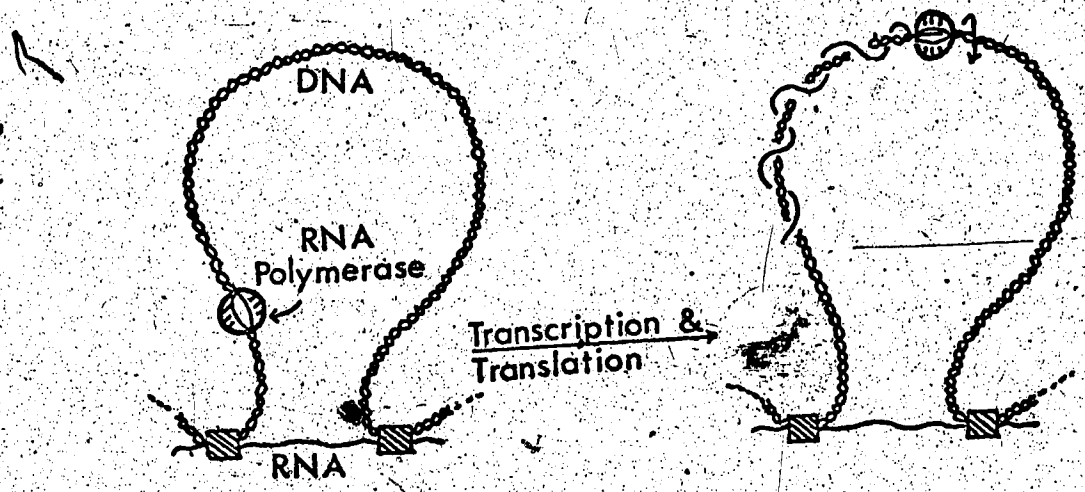


Figure 7. Possible functions of w.
RNA transcribed from a rigidly held topologically closed template must be wound around the template unless there is a mechanism for unswivelling the DNA ahead of the transcription site. In the absence of such a mechanism entanglement could occur. This is a special problem in eukayotic systems where RNA of molecular weight 10^7 can be transcribed as a single piece.

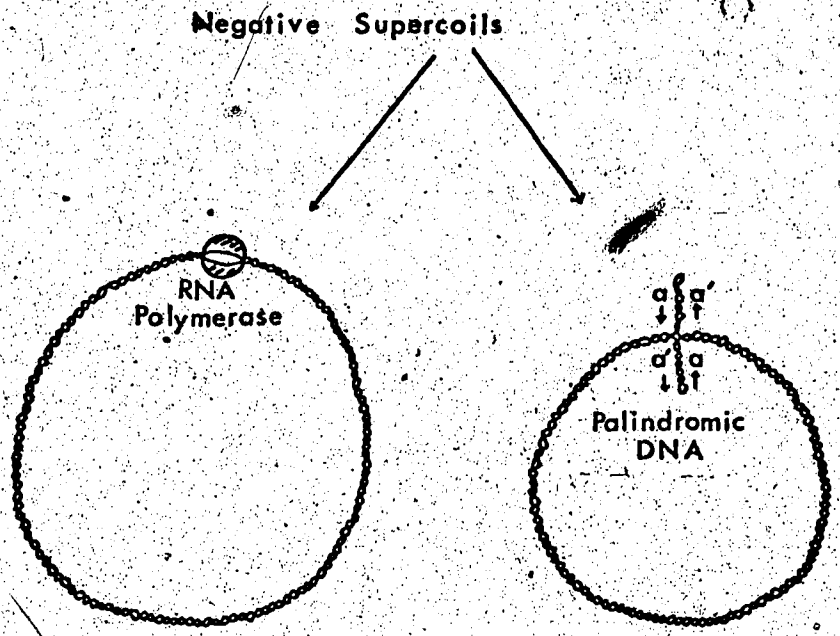


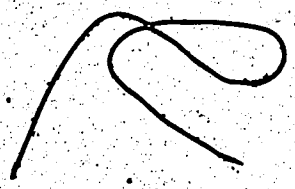
Figure 8.
a. Torsional strain due to negative supercoiling will facilitate opening of local regions of the helix promoting transcription by RNA polymerase.
b. Torsional strain due to negative supercoiling will promote the outfolding of palindromic regions of the duplex for interaction with regulatory proteins.

sites. For example regions of palindromic DNA have been found in the mammalian chromosome (Wilson and Thomas 1974) and are present in the few examples of bacterial chromosomal control elements that have been sequenced. These regions of the DNA can in principle loop out without extensive loss of hydrogen bonding. In a linear DNA molecule the free energy cost of such loops would place the equilibtium in the direction of the unlooped conformation, but in a molecule under the torsional strain of negative superhelical twisting the equilibrium could be forced in the opposite direction, increasing the availability of these sites to regulatory proteins. Purification of the w protein from calf thymus has been achieved in substantial measure as will be described in the results.

The protocol followed for the determination of the unwinding angle due to ethidium can be adapted to the study of the interaction between DNA and other ligands. If a complex is formed between a circular DNA molecule and a ligand (such as a protein) under conditions where the DNA is fully relaxed and the superhelix density is measured after removal of the bound ligand, the angular distortion imposed on the helix through binding of the ligand can be calculated. A version of this method was used by Saucier and Wang (1972) to show that E. Coli RNA polymerase causes a net unwinding of the DNA helix of approximately 120° (calculated on the basis of 12° unwinding by ethidium) when bound to

lambda DNA.

A long term objective of our research is to use a version of the same technique to study the interaction between purified histone fractions and closed circular DNA. For these experiments a purer preparation of w protein than that used by Champoux and Dulbecco was required since this was a relatively crude preparation of chromatin.



2. MATERIALS AND METHODS

Closed circular DNA

M13 RFI was prepared from M13 infected E. Coli AB257 by a modification of the method of Hayashi and Hayashi (1971). Cells grown in 30 litres of trypticase soy broth at 37°C were infected at 1/4 log phase with 10 pfu of M13 per cell. In some cases chloramphenicol (50 micrograms per ml.) which did not appear to influence the yield of RFI, was added 2 hours after infection. 3 or 4 hours after infection the cells were harvested and washed with 0.03 M Tris chloride, 5 mM EDTA, 0.14 M NaCl pH 8, and resuspended in 750 mls of the same buffer. 50 mg of lysosyme dissolved in 10 mls of buffer was then added to the cell suspension which was divided equally among 6 Beckman 19 rotor bottles. After standing for 20 min at room temperature 125 mls of 0.5% SDS in distilled water was added rapidly to each by means of a squeeze bottle with its tip held beneath the surface of the sphereoplast suspension. This ensures mixing without shearing of the viscous DNA solution. The lysate was gently stirred with a spatula and then centrifuged 5 hours at 19,000 rpm to pellet cellular DNA and other debris. The supernatants were combined and solid sodium perchlorate was added to a final concentration of 1 M. 300 mls of a 1 to 1 mixture (v/v) of freshly redistilled phenol and chloroform was added, and the

mixture was stirred slowly at room temperature for 1 hour, then centrifuged for 20 minutes at 10,000 rpm in a Sorvall GSA rotor. The supernatants were pooled and nucleic acid was precipitated overnight at 4°C by the addition of 2.5 volumes of 95% ethanol. The precipitate was collected by decantation and centrifugation, then redissolved in 100 mls 0.01 M Tris chloride, 0.1 mM EDTA pH 8. 5 mg of pancreatic RNase dissolved in 5 mls of the same buffer (heat treated at 80°C for 5 minutes) was added and the mixture was incubated at 37°C for 30 minutes. DNA was pelleted by centrifugation at 50,000 rpm for 5 hours in the 50Ti rotor. The pellets were resuspended overnight in 25 mls 0.01 M Tris chloride 0.1 mM EDTA pH 8, and 1 ml of ethidium bromide solution (10 mg per ml) was added followed by 24 gms of solid CsCl. The solution was then divided among 4 50Ti rotor tubes and centrifuged to equilibrium at 35,000 rpm for 40 hours at 20°C. The lower band was removed by syringe, extracted three times with water saturated butanol, and dialized against 0.01 M Tris chloride 0.1 mM EDTA pH 8. Typical yields from this procedure were 50 optical density units of M13 RFI from 30 litres of culture.

PM2 DNA

Bacteriophage PM2 was obtained by the method of Salditt et al. (1972), without modification. Purification of the bacteriophage was stopped after the first CsCl centrifugation. On some occasions the DNA was extracted by the phenol-SDS procedure of Mitra et al. (1967). The yields of closed circular DNA obtained by this method of extraction were irreproducible. When the procedure was modified by the substitution of a 3 to 1 mixture of chloroform and n-butanol for phenol a considerable improvement in yield was experienced. This method requires more extractions (4 or 5) than the standard phenol method (2) for removal of protein. Up to 200 optical density units of PM2 DNA were obtained from a single 30 litre batch of lysate.

lambda DNA

E. Coli M5107 (lysogenic for the temperature sensitive, repressor, lysis defective mutant of lambda $CI^{ts57}S^{7}$) was grown at 34°C in 30 litres of 1/2 strength soy broth (with salts and glucose added to final concentrations of 2.5 gm dipotassium hydrogen phosphate, 5 gm NaCl and 2.5 gm of glucose per litre) to an optical density at 610 nm of 0.25. The temperature of the medium was raised to 42°C for 15 minutes and then returned to 37°C, after which incubation was continued for 4 hours. The cells were harvested (20 gm

wet weight) and resuspended in 400 mls 0.1 M NaCl, 5mM magnesium chloride, 0.01 M Tris chloride pH 8. The cells were then lysed by stirring for 30 minutes at 4°C with 10 mls of chloroform. DNA and RNA were degraded by incubation of the lysate at 37°C with 0.5 mg pancreatic DNase and 5 mg RNase for 30 minutes. Particulate cell debris was removed by centrifugation at 10,000 rpm for 30 minutes in a Sorvall GSA rotor. The pellet was re-extracted with 100 mls of buffer and the combined supernatants were centrifuged at 18,000 rpm for 7 hours in the Beckman 19 rotor. The pellets from this centrifugation were resuspended in 50 mls 0.1 M NaCl, 0.01 M Tris chloride pH 8, and CsCl was added to a final density of 1.475 gm cm^{-3}. The solution was then centrifuged to equilibrium at 25,000 rpm for 24 hours in the Beckman 60Ti rotor. The phage band was collected by syringe and dialized against 0.1 M NaCl, 5 mM magnesium chloride, 0.01 M Tris pH 8. Lambda DNA was extracted by the same chloroform butanol method as used for PM2 DNA (yield approximately 200 optical density units from 30 litres of culture).

T7 DNA

Bacteriophage T7 was prepared by the method of Summers and Szybalski (1968) and DNA was extracted from the phage as described for PM2 DNA.

Water soluble carbodiimide

N-cyclohexyl-N'b(4-methylmorpholinium)-ethyl carbodiimide was obtained from Sigma as the p-toluenesulphonate salt. Before use in these experiments it was converted to the bromide salt according to the method of Metz and Brown (1969) with the minor substitution of Dowex 2 for Dowex 1, to facilitate preparation of the bromide salt of the resin. A thick slurry of Dowex 2x8 (200 to 400 mesh) was adjusted to pH 12 with sodium hydroxide to prepare the resin in the form of the free base. The resin was washed on a Buchner funnel with distilled water until the pH of the effluent was below 8, then resuspended in a small volume of water and acidified to pH 2 with 1 M HBr. The resin in the bromide form was thoroughly washed with distilled water before use. A column containing 200 mls packed bed volume of the resin is sufficient to convert 5 gms of the carbodiimide p-toluenesulphonate salt to the bromide form. After lyophilization of the solution the reagent was redissolved in absolute ethanol for storage at -20°C.

Reaction of DNA with Carbodiimide

During the course of experiments various procedures were applied to the condensation reaction between DNA and carbodiimide (see Ch. III). The procedure found to be most

satisfactory is described here.

The reaction mixture for DNA with carbodiimide contained 10% w/v of the carbodiimide as either the p-toluenesulphonate or the bromide salt, 50% v/v ethanol, 0.01 M sodium carbonate and 0.05 M sodium bicarbonate. The reaction was allowed to proceed at room temperature for varying periods up to 12 hours. Use of the bromide salt permits the reaction to be monitored spectrophotometrically at 290 nm. Reaction of denatured DNA under these conditions is rapid and complete within 5 minutes. Reaction of native linear DNA follows sigmoidal kinetics suggesting that the reaction proceeds via an unzippering mechanism from a few sites which increase in number as the reaction progresses (see Fig 9). Closed circular DNA reacts with the reagent partially under these conditions. This observation is similar to that of Dean and Lebowitz (1971) who showed that only a fraction of the bases in closed circular DNA react with formaldehyde under mild conditions (see Fig. 18).

After reaction the DNA was separated from excess reagent by chromatography on Bio Rad Agarose 50M equilibrated with 0.05 M potassium phosphate buffer pH 6.5, 0.1 mM EDTA. On some occasions large losses occured during this step, probably through irreversible adsorption to the resin. Ethanol precipitation of the reacted DNA was attempted in some cases but again large losses were experienced.

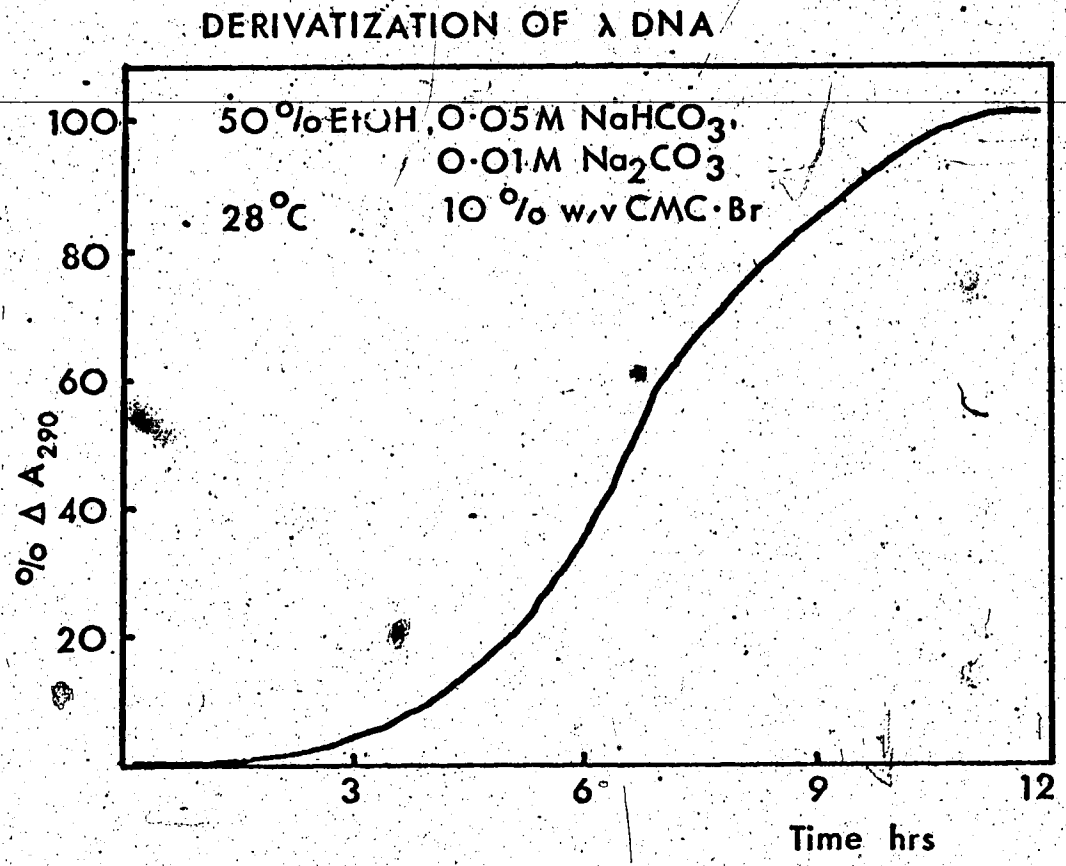


Figure 9. Kinetics of derivatization of lambda DNA monitored spectrophotometrically at 290 nm. Lambda DNA reacts more slowly than dTC-dGA, because of its high molecular weight and the small number of defects in the structure.

The extent of reaction of derivatized closed circular DNA was estimated by measuring the buoyant density shift in CsCl, using the calibration curve plotted in Fig (10).

The Dederivatization Reaction

In most cases the dederivatization reaction was carried out by dialysis of the derivatized DNA against two changes of 0.01 M sodium bicarbonate adjusted to pH 11 with sodium hydroxide at room temperature. Approximately 30 hours is required for completion of the reaction under these conditions. The kinetics of dederivatization of dTC and dGA at pH 12.8 are first order, as shown in Fig (11).

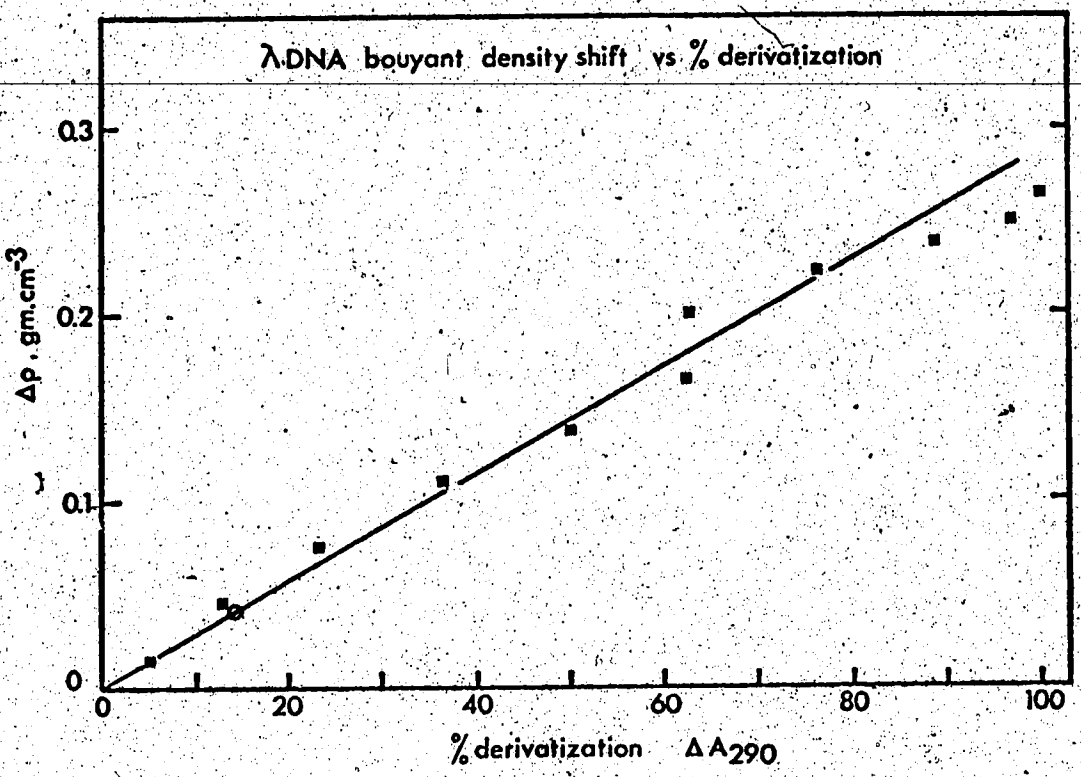


Figure 10. Buoyant density of lambda DNA plotted as a function of the fraction of bases derivatized. Samples were taken at various time points in the experiment shown in Figure 9. The reaction was stopped by the addition of 1 M potassium phosphate pH 6.5 and cooling to 0°C. DNA was then separated from excess reagent by chromatography on Agarose 50 M. Densities were calculated from analytical sedimentation equilibrium runs by the isoconcentration method, since standards are not available for the most highly derivatized samples (Ifft et al. 1961).

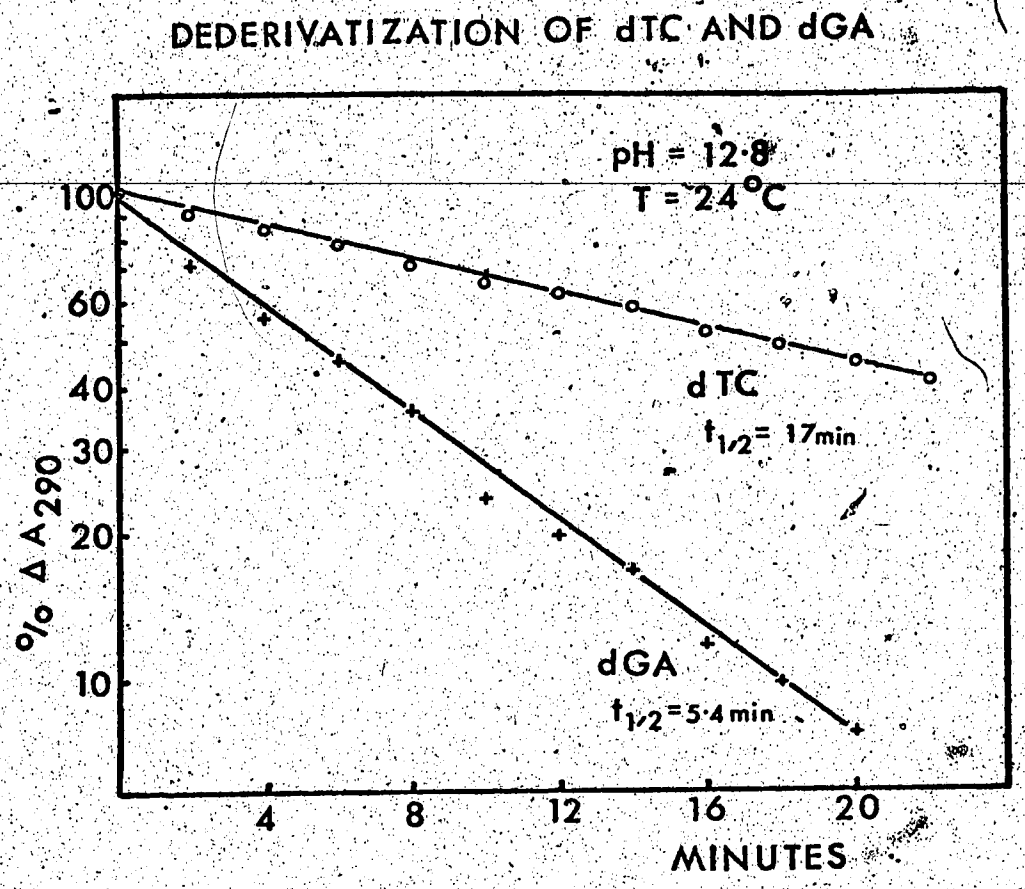


Figure 11. Dederivatization of dTC and dGA in 0.05 M NaOH monitored spectrophotometrically at 290 nm. The final decrease in optical density was 40% for dTC and 56% for dGA.

Electron Microscopy

Electron micrographs were prepared by the method of Davis et al. (1971), using 65% formamide in the hyperphase, 35% formamide in the hypophase for grids of M13 RFI, and 40% formamide in the hyperphase, with 10% formamide in the hypophase for electon micrographs of PM2 DNA. Grids were stained with uranyl acetate and rotary shadowed with 20 A of platinum carbon.

Analytical Ultracentrifugation

In early experiments sedimentation velocity centrifugation of DNA was carried out as described by Studier (1965), in 1 M NaCl, 10 mM Tris, 0.1 mM EDTA pH 8, using a Vinograd type 1 band forming centerpiece. For the titration of superhelical turns 2.83 M CsCl was used as the sedimentation medium as described by Ghay et al. (1971). This was found to have the advantage of forming sharper sedimenting bands and was used throughout for later experiments. All sedimentation velocity runs were performed at 44,000 rpm, 20°C with 8 minute photographs. Sedimentation coefficients of DNA in the CsCl runs were calculated using the expression $S^{o}_{20,*} = d(\log r)/dt \times 27480$ where 27480 includes $1/w^2$ and correction coefficients of 1.16 for solvent viscosity and density, and 1.31 for the $S_{20,w}$ of CsDNA cf NaDNA. Sedimentation equilibrium experiments were carried out using 0.5 to 1 microgram of DNA per ml in CsCl solutions of known initial density. Most runs were performed at 44,000 rpm and

25°C, allowing 17 hours or longer for the solution to come to equilibrium. Buoyant densities were calculated either from the position of the band relative to a reference standard, or, where a standard was not available by the isoconcentration method (Ifft et al. 1961).

Acrylamide Gel Electrophoresis

1. Purification of acrylamide.

On one occasion acrylamide was purified by recrystallization from chloroform as described by Loenig (1967). The yield obtained was poor (50%) and because of the high toxicity of acrylamide the manipulations required for collecting and drying the crystals were hazardous. It was found that acrylamide can be adequately purified for electrophoresis of proteins by stirring a 30% w/v solution with 5 gms per 100 mls of activated charcoal, and filtering under vacuum through a millipore filter. Attempts to purify more concentrated solutions (60% w/v) by this method were unsuccessfull because of spontaneous polymerization of the acrylamide. Two methods of polyacrylamide gel electrophoresis, the acid gel system of Panyim and Chalkley (1969), and the SDS gel system of Lemmli (1967), were used during the course of these experiments, with minor modifications to allow some of the same solutions to be used for both systems.

Solutions for acid gels:

1. 30% Acrylamide 0.2% methylenebisacrylamide (Bis) (w/v)

2. 4% TEMED in 43% acetic acid v/v in water.

3. 6.7 M Urea containing 0.3% ammonium persulphate in water (prepared fresh daily).

4. 5% acetic acid in water (running buffer)

Solutions 1 and 2 can be stored indefinitely at 4°C. Gels are prepared by mixing 4 parts of solution 1 with 3 parts of solution 2 and 1 part of solution 3 under vacuum. 30 mls total of solution is sufficient to form 12 gels in tubes 15 cm long x 0.5 cm internal diameter. After pouring, gels are overlaid with water and allowed to set for at least 1 hour. It was found that the fraction of broken gels can be reduced if the gel tubes are rinsed before use in a dilute solution of a non-ionic detergent (such as 0.1% Nonidet P40 or any commercial dishwashing liquid) and dried at 70°C.

Before use the gels are pre-electrophoresed for 4 to 8 hours at 2 ma per gel with the anode at the top, against the running buffer.

Samples containing 10 to 50 micrograms of protein in 10% sucrose are applied to the upper surface of the gel and electrophoresed at 2 ma per gel until a band of methylene blue marker dye has migrated to the lower surface. Following electrophoresis the gels were stained using one of the methods described below.

SDS gel electrophoresis

Solutions:

1. 30% acrylamide 0.2% Bis (as for acid gels).

2. 48 mls 1 M HCl, 36.6 gms Tris base diluted to 100 mls with water.

3. 2% Bis in water

4. 15.1 gm Tris base, 16.5 ml 6 M HCl, diluted to 100 mls with water.

5. 10% w/v SDS

6. (10x strength running buffer) 30.25 gm Tris, 144 gm glycine, 10 gm SDS made up to 1 litre with water.

30 mls of a 10 % gel solution is prepared by mixing 10 mls of 1 with 3.75 mls of 2, 3 mls of 3, 0.3 mls of 5 and 13 mls of water containing 10 mg of ammonium persulphate and 15 microlitres of TEMED. Gels are overlaid with water until polymerization is complete (approximately 1 hour). The water is then removed using a pasteur pipette and the gels are overlaid to a depth of 1.5 cm with the stacking gel solution (2mls of 1, 0.6 mls of 3, 1 ml of 4, 0.1 mls of 5 and 6 mls of water containing 10 mg of ammonium persulphate and 10 microlitres of TEMED) This in turn is overlaid with water. Samples containing 10 to 50 micrograms of protein are prepared for electroproresis by heating to 100°C for 2 minutes with 0.75 volumes of a cocktail containing 0.4 ml solution 4, 30 microlitres of 0.05% bromphenol blue, 0.1 ml 2-mercaptoethanol, 0.35 ml solution 5, and 0.2 ml glycerol.

The samples are underlayered onto the upper gel surface and electrophoresed at 3 ma / gel with the anode at the bottom, until the bromphenol blue marker dye has reached the lower surface of the gel.

Staining

Gels were stained overnight in either 0.05% Coomassie Brilliant Blue in methanol: acetic acid: water, 5:1:5 or for 4 hours in 1% Amido Black in 7% acetic acid, 30% ethanol. Destaining was accomplished electrophoreticaly using the Canalco destainer and 7.5% acetic acid in 30% methanol as the destaining solvent.

Polynucleotide ligase

Polynucleotide ligase was prepared by the method of Weiss et al. (1968) from T4 infected E. Coli . In one preparation T4AmN82 was used instead of wild type T4. although the concentration of ligase in the crude extract was higher than for wild type T4 the purification did not give a significantly improved yield of enzyme. During purification the enzyme was assayed by the method of Modrich and Lehman (1970). Exonuclease III required for this assay was a gift of W. Flintoff. This assay, which depends upon the ability of ligase to form covalently closed single stranded circles from ^{3}HdAT suffers from one defect in that it is relatively insensitive to contamination of the ligase by exonuclease. An exonuclease copurifys with the ligase forming a sharp peak at the centre of a broader ligase peak

on phosphocellulose. Rechromatography of the pooled ligase fractions on phosphocellulose, pooling only the outer fractions of the ligase peak was necessary before the enzyme was sufficiently free of nuclease to be of use in reseaing double stranded circular DNA containing single stranded breaks.

Fluorimetric assays

A Turner model 430 spectrofluorimeter was used for these assays. The fluorimetric assay for covalently linked complimentary strands of defined synthetic polynucleotides has been previously described (Morgan and Paetkau 1970). A modification of this procedure was devised for the assay of covalently linked complimentary strands in natural DNA's, and for covalently closed circular DNA.

Under the low ionic strength conditions of the assay complimentary single strands do not reanneal, however single stranded natural DNA's have sufficient internal complimentarity to give 50% of the fluorescence enhancement of an equivalent amount of native DNA after the thermal denaturation step in 10 mM Tris pH 8. This is near the fraction predicted for a random polymer (Grala and DeLisi 1974). If the assay is performed with a solution containing 20 mM tripotassium phosphate (pH 12), 0.1 mM EDTA 0.5 micrograms per ml of ethidium bromide the short bihelical regions in denatured natural DNA's no longer give any fluorescence enhancement. Native DNA does not denature under

these assay conditions, and so gives normal fluorescence enhancement.

In the new procedure the fluorescence of a solution containing 0 to 1 microgram of DNA is measured against the fluorescence of a standard containing no DNA (0% relative fluorescence) and the fluorescence of a solution containing a known concentration of DNA. The fluorescence is excited at 525 nm and read at 600 nm. The mixture is then heated to 96°C in a Temp Bloc for 2 minutes or longer and cooled to room temperature before rereading against the same standards. With linear cross linked DNA the fluorescence after heating relative to that before heating is directly proportional to the fraction of double stranded DNA molecules that are cross linked. With covalently closed circular DNA a correction factor must be applied for the difference between the fluorescence enhancement of broken circles and that of closed circular DNA. The fluorescence of the closed circle relative to a linear DNA is a function of both the superhelix density and the ethidium bromide concentration. This difference has previously been utilized in a less sensitive assay for ligase and nuclease by Paoletti et al. (1971).

The assay can be applied to numerous problems of biological interest. In these studies it has been used to monitor the nicking and resealing reactions of closed circular DNA's, and for the assay of w protein activity.

(see Fig 12).

Nicking of closed circular DNA

The nicking reaction required in the protocol for determining the sense and absolute magnitude of supercoiling was carried out at room temperature by making a solution of derivatized DNA 5 mM in magnesium chloride and 0.05 M potassium phosphate pH 6.5. 10 ng Pancreatic DNAse I, or 100 ng of E. Coli endonuclease I (Gift of W. Flintoff) per ml of reaction mixture were then added and, the reaction was followed fluorimetricaly as described above, and terminated by the addition of 10 mM (final concentration) EDTA when 70% of the molecules had received single stranded breaks. The reaction mixtures were prepared immediately for preparative ultracentrifugation by making the volume up to 8 mls with 0.05 M potassium phosphate buffer pH 6.5 containing 0.5 mg/ml ethidium bromide, followed by the addition of 7.5 gms of solid CsCl, and centrifugation to equilibrium at 37,000 rpm, 4°C for 40 hrs. The upper band containing nicked and linear molecules was collected and extracted with water saturated butanol three times, then dialized in the cold against two changes of 0.05 M potassium phosphate buffer pH 6.5, 0.1 mM EDTA.

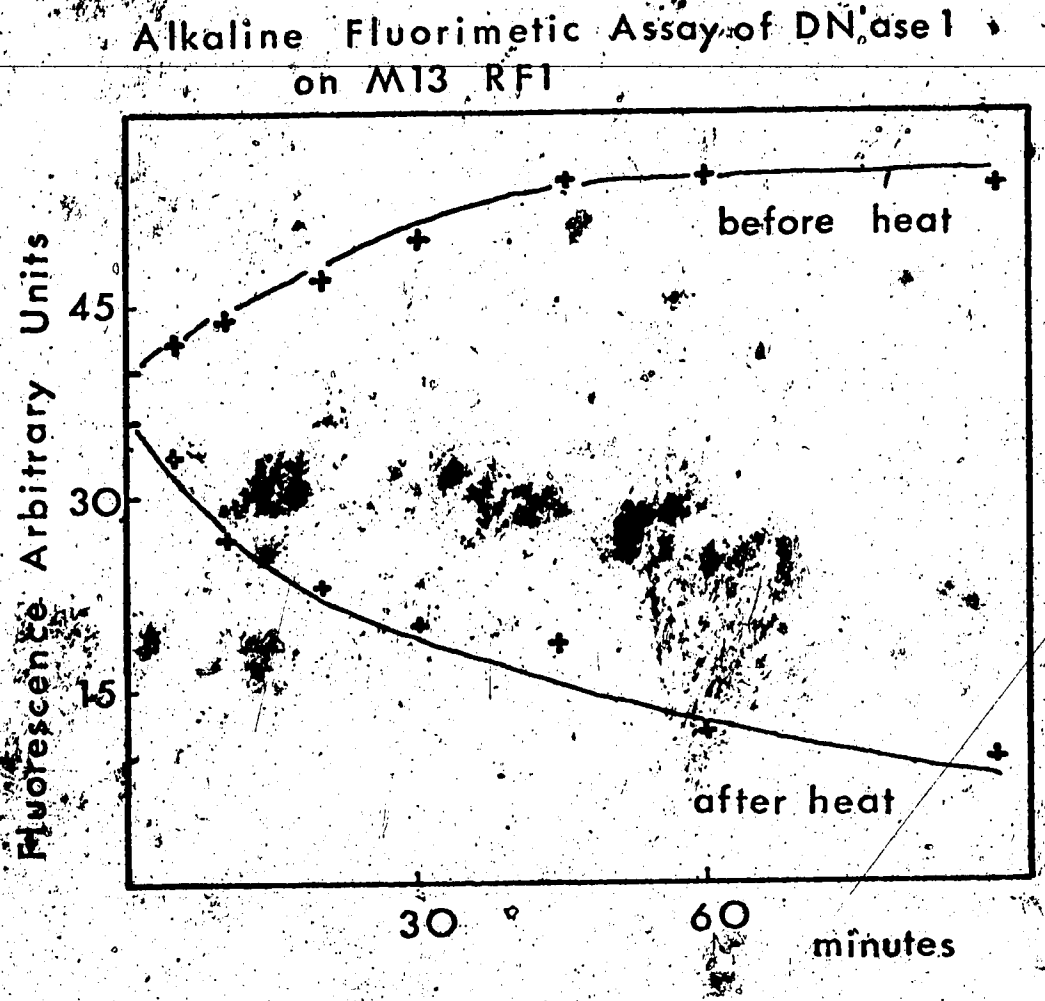


Figure 12. Fluorimetric assay of the action of DN'ase 1 on M13 RFI. 1 microlitre of a solution containing 0.1 microgram per ml of DN'ase 1 was added to 0.5 mls of reaction mixture containing 0.5 O.D. units of RFI/ml, 10 mM Tris, 0.1 mM EDTA, 5mM magnesium chloride, pH8. 50 microlitre samples were taken at the time points shown and assayed fluorimetrically in 20 mM tri-potassium phosphate, 0.1 mM EDTA, .1 microgram per ml ethidium bromide, pH 12.

Resealing of Nicked circular DNA

This reaction was carried out under the same conditions as the nicking reaction except that 0.1 mM ATP and 1 mM DTT were included. Approximately 1 unit of polynucleotide ligase per ml of reaction mixture was added and the reaction was monitored fluorimetrically. It was terminated after the fraction of closed circles had become constant by the addition of 10 mM EDTA (final concentration).

W Protein

For the experiments on the ethidium unwinding angle w protein was used in a crude extract prepared from secondary mouse embryo cells as described by Champoux and Dulbecco (1972). For this reaction a solution of derivatized DNA was made 0.2 M in NaCl and 1/50'th of the volume of the enzyme extract was added. Where possible the reaction was followed fluorimetrically. A further volume of enzyme equal to the first was added when the reaction first appeared to level off, and the reaction was allowed to proceed for an additional two hours to ensure its completion.

Purification of w

The purification of w protein from calf thymus is considered in general terms on p.119. The detailed procedure described here represents the state of the art at the time of writing.

100 grams of calf thymus (stored frozen at −20°C) was homogenized at full speed for 1 minute in a Sorvall Omni Mixer cup with 500 mls of 0.1 M NaCl, 0.05 M sodium phosphate buffer pH 6.0, 1 mM EDTA (extraction buffer) Chromatin and connective tissue debris were collected by centrifugation at 5,000 rpm for 10 minutes in a Sorvall GSA rotor. The supernatant was discarded and the pellet was washed by homogenization followed by centrifugation 3 x with 500 mls of the extraction buffer. It was then resuspended in

300 mls of 0.1 M NaCl, 0.05 M sodium phosphate pH 6.8, 1 mM EDTA containing 1% of a 0.1 M solution of benzyl sulfonyl fluoride in ethanol (this reagent is also commonly referred to as phenylmethylsulfonylfluoride [PMSF], and alpha-toluene sulfonyl fluoride) in order to inhibit nuclear proteases. After incubation for 15 minutes at room temperature 55 mg of iodoacetamide dissolved in 5 mls of water was added (1 mM final concentration) and the incubation was continued for a further 45 minutes. The chromatin was then pelleted by centrifugation and washed once with 500 mls of 0.1 M NaCl in 0.05 M sodium phosphate pH 6.8, 1 mM EDTA (Buffer B). The pellet was resuspended in 500 mls of buffer B and 500 mls of 2 M NaCl, 0.05 M sodium phosphate was added with rapid stirring. The exceedingly viscous chromatin solution was sheared in 50 ml batches in a small Omni mixer cup at full speed for 15 seconds and the sheared solution was added with rapid stirring to 7 litres of 0.05 M sodium phosphate pH 6.8.

A stringy precipitate forms which was collected on a glass rod and homogenized with 300 mls of buffer B, 300 mls of 1.6 M NaCl, 0.05 M sodium phosphate pH 6.8 was then added with rapid stirring followed by 60 grams of polyethlyeneglycol 6000. Phase separation occurs within 15 minutes. The suspension was centrifuged at 10,000 rpm for 10 minutes in the Sorvall GSA head in order to compress the pellet and clear the supernatant. The supernatant was saved

(w fraction 1). In this experiment, as in previous ones a second extraction of the DNA pellet was carried out with 2 M (final concentration) NaCl. This extract contains about the same amount of w activity as the first one.

For reasons which are not clear the w in this second extract has not been sucessfully chromatographed from chromatin preparations which have been treated with protease inhibitors, although it is present in the crude extract. The w fraction 1 was diluted with 3 volumes of 0.05 M sodium phosphate pH 6.8, 1 mM EDTA (final NaCl concentration 0.2 M) and 30 mls packed bed volume of Whatman P11, previously equilibrated against the same buffer was added. The suspension was stirred rapidly for 1 hour and the phosphocellulose was collected by decantation and filtration, washed briefly with 0.05 M sodium phosphate pH 6.8, 1 mM EDTA 1 mM EDTA, 0.2 M NaCl (starting buffer) and resuspended in 50 mls of the same. The slurry was layered over a previously prepared column (150 ml packed bed volume) of P11 and the activity was eluted from the column with a 500+500 ml linear salt gradient from 0.2 to 1.5 M NaCl, 0.05 M sodium phosphate pH 6.8. The elution profile is shown in fig. 13.

W containing fractions from phosphocellulose were pooled and concentrated by ultrafiltration to approximately 3 mls using a PM10 or PM30 membrane. The concentrate was then applied to a Sephadex G100 or G150 superfine column

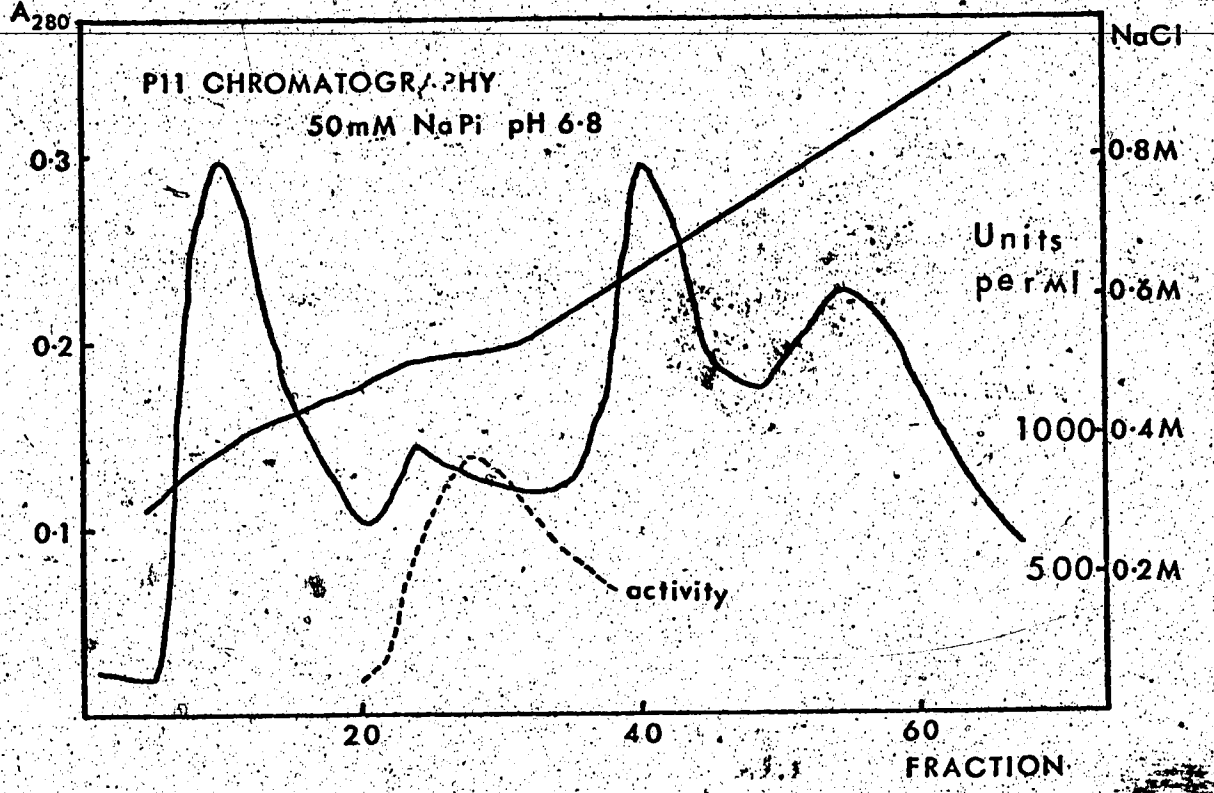


Figure 13. Elution profile of w from phosphocellulose. W was bulk adsorbed to 30 mls packed bed volume of Whatman P11 from 0.2 M NaCl, 0.05 M sodium phosphate pH 6.8, and layered over a previously prepared column of Whatman P11 (150 mls packed bed volume). The activity was eluted by a gradient from 0.2 to 1.5 M NaCl in 0.05 M sodium phosphate pH 6.8.

(100 mls bed volume) with 0.5 M NaCl, 0.05 M sodium phosphate pH 6.8 as the eluting medium. 1.7 ml fractions were collected. The elution profile from Sephadex G100 is shown in Fig. 14. Chromatography on Sephadex G150 gave better resolution of the w activity from the excluded peak, at the cost of failing to resolve the enzyme peak completely from the residual lysine rich histone KAP. SDS gel electrophoresis of the active fractions from G100 showed three main components to be present with molecular weights of 53,000, 51,000 and 32,000 fig. 29. Minor components of higher molecular weight were present. However these were also present in the inactive excluded peak and so are unlikely to be the enzyme.

Assay of W

The assay cocktail contains 50 micrograms / ml of PM2 DNA dissolved in 0.2 M NaCl, 2 mM EDTA, 10 mM Tris pH 8. The enzyme was assayed by adding from 1 to 5 microlitres of enzyme solution, diluted if necessary with 0.2 M NaCl, 10 mM Tris pH 8, to 30 microlitres of the assay cocktail followed by incubation at room temperature (24°C) for 15 minutes. The entire assay mixture was then rinsed into 1.7 mls of the alkaline ethidium bromide solution (see Fluorimetric assays). Under these conditions complete relaxation of the superhelical DNA results in a 33% decrease in the fluorescence enhancement of closed circular PM2 DNA. The contribution from contaminating nicked circles was

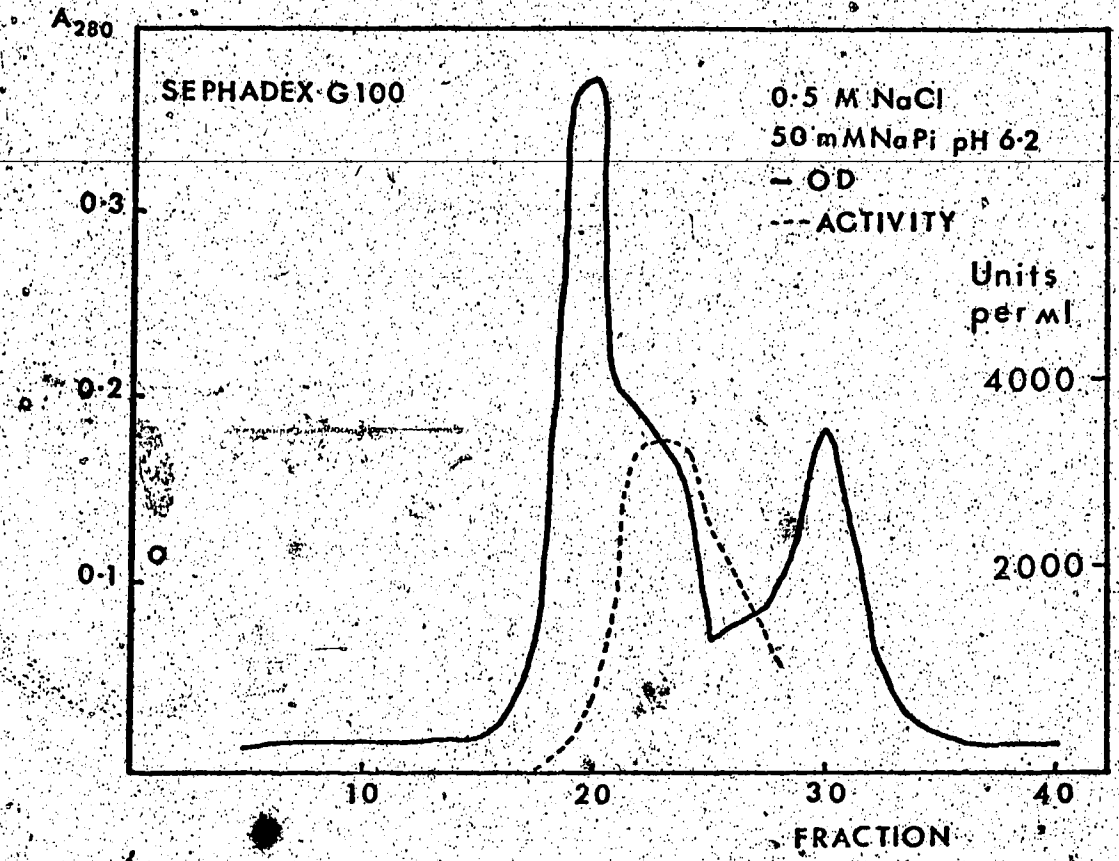


Figure 14. Elution profile of w from Sephadex G 100. Peak fractions containing w from phosphocellulose were pooled and concentrated to 3 mls by ultrafiltration on a PM 30 membrane then applied to a column of Sephadex G 100 superfine and eluted with 0.5 M NaCl, 0.05 M sodium phosphate pH 6.8, 0.1 mM EDTA.

eliminated by the heat step. One unit of enzyme activity was operationally defined as the amount of enzyme required to completely relax 1.5 microgram of superhelical PM2 DNA (the amount present in a 30 microlitre aliquot of assay mixture) in 15 minutes at 24°C. Since the enzyme kinetics are neither linear, nor exponential exact quantitation is uncertain unless a series of enzyme concentrations is assayed, with the end point taken where no further decrease in fluorescence enhancement occurs.

An additional problem in the quantitation of w that went unrecognized until relatively recently is that the enzyme is inactivated when the protein concentration in the reaction mixture falls below 1 microgram per ml (possibly because of adsorption to the glass walls of the reaction vessel). This effect is demonstrated in the kinetic studies shown in fig 15a,b. The effect is partially but not completely overcome by the addition of 300 micrograms per ml of gelatin to the reaction mixture (fig 15c).

Artifactual decreased fluorescence enhancement was occasionally observed in samples containing large amounts of the lysine rich histone KAP. This is due to the binding of protein to DNA with consequent exclusion of ethidium. Other histone fractions interfere with the assay if carried out at pH 8 but not when alkaline conditions are employed.

Where this artifact was suspected the reaction of DNA

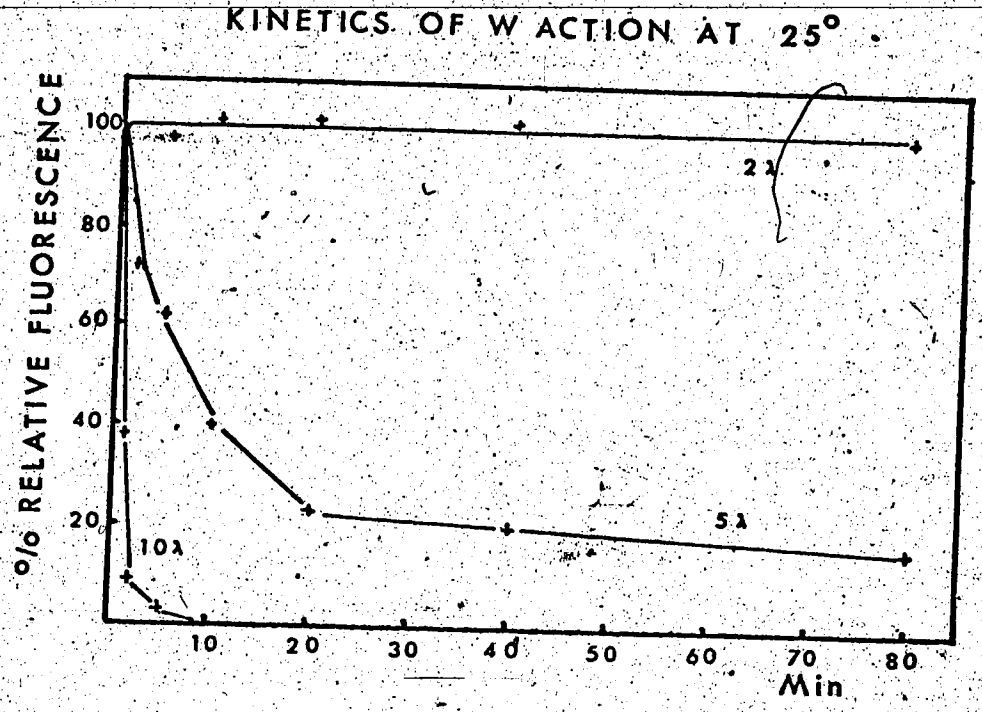
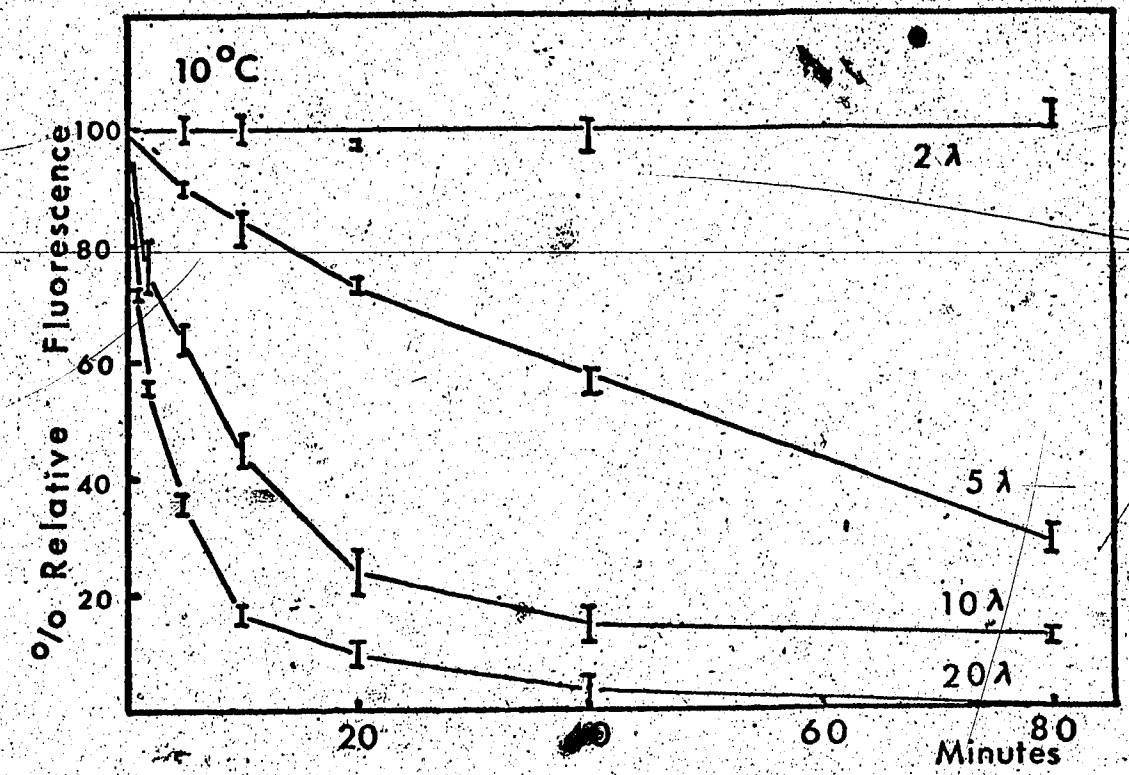
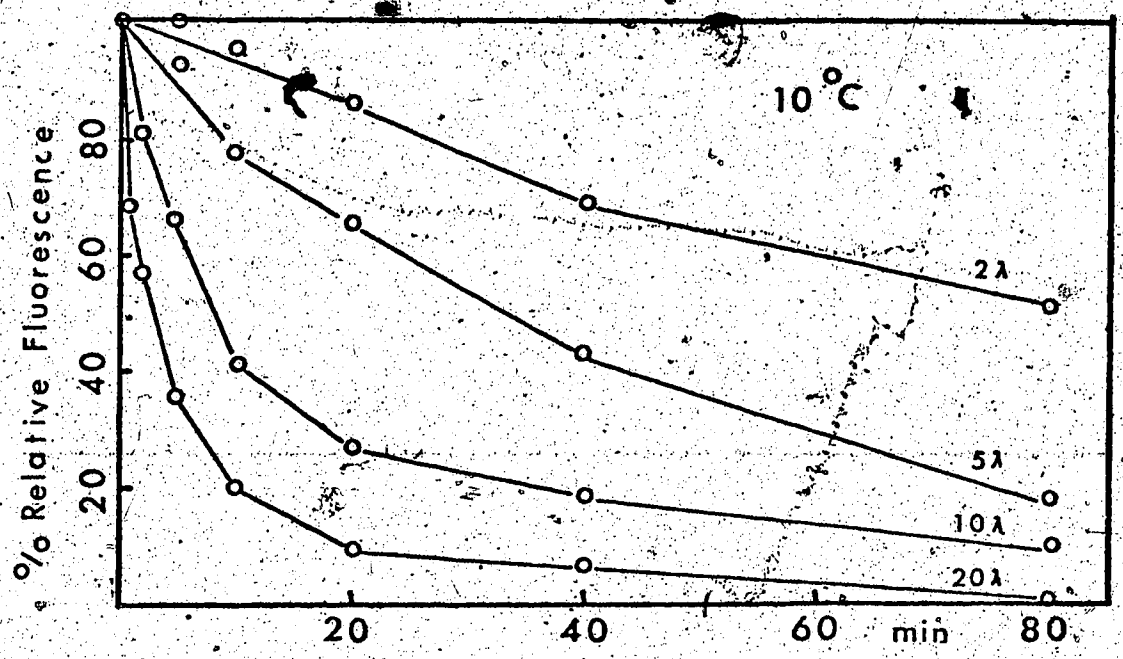


Figure 15. Assay of purified fractions of w with PM2 DNA in 0.2 M NaCl, 10 mM Tris, 2 mM EDTA, pH8 at 24° C. 0.32 mls of assay mixture was mixed with varying amounts of diluted enzyme solution, at 0 time 50 microlitre samples were taken for assay in alkaline assay mixture. The relative fluorescecnce is plotted as a function of the final decrease in fluorescence that occurred with the highest level of enzyme.



15 b. The effect of lowering the tempefature to 10°C. The conditions of reaction are otherwise the same as in 15 a. The rate of reaction is approximately 1/5'th that at 24°C.



15 c. Assay of w at 10°C under the same conditions as 15 b except that the reaction mixture contained 100 micrograms per ml of gelatin. The rate of reaction is still not linear with respect to enzyme concentration.

with w was stopped by the addition of 3 micrograms of trypsin (stored as a 1 mg per ml solution in 1 mM HCl) followed by a 10 minute incubation at 24°C. Under these conditions the activity of w is destroyed almost instantaneously, and the histones are degraded to a point where they no longer interfere with the fluorescence enhancement of DNA.

The Fluorimetric titration of superhelical turns in closed circular DNA

These experiments were carried out over a range of salt concentration from 0.05 M to 0.5 M NaCl, buffered with 1 mM Tris pH 8, and 0.1 mM EDTA. The protocol was the same in all experiments. A series of experimental tubes containing 2 mls of solution at the same salt concentration, but with different concentrations of linear calf thymus DNA, or closed circular DNA was prepared. In addition two standard tubes were included, one without DNA, the other containing 0.75 mg/ml of calf thymus DNA. The sample DNA concentrations ranged from 0.8 to 25 micromolar in phosphate depending upon the salt concentration employed. The standards were used to set the 0 and 100% relative fluorescence points on the fluorimeter scale. The excitation wavelength was set at 510 nm and the emission was measured at 600 nm. Equal volumes of ethidium bromide in the same salt solution as the DNA were added to all tubes in the series by means of a calibrated micropipette. The extinction coefficients used for DNA at

260 nm were 6600 for calf thymus and PM2 DNA's (approximately 40% GC content) and 6740 for M13 and PhiX174 RFI (approximately 50% GC content). Concentrations of ethidium were calculated on the basis of an extinction coefficient at 487 nm of 5450 (Waring 1965).

The fraction of the dye bound to the DNA was determined by measuring the fluorescence of the sample solution relative to the two standards. Binding constants for the dye were calculated using an Olivetti Programma 101 calculator and the program listed in the appendix. See page 111 for the theoretical basis and results of these experiments.

III. RESULTS

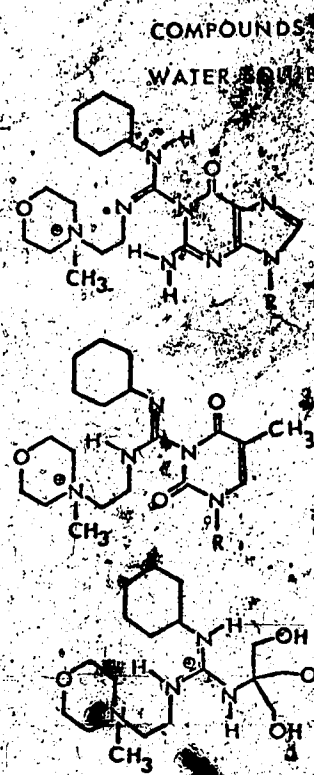
The separation of dTC from dGA.

The replication of natural DNA and the synthetic polymer dTG:dCA in vitro by E. Coli DNA polymerase I is linkage of product strands so that they cannot be separated by alkaline buoyant density centrifugation of other means (Schildkraut et al. 1964, Paetkau 1969). The cross linking reaction can be prevented by including a factor in the synthesis mixture that is present in crude fractions of D and RNA polymerases (Flintoff and Paetkau 1974). covalently linked product can be detected and assayed by simple fluorimetric procedure using ethidium bromide (Morgan and Paetkau 1972).

Another synthetic polymer dTC:dGA does not show strand separation when banded at buoyant equilibrium in alkaline CsCl, because the two strands have similar buoyant densities. It was of interest to show whether or not the strands of this polymer are covalently joined when synthesized in the absence of the factor required for strand separability of dTG:dCA. That they were probably not joined was shown by applying the assay developed for the detection of covalently linked sequences in dTG:dCA to dTC:dGA. No return of fluorescence after heating under the conditions of the assay was observed. However the structural differences between the two polymers meant that an independent control

was necessary to ensure that the assay could be legitimately be applied to dTC:dGA. The separated strands of dTC:dGA were also required for physical studies of this polymer which has several interesting properties (Morgan and Wells 1968, Johnson et al. 1974)

N-cyclohexyl-N'b-(4-methylmorpholinium) ethyl carbodiimide reacts specifically with guanine and thymine residues of denatured DNA (Ho and Gilham 1967, Metz and Brown 1969, Drevich et al. (1967) forming the products shown in Fig. 16



N-1 of guanine and N-3 of thymine are blocked in the reaction so that specific Watson-Crick hydrogen bonds cannot form with the complimentary bases. The products are stable at neutral pH, but decompose readily under alkaline conditions to give back the original base. It was expected that the reagent could be used to prevent the strands of dTC:dGA from annealing under conditions where they can be separated.

Conditions had to be found where the reaction of the polymer could occur without degradation. In order to monitor the reaction at 290 nm (Metz and Brown 1969) the reagent had first to be converted from the p-toluenesulphonate salt to the bromide form. Initial attempts to carry out the reaction by melting the polymer at elevated temperature were hindered by the high ionic strength of solutions containing the reagent (requiring excessively high temperature to melt the polymer), and by side reactions which caused decomposition of the polymer. It was eventually discovered that conditions similar to those employed by Gilham (1962) for isolating ribotrinucleotides terminating in cytosine from RNA (using 7 M urea as solvent), could be used for the reaction of the polymer with the carbodiimide, however problems were experienced in controlling the reaction. An essential factor in determining the rate is the pH of the reaction mixture. High pH facilitates denaturation of the DNA, catalyses both

foreward and reverse reactions as well as hydration of the reagent. It was found that when Tris was used to buffer the reaction the pH of the mixture increases with time, probably because of reaction between Tris base and the reagent, with the formation of a strongly basic guanido compound (a suggested formula is shown in fig. 16). Omission of buffer, or replacement with bicarbonate overcame the difficulty. Another problem was recognized when attempts were made to derivatize T7 DNA by this procedure. The product sedimented slowly after dederivatization indicating extensive cleavage of the DNA strands under the conditions of the reaction. Various denaturing solvents other than 7 M urea were tried in attempts to overcome this problem. Results with dimethyl formamide and dimethyl sulphoxide were disappointing. The former reacts with the reagent to form a product which absorbs strongly at 290 nm, the latter was effective in promoting the reaction, but only when used at inconveniently high concentrations (>80%). Problems were experienced with rapid hydrolysis of the reagent in this solvent so that the reaction could not be controlled effectively. The solvent that was eventually found to be most suitable was 50% ethanol adjusted to pH 9.5 with 0.01 M sodium carbonate, 0.05 M sodium bicarbonate. Ethanol has the added advantage of being a suitable solvent for storage of the bromide salt of the carbodiimide after conversion from the p-toluenesulphonate. The kinetics of reaction of double stranded linear DNA under these conditions are sigmoidal

(see fig. 9), suggesting that the reaction proceeds via local denaturation around the initial reaction sites, which increase in number with time and eventually lead to the total collapse of the bihelical structure. After treatment with carbodiimide the strands of dTC:dGA can be separated by buoyant density centrifugation in CsCl of average density of 1.4 gm. cm^{-3} (Figure 17). Derivatized dGA has a buoyant density of 1.43 gm cm^{-3} while derivatized dTC has a buoyant density of 1.38 gm cm^{-3} (determined by preparative gradient centrifugation).

Identification of the strands was based on the more rapid dederivatization of dGA than dTC (see fig. 10) (Ho and Gilham 1967), effective transcription of the dTC strand to rGA by RNA polymerase (dGA does not appear to be a suitable template for this enzyme, probably because of aggregation), and the spectra of the dederivatized strands. Individually the separated strands do not give fluorescence enhancement with ethidium bromide, but when mixed and annealed normal fluorescence enhancement was obtained.

Under the conditions of reaction and dederivatization employed in these experiments approximately 30% of the single strands of T7 DNA were broken. In later experiments with PM2 DNA it was demonstrated that this side reaction occurs mainly during the alkaline dederivatization step.

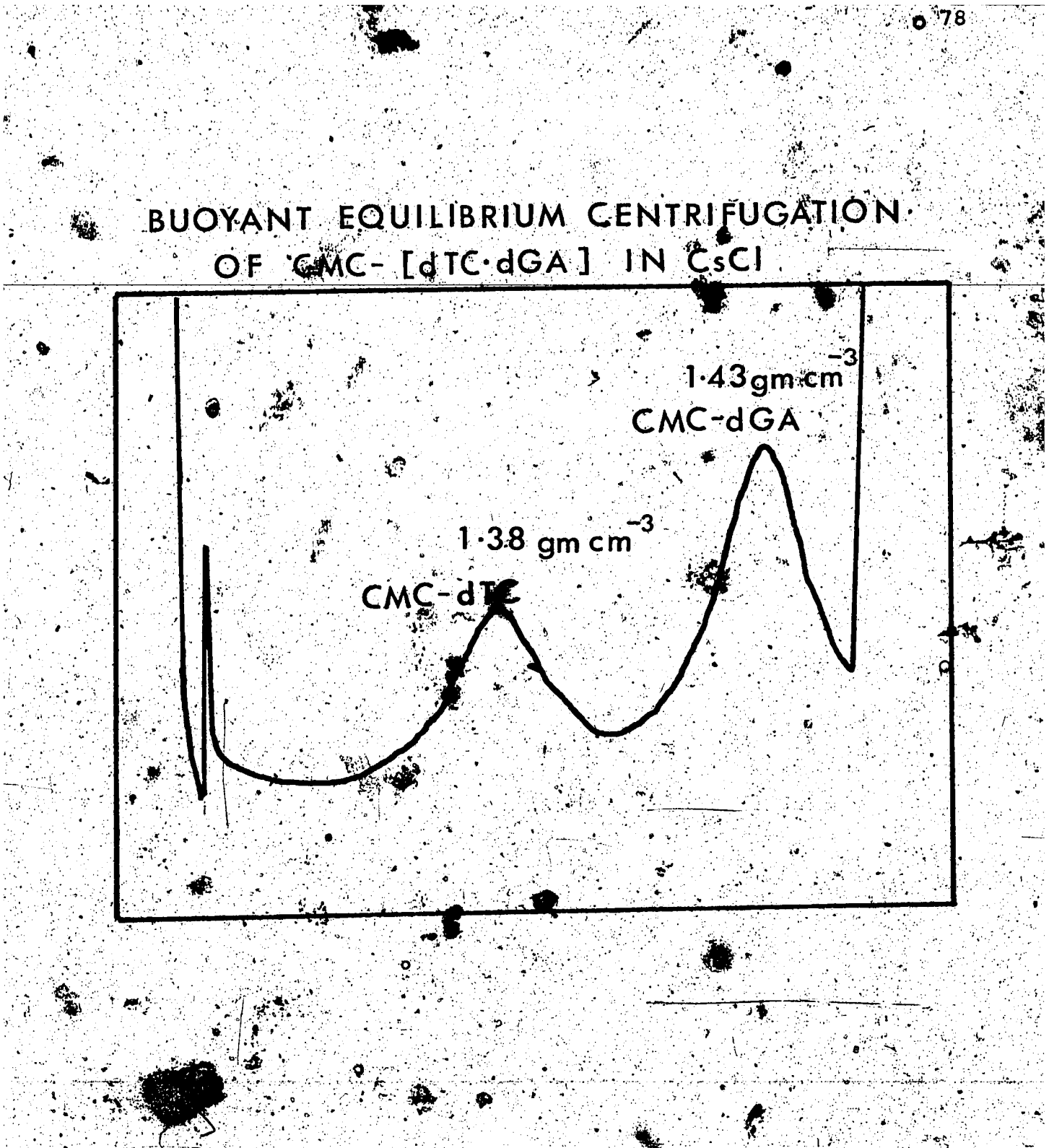


Figure 17. Buoyant equilibrium of derivatized dTC:dGA in CsCl (initial density 1.4 gm cm^{-3}). The sample was prepared by treating dTC:dGA with carbodiimide as described in methods. Centrifugation was at 52,000 rpm, 25°C for 17 hrs. Buoyant densities were obtained from a parallel preparative centrifugation.

The sign of supercoiling of natural closed circular DNA.

The experience gained with the carbodiimide suggested it would be an ideal reagent for application in the approach to determining angle change due to ethidium binding proposed in the introduction. Closed circular DNA was initially prepared from PhiX174 infected E. Coli. Problems were encountered in obtaining sufficient quantities of viable bacteriophage to infect the cultures. It was later found that RFI could be prepared with equal facility from M13 infected E. Coli, but PM2 phage grown on Pseudomonas BAL31 proved to be the best source of closed circular DNA for these experiments.

The reaction between carbodiimide and closed circular DNA is self limiting (figure 18), being similar to the reaction between formaldehyde and closed circular DNA when the reaction is carried out under mild conditions (Dean and Lebowitz 1971). This result was encouraging since it ensured that large stretches of unmodified DNA would be available for endonuclease and ligase reactions. Pancreatic DNase I nicks derivatized RFI slowly, and the product cannot be resealed with ligase, suggesting that this enzyme attacks derivatized regions preferentially. This illustrates the reason for starting the sequence of reactions with a closed circular molecule rather than a nicked circle since the reagent would otherwise react at the exposed ends, preventing resealing of the circle by ligase. The problem

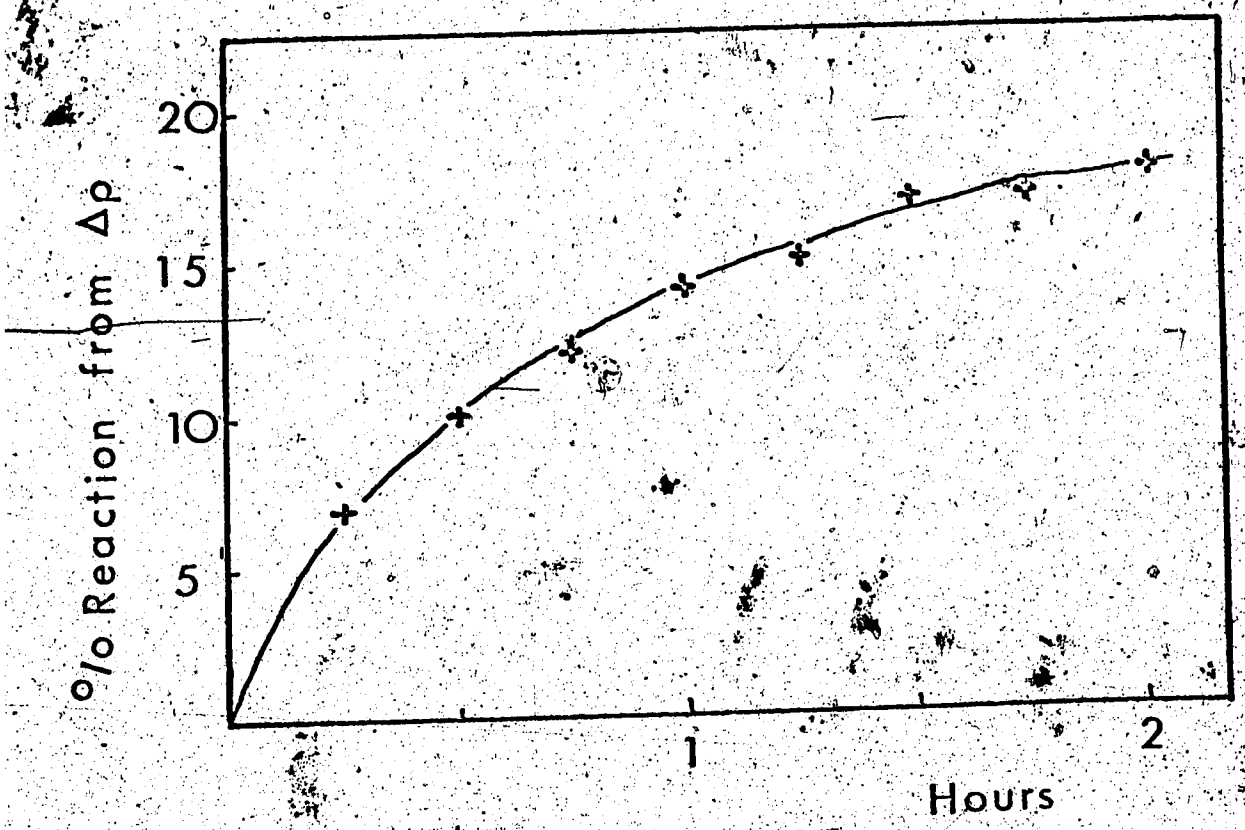


Figure 18. Kinetics of derivatization of PM2 DNA with carbodiimide at 0°C. The fraction of bases derivatized was calculated from the buoyant density shift in CsCl using the calibration curve shown in Figure 10.

was not encountered with E. Coli endonuclease I, although it does have a tendency to cause double stranded breaks when used at 37°C, this is less of a problem at 25°C.

In order to avoid excessive cleavage of the DNA the reaction was stopped when approximately 70% of the molecules had received single stranded breaks. Nicked molecules were separated from the remaining closed circles by buoyant equilibrium centrifugation in CsCl in the presence of ethidium bromide. On one occasion propidium diiodide was used instead of ethidium to improve the separation between the two bands of DNA, but difficulty was experienced in removing this dye from the DNA after the centrifugation. Niether water-saturated butanol (used to remove ethidium from the CsCl solution after centrifugation), nor dialysis against 0.05 M potassium phosphate, pH 6.5, followed by passage through a column of Dowex 50 ion [illegible] were effective in removing the last traces of [illegible] were resealed using T4 ligase and finally [illegible] dialysis against dilute alkali. Yields of [illegible] circular DNA from this cycle of treatments were low. In several experiments starting with 20 optical density units of M13 RFI no yield of material was obtained after the cycle of treatments, the main losses occurred during purification steps since DNA with bound carbodiimide is exceedingly sticky. However sufficient DNA (0.5 optical density units) was obtained from one experiment using M13 RFI to give the

results shown in Figure 19. The deep minimum in the titration curve clearly resolves the question of sign of natural superhelices in favour of negative, disproving Paoletti and LeRecq's contention that ethidium overwinds the duplex.

The conclusion was supported by an independent proof obtained from micrographs of partially derivatized PM2 DNA. The micrographs were prepared using the modified Kleinschmidt procedure of Davis et al. (1971) with the expectation that it would be possible to visualize the denatured, derivatized regions of the molecule. Slightly (less than 20%) derivatized circular molecules did not show open loops, probably because the derivatized regions are too small (highly derivatized lambda DNA does show open loops - see Plate 1).

Occasionally M13 RFI adopts a striking convoluted spiral conformation on the grid when spread by the formamide technique (Plate 2). This type of conformation has not been observed with native PM2 DNA but does occur when the DNA has been derivatized lightly (approximately 5%) (Plate 3). Increasing the level of derivatization to 12% (plate 4) leads to a somewhat more open conformation and the spiral molecules become more frequent; at 20% derivatization (Plate 5) most of the molecules are in this form.

An important characteristic of this form is the

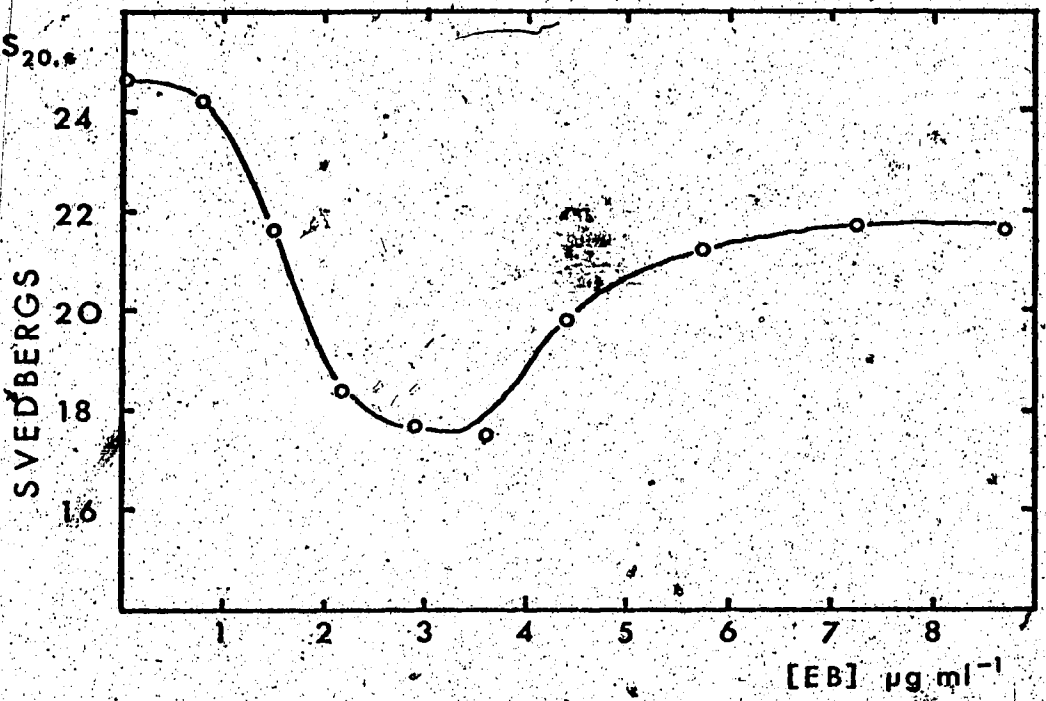


Figure 19. Titration of superhelical turns in M13 RFI which had been derivatized, nicked, resealed and dederivatized according to the protocol in Figure 5. At the time of ring closure 4.5% of the available residues in the DNA were derivatized.

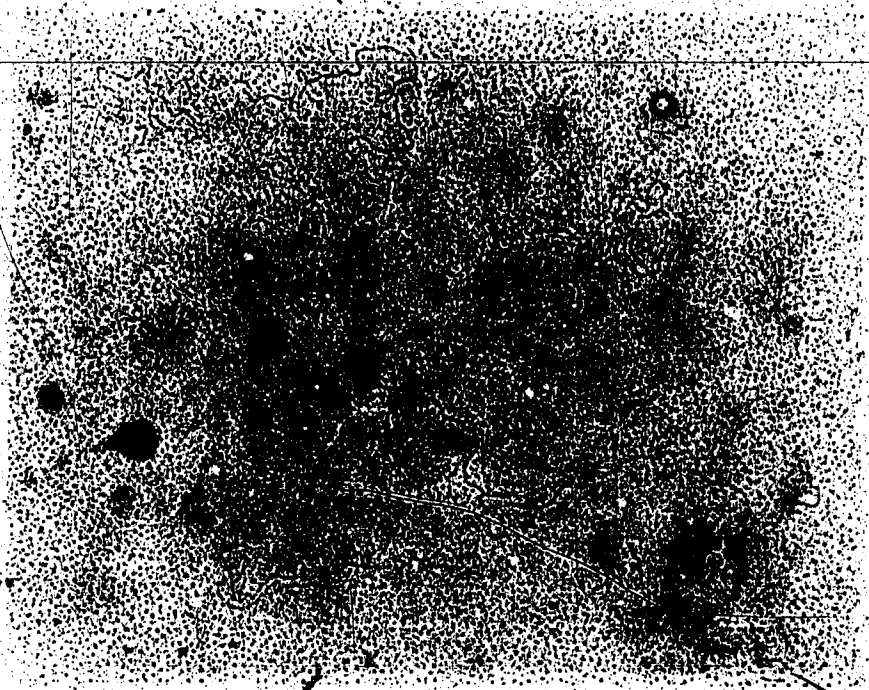


Plate [illegible] Lambda DNA in which 75% of the available T and G residue[illegible]ave been modified by the carbodiimide reagent. (M=27,[illegible]x).

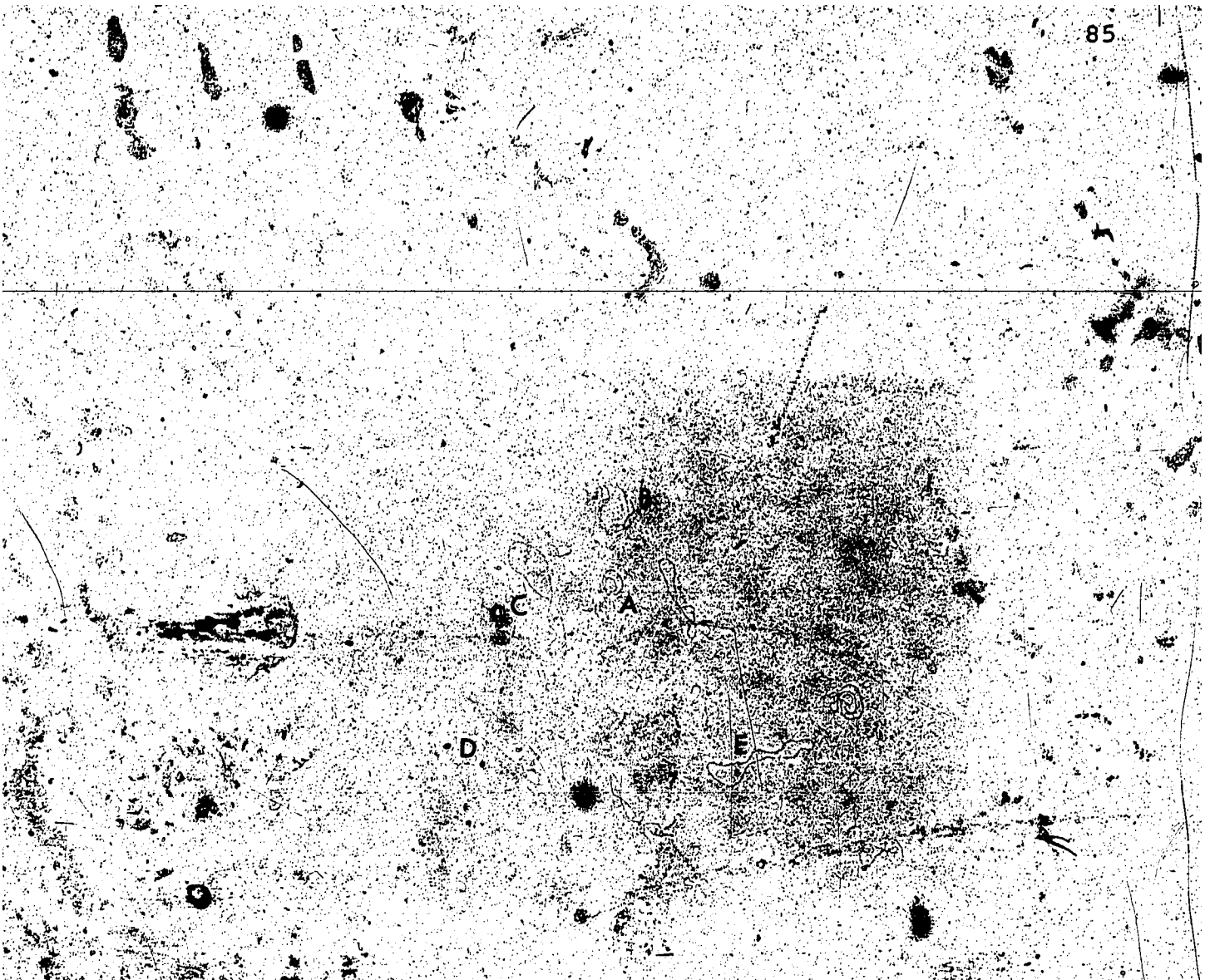


Plate 2. (A) M13 RFI in the right handed spiral conformation spread as described in the text. Also present in the same field are (B) M13 RFI in the interwound form usually observed in superhelical DNA, (C) a replicating molecule, (D) a single stranded M13 DNA circle and (E) a nicked-double stranded circle. This is a unique photograph in that all the possible forms of M13 DNA are present in the same field.

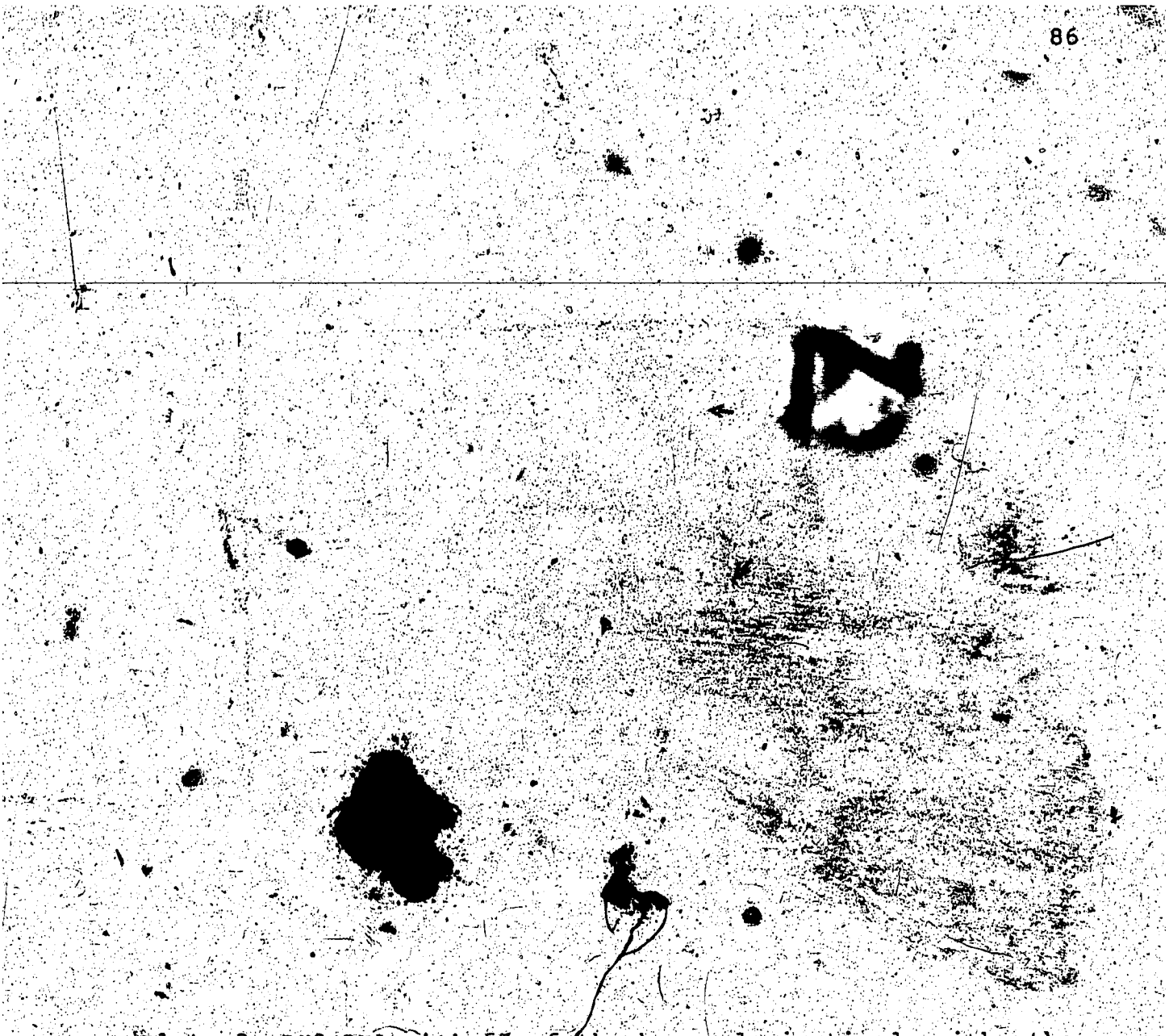


Plate 3. PM2 DNA with 5% of the bases derivatized with the carbodiimide. Arrows show right handed spiral forms. (M=17,000X)

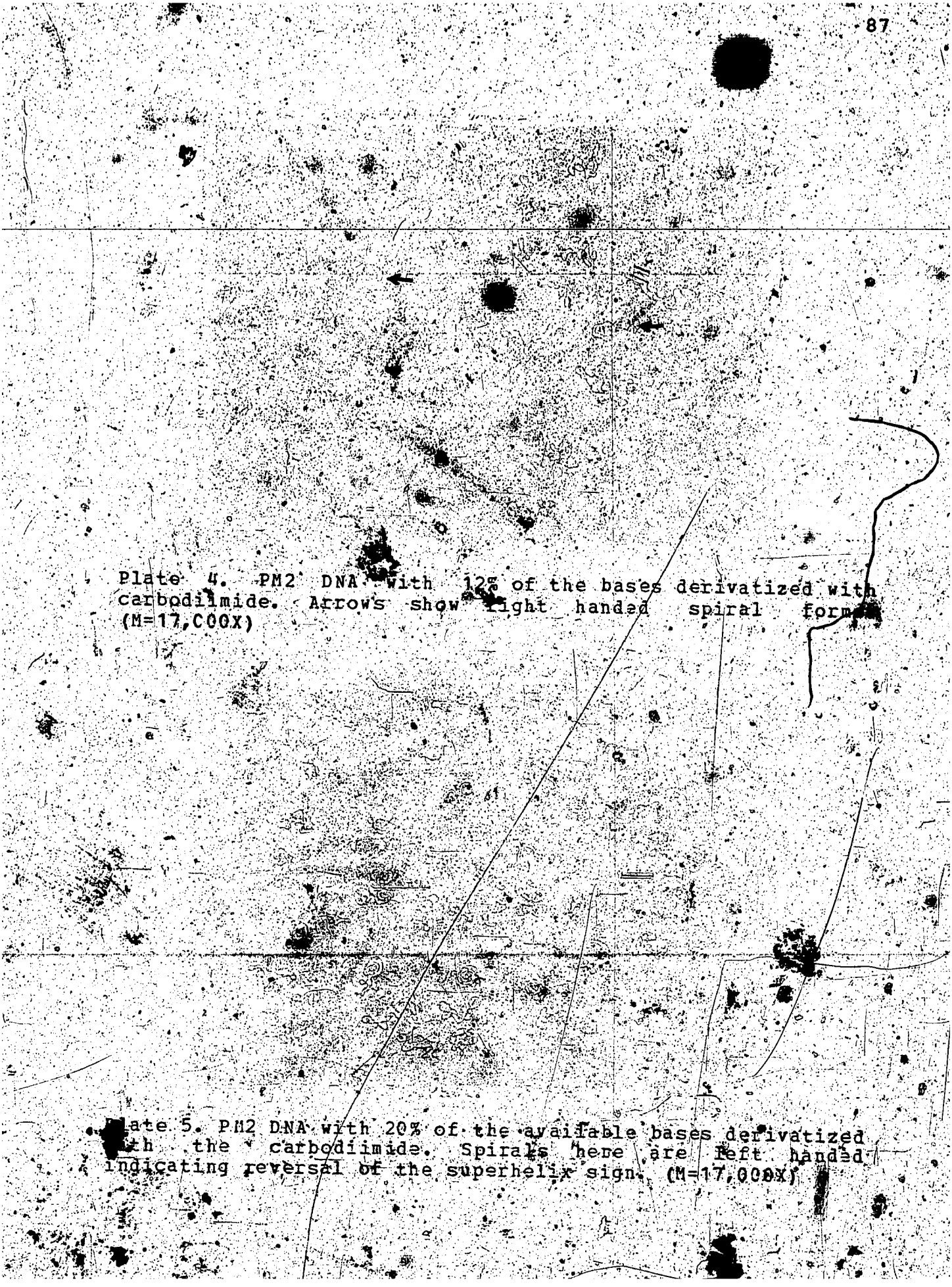


Plate 4. PM2 DNA with 12% of the bases derivatized with carbodiimide. Arrows show right handed spiral form. (M=17,000X)

Plate 5. PM2 DNA with 20% of the available bases derivatized with the carbodiimide. Spirals here are left handed indicating reversal of the superhelix sign. (M=17,000X)

handedness of the spiral which is always the same within a given preparation. All photographs were taken with the cytochrome C film below the grid, and the film emulsion facing upwards. Image reversal occurs during preparation of the negative, this is corrected during printing by placing the negative emulsion downwards in the enlarger. The final print shows the spiral form as it would be viewed from above. With native M13 RFI and lightly derivatized PM2 DNA (<12%) the spirals are always right handed when observed in an outside towards inside direction. With heavily derivatized PM2 DNA the spirals are always left handed from the same viewpoint. Since the reversal of handedness corresponds approximately to the minimum in the sedimentation coefficient when measured against the fraction of bases derivatized (Figure 20), it was reasoned that the handedness of the spiral is related to the sign of supercoiling. Campbell and Jolly (1973) suggested from light scattering experiments that closed circular DNA of low superhelix density can adopt a toroidal conformation in solution rather than the interwound form usually observed in electron micrographs (see fig 4). The two forms of the superhelix are topologically equivalent, but appear at first glance to have opposite handedness of supercoiling because of a 90° rotation of the superhelix axis that occurs during conversion of one form to the other (Vinograd et al. 1968). The highly ordered arrangement observed in our micrographs may arise by progressive attachment of a toroidal molecule

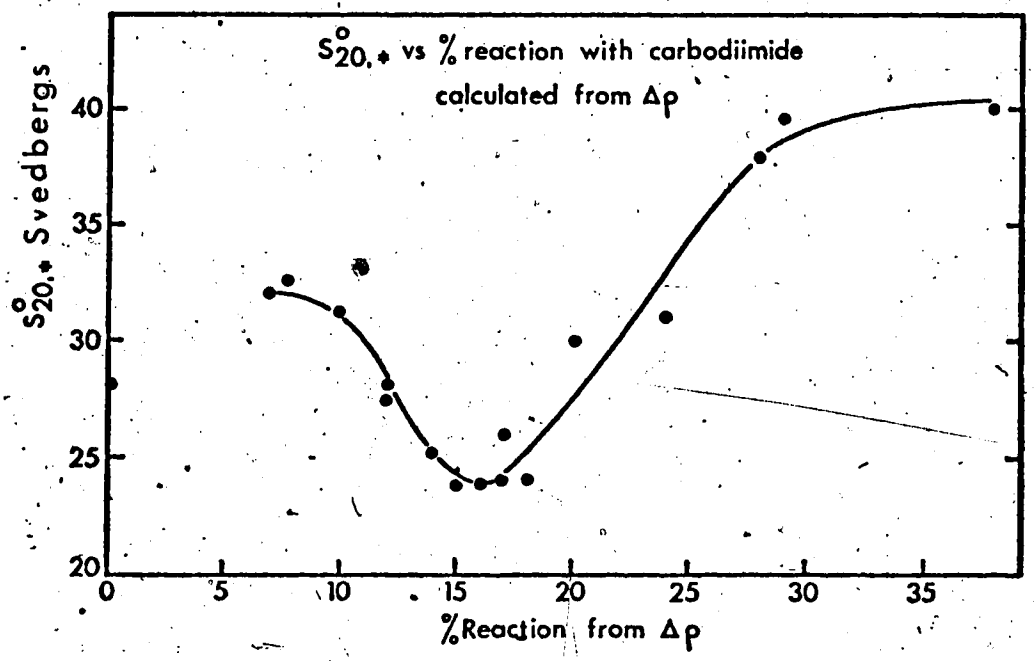


Figure 20. Sedimentation coefficient of PM2 DNA plotted as a function of the percent derivatization determined by the buoyant density shift. PM2 DNA derivatized to varying extents was obtained by taking aliquots from standard reaction mixtures after different periods of incubation. Very early points were not obtained because of the rapidity of the initial reaction.

to the cytochrome C film. Negative superhelical turns in a torcidal molecule will cause the molecule to curve in a right handed direction as attachment to the film proceeds, while positive supertwists will cause left handed curvature (Figure 21). If the superhelical turns are at the same time forced into the part of the molecule not yet attached to the film, the superhelix density of the unattached portion of the molecule will increase, as will the degree of curvature. Therefore a negatively supertwisted molecule will spiral inwards in a right handed direction, while a positively supertwisted molecule will form a left handed spiral. Since native M13 RFI and lightly derivatized PM2 DNA molecules form right handed spirals the supertwists are negative. Highly derivatized PM2 DNA is positively supertwisted since the molecules adopt a left handed spiral conformation.

Spiral forms are occasionally found in micrographs of high molecular weight linear DNA. These probably arise when initial attachment of the molecule occurs at two different sites, forming a topologically closed unit. A small excess or deficiency of helical turns in this region can then be manifested as a spiral. In linear molecules right and left handed spirals are observed with equal frequency.

Beyond proving the handedness of natural superhelices the method of synthesizing negative supercoils offers a simple method for determining the value of the unwinding angle due to binding of ethidium. In order to make this

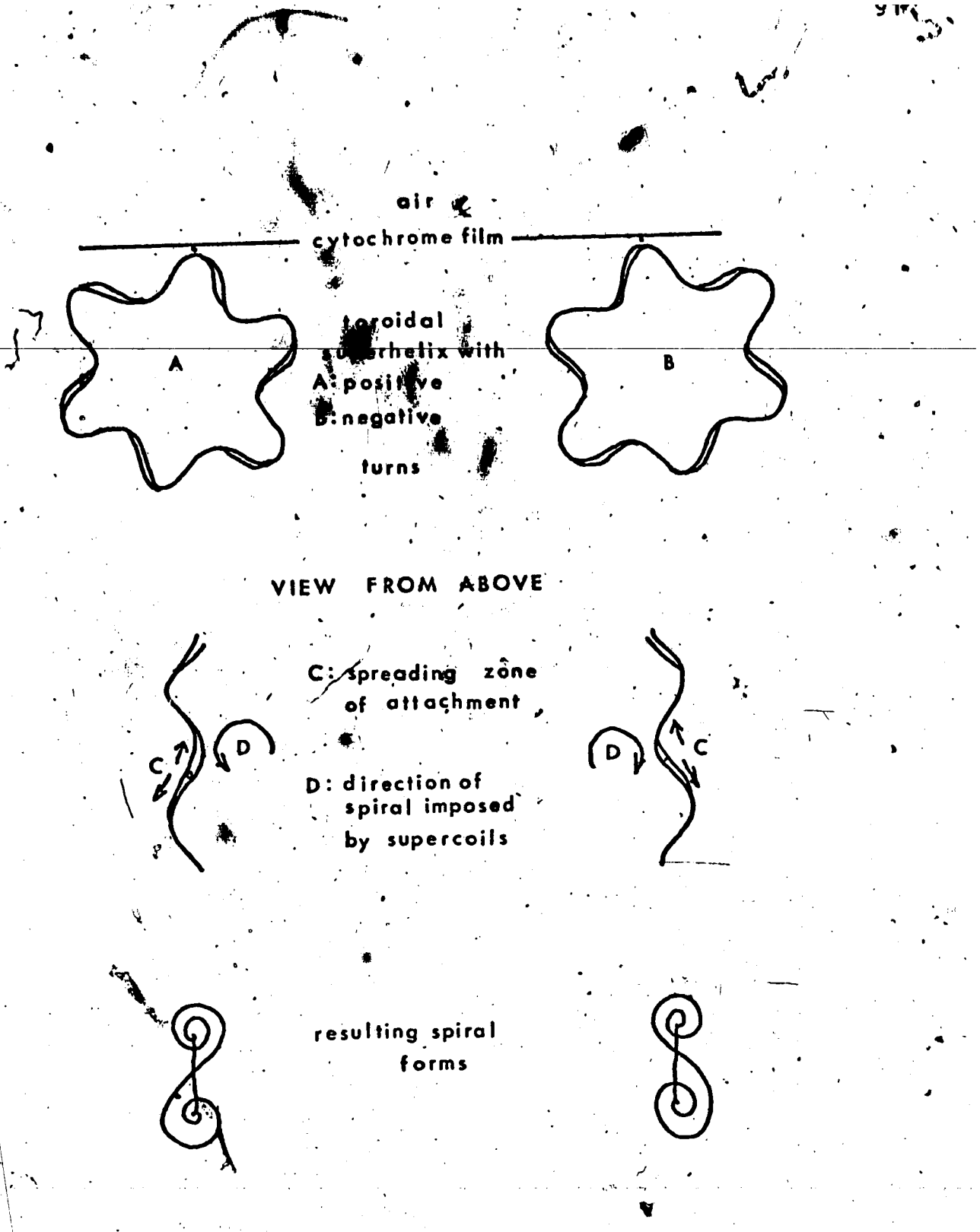


Figure 21. Illustration of the proposed mechanism of spiral formation by progressive attachment of a toroidal supercoiled molecule to a cytochrome monolayer. The spirals formed are viewed from above as in the electron microscope.

calculation it is necessary to know the exact fraction of available bases in the duplex that are derivatized at the time of ring closure (in natural DNA a small fraction of the thymine and guanine residues cannot be derivatized because of steric hindrance, (Metz and Brown 1969)).

Buoyant density shift was an appropriate parameter to measure since the change upon complete derivatization is large and can be determined accurately. PM2 DNA and M13 RFI are unsuitable for the preparation of standard curves since it is necessary to nick the DNA before it will react completely with the reagent, single stranded breaks are probable starting points for the reaction of the reagent with DNA, and the partially reacted products of two molecules nicked a different number of times could have widely differing degrees of reaction. This difficulty does not arise if a monodisperse linear DNA is used to prepare the standard curve. Lambda DNA was chosen for this purpose.

Reaction of DNA with the carbodiimide results in a large increase in the optical density at 290 nm. Since this wavelength is in a sharply sloping part of the absorption spectrum for both the starting material and the product, the absolute change in optical density cannot be used reproducibly to determine the extent of reaction. If a parallel control is allowed to react completely with the reagent it can be used to determine the fraction of bases derivatized at intermediate stages in the reaction. This

method of determining the derivatized fraction of bases cannot be utilized for the experiments as outlined since the important quantity is the fraction of bases derivatized at the time of ring closure. Any loss of bound reagent during the initial steps in the procedure would not be detected. Furthermore closed circular DNA does not show an increase in optical density early in the reaction even though a buoyant density shift can be detected. This may be due to a conformational change in the unreacted portion of the molecule with an associated optical change. When the fraction of final change in optical density for lambda DNA is plotted against the bucyant density shift a linear graph was obtained (Figure 9).

When the sedimentation coefficient of PM2 DNA was plotted against the fraction of bases derivatized a deep minimum appears in the curve, at 16% reaction, (see Figure 20). Assuming that the derivatized portions of the molecule are completely unwound this implies a superhelix density of -0.16, three times that obtained using the assumption of 12° unwinding by ethidium (Gray et al. 1971). While the first assumption may not be valid, for the reasons discussed in the introduction, the required degree of reaction is similar to that shown by Dean and Lebowitz (1971) to be necessary before a minimum sedimentation velocity is reached for PM2 DNA treated with formaldehyde.

It was necessary to show that the dederivatization

reaction could be accomplished without leaving a significant number of defects in the molecule, since these could cause anomalous results when final superhelix densities were measured. PM2 DNA which had been heavily treated (approximately 25%) with the reagent, but not nicked or resealed, was dederivatized under the conditions used in the other experiments. Samples taken at various times during the dederivatization showed that the sedimentation coefficient undergoes a reverse sequence of changes to those observed during the derivatization reaction attaining, after 24 hours the initial sedimentation coefficient, (27.8s) of underivatized PM2 DNA (Table 1). A small number of defects persisting in the DNA after the dederivatization would lead

Table 1.

time	S
0 hrs.	27.5
2 hrs.	25.6
5 hrs.	26.4
14 hrs.	34.8
28 hrs.	27.9

Dederivatization of PM2 DNA. PM2 DNA with approximately 25% of the available T and G residues derivatized was incubated at pH 10.9 in 0.2 M sodium carbonate/bicarbonate buffer at 24.5°C. Samples taken at the time indicated were stored at −20°C after lowering the pH to 6.5 with 0.5 M potassium phosphate buffer.

to an increase in the sedimentation coefficient analogous to that which occurs after slight reaction with formaldehyde (Dean and Lebowitz 1971), and carbodiimide (Figure 20).

During the dederivatization reaction approximately 50% of the molecules receive single stranded breaks.

If it is assumed that DNA in the derivatized regions is completely unwound at the time of ring closure the superhelix density of the dederivatized molecule should be the same as the fraction of bases derivatized at the time of ring closure. Using this assumption, and making a correction for the superhelical turns introduced by the change in salt concentration between resealing and sedimentation conditions, the unwinding angle due to ethidium binding was calculated to be -33^{o} for the experimental data shown in Figure 19; (see below for details of the calculation). This is almost three times the unwinding angle of -12^{o} that has been used in previous calculations of superhelix density, and agrees with the high degree of derivatization necessary before superhelical turns naturally present in PM2 DNA are released by derivatization.

In an additional experiment to confirm this result the mammalian w enzyme (Champoux and Dulbecco 1972) was used to relax PM2 DNA molecules which had been derivatized to varying extents. As noted in the introduction this enzyme combines the functions of the endonuclease I and ligase treatments of the original experiment, with a large saving in time, and much higher yields of relaxed material. After treatment with enzyme the PM2 DNA was dederivatized and the superhelical turns were titrated (Figure 22). The unwinding

angle due to bound ethidium was calculated in the following manner: the ratio of bound dye molecules to phosphate residues (v'), corresponding to the minimum in the sedimentation coefficient was calculated using the relationship $v' = C \times 2.45 \times 10^4 (0.241 - v')$, where C is the molar concentration of ethidium (Gray et al. 1971a). For the molecule that had 12% of its residues derivatized v'=0.103 dye molecules bound / phosphate. For the molecule that had been relaxed, but not derivatized v'=0.0206. The difference between these numbers, 0.0824 is the number of dye molecules required to compensate for the superhelical turns formed during the dederivatization reaction. This is equivalent to 1.65 dye molecules / turn of primary helix. The unwinding angle due to derivatization was $0.12 \times 360° = 43°$ therefore 1 dye molecule unwinds the helix by $43°/1.65 = 26°$. In the case of PM2 DNA which had been 7% derivatized the calculated unwinding angle was also found to be 26°, within experimental error of the value obtained in the previous experiment, lending further support to the contention that accepted values of superhelix density are too low by a factor of 2 or 3.

In addition to the lines of evidence already discussed there are several others which point to the possibility that the accepted value of the unwinding angle of $-12°$ is too low. Denhardt and Kato (1973) have shown that ultraviolet irradiation of PhiX174 RFI results in a decrease in the

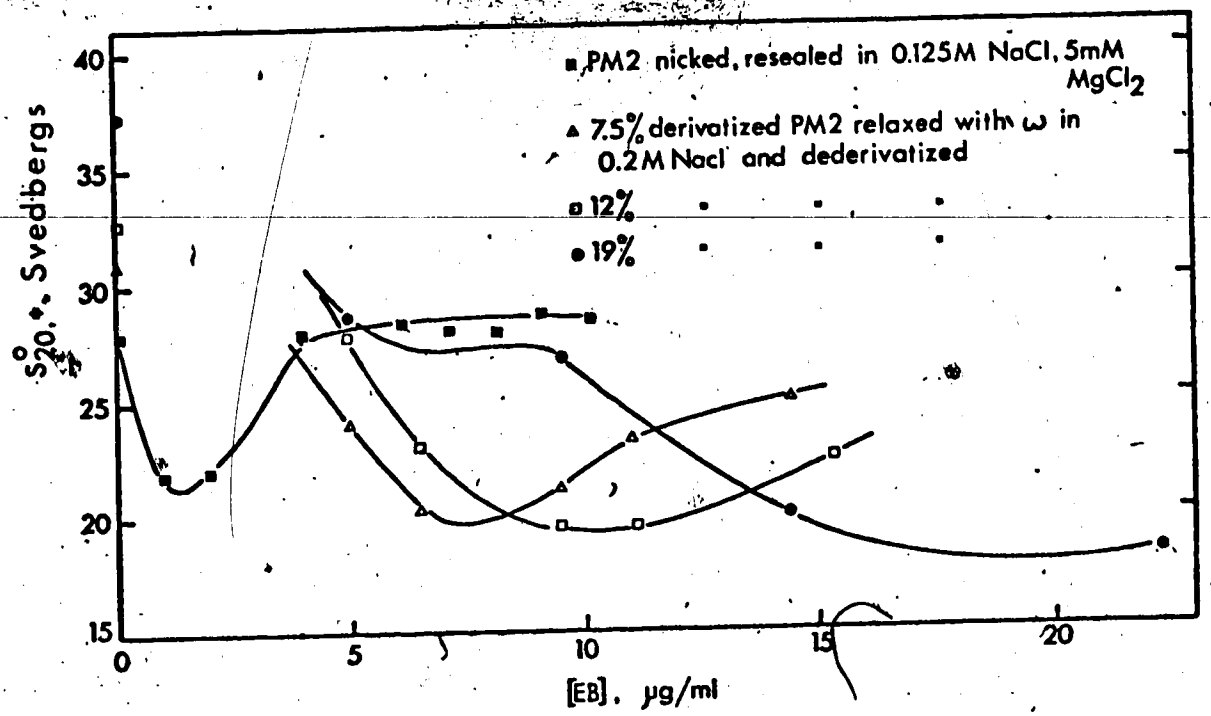


Figure 22. Titration of superhelical turns in PM2 RFI that had been derivatized then relaxed with mammalian ω enzyme before dederivatization (see p. 62 for details).

superhelix density. They calculated on the basis of 12° unwinding by ethidium that the decrease in winding angle caused by the formation of each pyrimidine dimer was 5.5°, less than the 9° unwinding angle predicted if the structure of the pyrimidine dimer is the same as that studied by Camerman and Camerman (1968). The value of 9° unwinding is more likely to be an underestimate than an overestimate because hydrogen bond breakage and base unstacking may occur in the region of a pyrimidine dimer.

Kasamatsu et al. (1971) have shown that heat treatment of D-looped mitochondrial DNA increases the average apparent superhelix density from −0.012 to −0.021. In the heating process a single stranded piece of DNA corresponding to approximately 3.3% of the genome is lost. The predicted increase in superhelix density is therefore −0.033, more than three times that calculated from the experimental results on the basis of −12° unwinding by ethidium.

Delius et al. (1972) studying the interaction of the T4 gene 32 protein with SV40 DNA were able to observe open loops of denatured DNA with bound protein after glutaraldehyde fixation. These loops are variable in size, but all examples shown were much larger than the 3.9% of the genome length predicted from the published superhelix density (Gray et al. 1971).

Wang (1969) and Ivanov et al. (1973) have noted a

difference between the observed and predicted CD spectra of superhelical DNA at various ionic strengths. Ivanov et al. (1973) explained this in terms of a variable unwinding angle due to ethidium the result could alternatively be explained if the unwinding angle due to ethidium is greater than 12° at all ionic strengths.

The observation of Dean and Lebowitz (1971) that reaction of PM2 DNA with small amounts of formaldehyde results in an apparent increase in the superhelix density presents a puzzling anomaly. Similar effects have been noted by Denhardt and Kato (1973), with PhiX174 RFI which contained a small number of pyrimidine dimers, by Beerman and Lebowitz (1973), in closed circular DNA which had reacted partially with methylmercury, and by ourselves with the reaction between carbodiimide and M13 RFI (Figure 23). A possible explanation of this effect has its origin in the difference between titrable and true superhelical coiling noted in the introduction. Freifelder (1971) found that intercalation of ethidium into DNA causes a marked stiffening of the duplex. The effect appears to be a general property of intercalative dyes since Lerman (1961) has shown a large increase in the intrinsic viscosity of DNA-acridine dye complexes relative to that of uncomplexed DNA. The stiffening effect may play a secondary role in the removal of superhelical turns from non-denatured closed circular DNA. Partial denaturation of the closed circle would

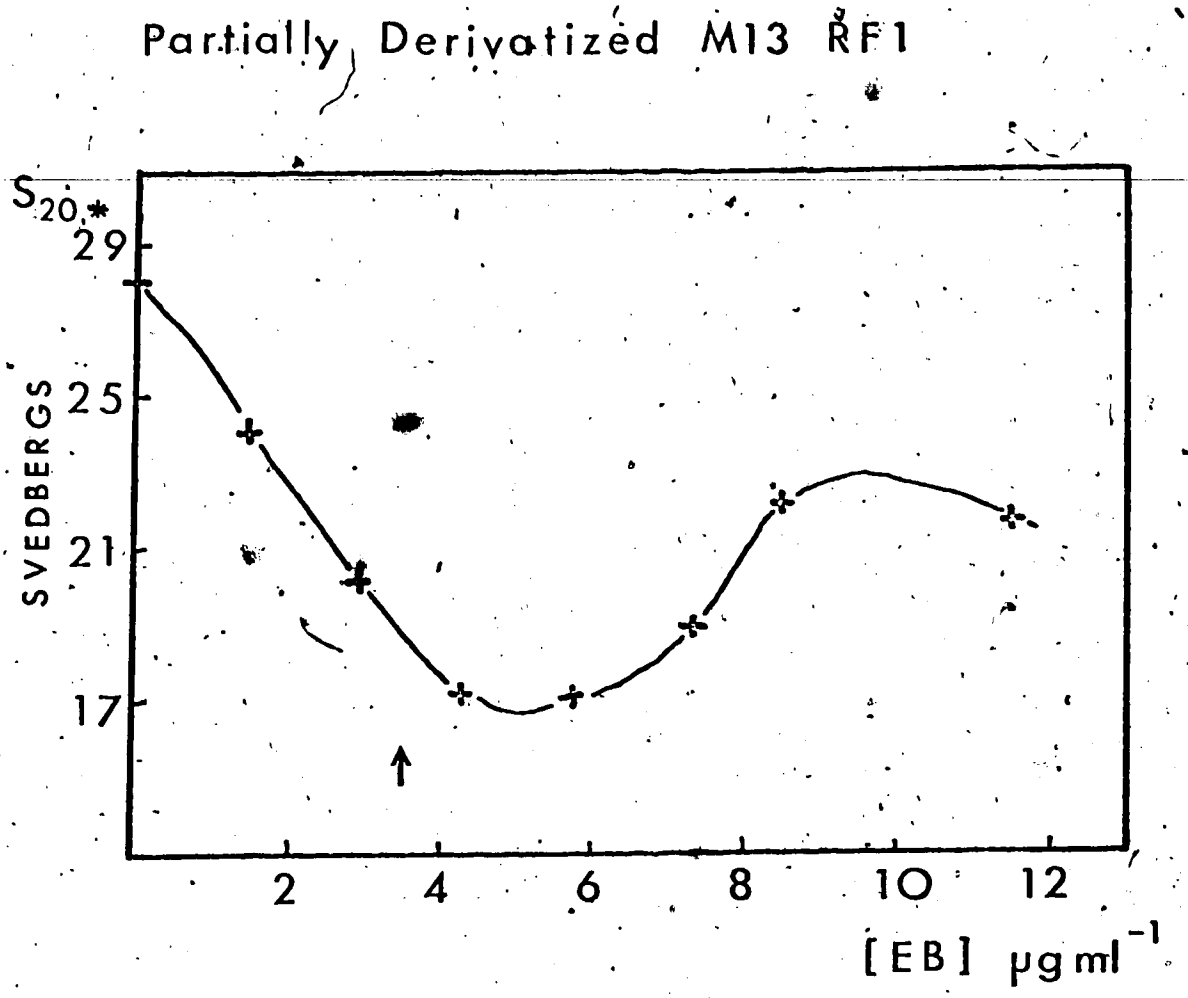


Figure 23. Sedimentation coefficient of M13 RFI as a function of ethidium bromide concentration after light-derivatization with carbodiimide, showing an increase in the apparent superhelix density relative to native M13 RFI. The position of the minimum in the titration curve for native M13 RFI is indicated by the arrow. The degree of reaction of this DNA with carbodiimide was not recorded.

alleviate the effect by permitting DNA to bend freely at the denaturation sites, leading to an apparent decrease in the unwinding angle due to ethidium. Evidence in support of this explanation can be derived from viscometic studies of DNA at varying ionic strength (e.g. Rosenberg and Studier 1969), which show a considerable increase in the stiffness of DNA as the ionic strength is lowered. Wang (1969) has shown that the apparent superhelix density of superhelical DNA measured by ethidium bromide titration decreases as the ionic strength is lowered. The common origin of the two effects suggests (but does not prove) a close relationship.

Reversible reaction of DNA with reagents other than water soluble carbodiimide

Because the water soluble carbodiimide is positively charged it is conceivable that ionic interactions could exist between the derivatized strands, even though these must be small (since the strands of dTC and dGA can be separated buoyant density equilibrium centrifugation and open loops are observed in highly derivatized linear DNA by electron microscopy). Since we had no strong evidence that the derivatized strands of the closed circular DNA in fact adopted the entropically favourable unwound conformation after derivatization a number of reagents capable of reversible reaction with DNA were examined for possible use in control experiments with the same experimental design as those using the carbodiimide.

Formaldehyde reacts with mononucleotides with kinetics suggesting that the reaction is freely reversible (Grossman 1968). The product formed with thymidine is unstable and cannot be isolated, but those formed with cytosine, adenosine and guanosine are moderately stable and can be separated by thin layer chromatography (Figure 24) Previous attempts to renature DNA after prolonged dialysis to remove the formaldehyde were unsuccessful (Grossman 1968), probably because of an irreversible reaction with DNA which could not be detected spectrophotometrically. It was hoped that by manipulating the conditions of the initial reaction the

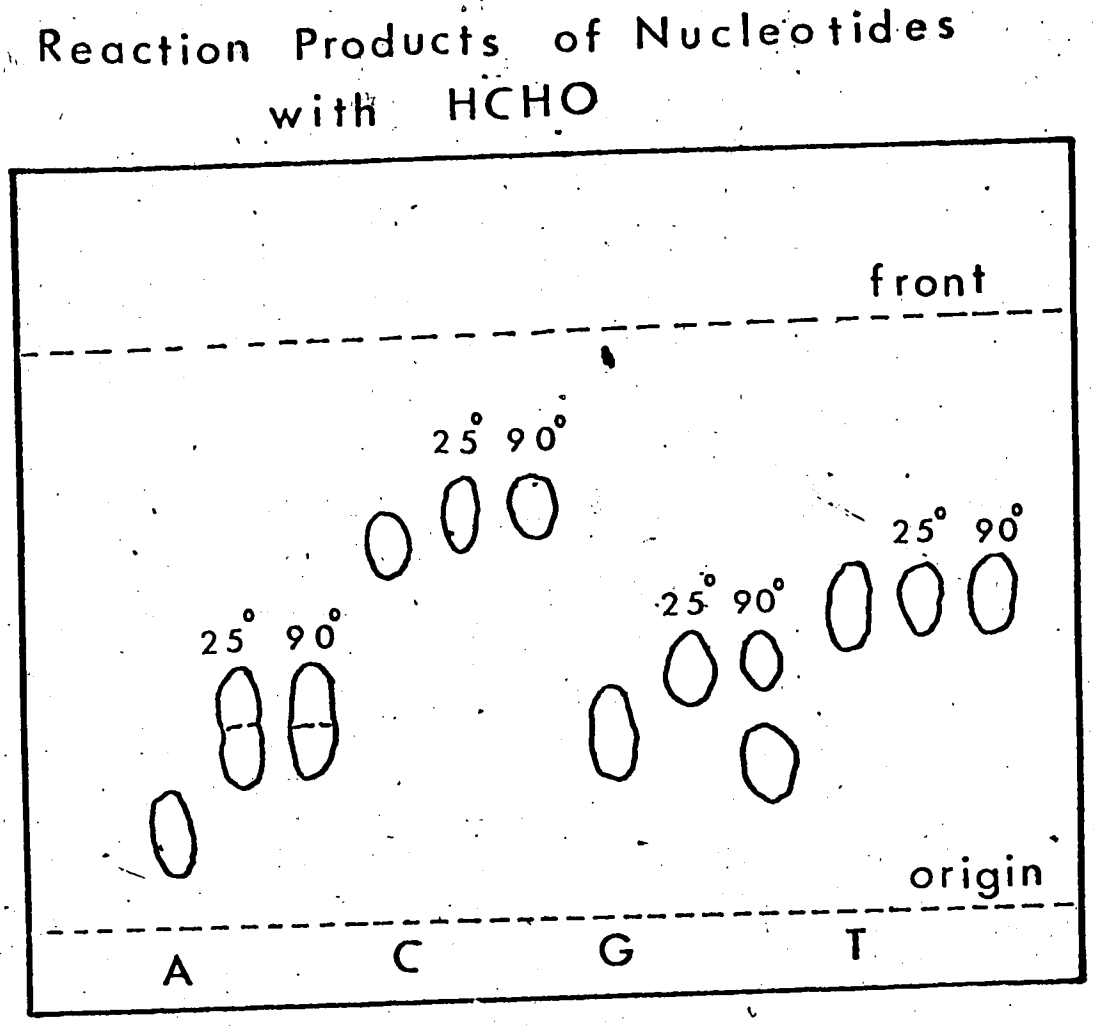


Figure 24. Separation of the products formed by the reaction of HCHO with 5'monodeoxynucleotides. Reactions were carried out in sodium carbonate/sodium bicarbonate buffer pH 10.9 with 7% HCHO freshly depolmerized by heating to 100°C for 15 minutes, in solutions containing 10 mg/ml of mononucleotide for 100 minutes at 25°C and 90°C respectively. 1 microlitre samples were chromatographed on cellulose thin layers, using saturated ammonium sulphate: 0.1 M sodium phosphate pH 6.8: 2-propanol 100:60:2 as solvent. Resolution of the reaction products of dAMP can be improved by diluting 70 mls of this solvent with 30 mls of water.

irreversible reaction could be eliminated.

Cytosine forms a single product with formaldehyde under all conditions examined, and so presumably was not the site of irreversible reaction, however purine nucleosides each form two different products. The temperature of the reaction strongly affects the nature of the products formed by dGMP but there is no effect on the ratio of products formed by dAMP. Attempts to purify and further characterize the products formed were only partially successful. The compounds with dAMP appeared to decompose slowly to the parent compound, or one with a similar spectrum. This was also true for the faster moving product with dGMP, however the slower migrating product did not decompose at neutral pH. This compound has a higher optical density at 290 nm than dGMP which suggests by analogy with the carbodiimide adduct that the site of reaction is N-1. This compound decomposed to dGMP upon incubation at pH 11, leading us to expect that previous failures to renature derivatized DNA after dialysis may have been due to the presence of guanylate residues modified in this way, however when T7 DNA which had reacted with formaldehyde at 25°C (in 50% ethanol) was exhaustively dialyzed against dilute alkali the fluorescence enhancement in alkaline assay mixture of a sample of this DNA was approximately 70% that of an equivalent sample of native T7 DNA. A large fraction of this (50%) was cross linked as shown by recovery of fluorescence

after heating. The thermal melting point of the formaldehyde treated DNA in 0.1 M SSC was found to be 6°C lower, and somewhat broader than that of native T7 DNA. The experiment showed that although DNA can be renatured after treatment with formaldehyde by dialysis at alkaline pH, a large number of defects persist. A possible site of irreversible reaction is C-2 of adenylate residues. This might not be detected spectrophotometrically since the spectrum of C-2 methyl adenosine is very similar to that of unmodified adenosine (Hall 1971).

Glyoxal and other 1,2 dicarbonyl compounds are capable of reacting reversibly with guanine residues in denatured DNA (Shapiro and Hachman 1966). Unfortunately the acid conditions required for reaction of glyoxal itself, and maintenance of the derivatized product are unsuitable for use with high molecular weight DNA, since depurination with subsequent hydrolysis of the phosphodiester backbone occurs.

This objection does not apply to the reaction of DNA with glyoxylate. Experience with this compound showed it to be unsuitable for use in the intended experiments, but the reaction with DNA will be discussed since it has not previously been reported. Glyoxylate is specific for the purine bases. A single product is formed with adenosine which is unstable, and decomposes after separation from free glyoxylate. Deoxyguanosine and dGMP form a series of compounds depending upon the conditions of reaction. At pH

6.5 (25°C in 1M gyoxylate) both formed a single product which fluoresced blue when excited by long wavelength UV light, the spectrum of the compound formed by dGMP is shown in Figure 25. The compound formed by guanosine has a negative charge (shown by paper electrophoresis), implying the presence of a free carboxyl group. At pH 10.5 the main products are non-flucrescent, neutral compounds, with the spectra shown in Figure 26. Since there is no immediate charge in the spectra of these compounds at pH 13 N-1 of the guanine ring system is probably blocked. Even after prolonged reaction at pH 6.5, or 10.5 some free deoxyguanosine remained, suggesting that the reaction with glyoxylate is reversible. However when T7 DNA which had been fully derivatized with glyoxylate was dialyzed exhaustively against dilute alkali the resulting material had a thermal melting point 4°C below that of native T7 DNA. Glyoxylate therefore compares favourably with formaldehyde with regard to the reversability of its reaction with DNA, but is still unsuitable for use in the proposed experiments.

The reaction of DNA with acetic anhydride was also investigated, however depolymerization of the DNA resulted from reaction with this reagent even under mild conditions (pH 7 maintained by pH-stat).

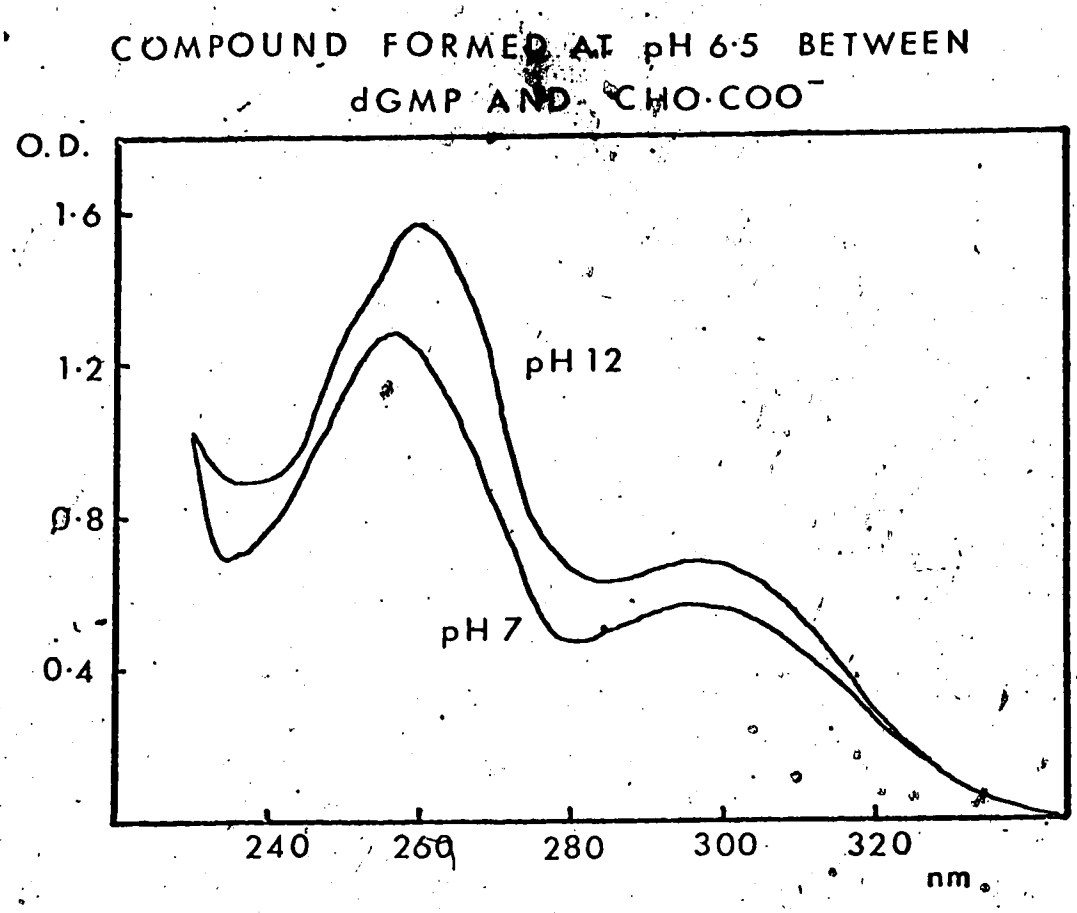


Figure 25. Spectrum of the compound formed at pH 6.5 between dGMP and potassium glyoxylate. A solution containing 10 mg/ml of dGMP, 2 M potassium glyoxylate 0.1 M potassium phosphate buffer pH6.5, was allowed to react for 72 hours at 24°C. The product was separated from excess glyoxylate by chromatography on Sephadex G10 in distilled water. This compound is highly fluorescent under long wavelength UV light.

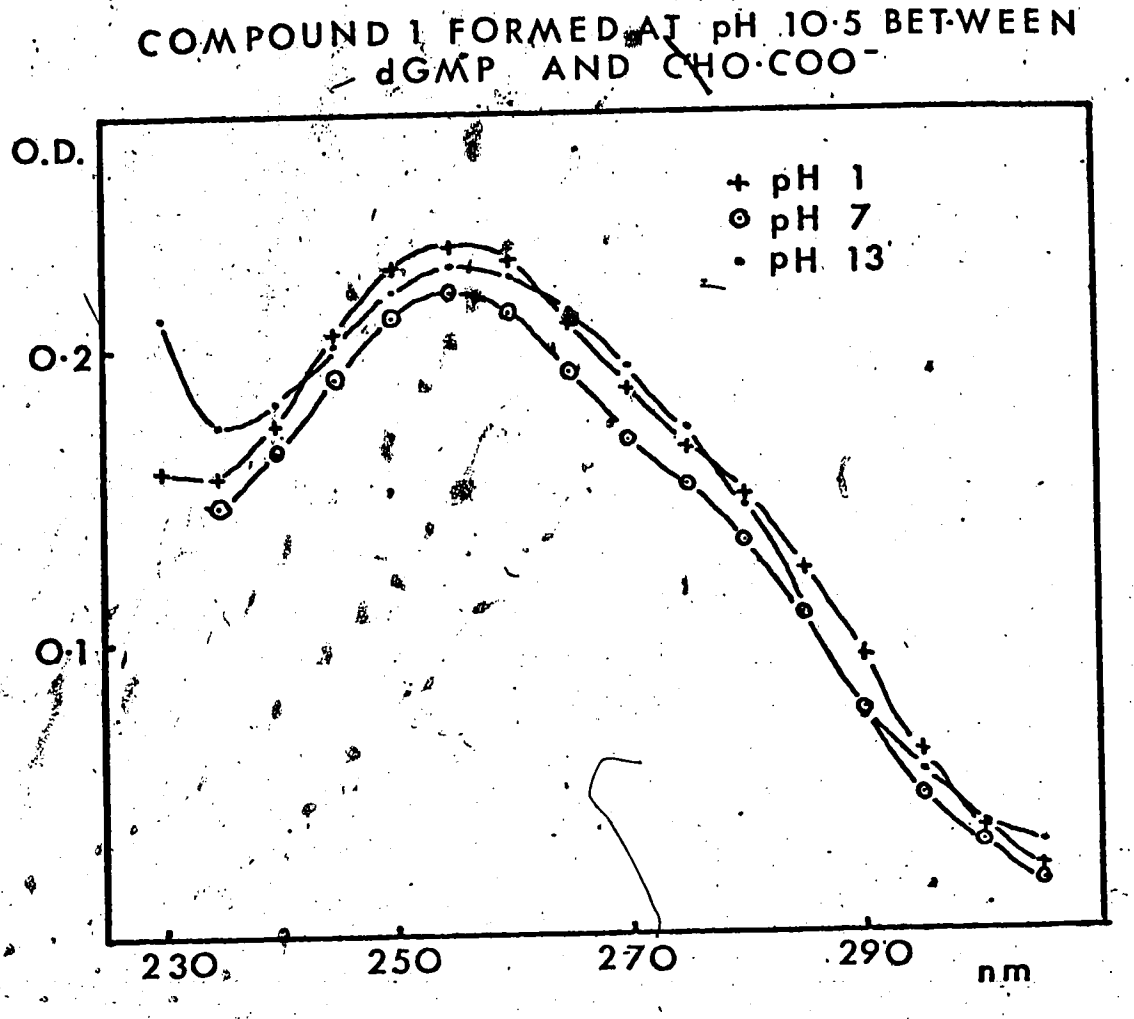


Figure 26. a. The spectum of compound 1 formed between guanosine and glyoxylate at pH 10.5. A solution containing 10 mg/ml of guanosine, 2 M potassium glyoxylate was adjusted to pH 10.5 with 1 M NaOH and allowed to react at room temperature for 48 hours. This product was the leading peak from Sephadex G10. It is slightly fluorescent under short wavelength UV light.

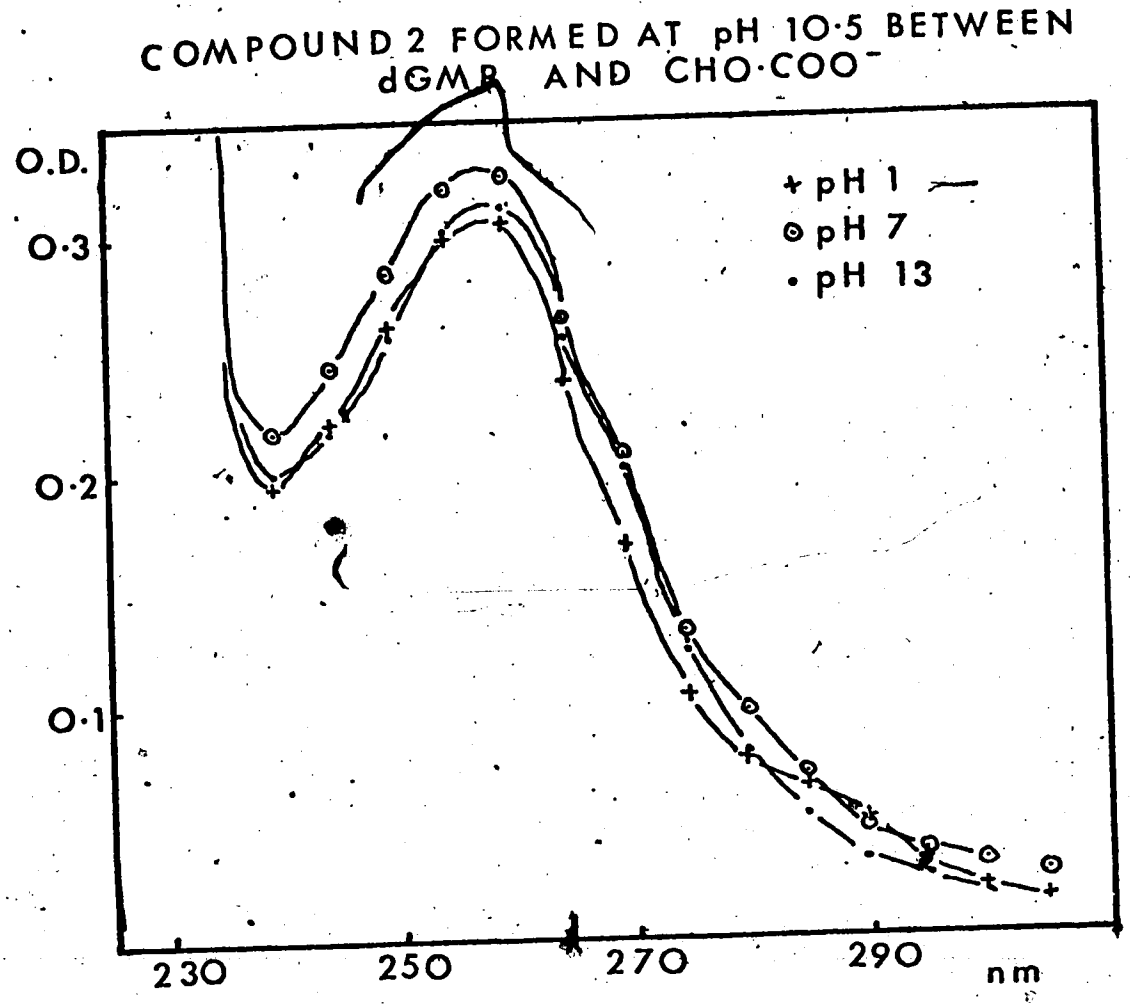


Figure 26. b. Spectrum of compound 2 obtained from the same reaction mixture as compound 1. This eluted with the salt peak from sephadex G10, requiring a second purification using Dowex-2 Cl^- to remove residual glyoxylate.

Consequences of an increased unwinding angle due to bound ethidium.

All natural closed cicular DNA's that have been examined have been found to have superhelical turns of the same sense, which has been shown to be negative both by the results discussed here and by Schmir et al. (1974). The range of values found using the formerly accepted value for the unwinding angle due to ethidium binding of -12° is quite narrow, from -0.012 to -0.058 with the majority falling around -0.03. According to the results discussed previously these values should be increased to lie between -0.03 and -0.15, with the majority lying between -0.08 and -0.1. This is the magnitude of supercoiling predicted by the models of chromatin structure discussed in the introduction. The negative sign implies a left handed first order superhelix providing there is only one DNA duplex is coiled about the superhelix axis in the chromatin fibre[1].

Direct evidence in favour of a left handed primary superhelix comes from the observation that vegetative SV40 DNA is associated with histones and other chromosomal proteins in a fast sedimenting complex (Levene 1974) and has

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1 It would imply a right handed supercoil if two duplexes are coiled about a common axis (see Figure 4), conceivably this situation could apply in late interphase if daughter chromosomes remain associated prior to metaphase condensation.

a negative superhelix density which is either 75% that of viral DNA (Eason and Vinograd 1971) or the same as viral DNA (Mayer and Levine 1972), depending upon the method of isolation (Partial relaxation by w during gentle isolation may be responsible for the lower apparent superhelix density in the case of Eason and Vinograd's preparation). If ethidium unwinds the duplex by 30° the superhelix density of the intracellular viral DNA is −0.1. the value predicted for left handed versions of Pardon and Wilkins' model (1972) and that proposed on page 23, but not the model of Bram and Ris (1971) (unless there is a net overwinding of the primary helix, which is quite conceivable since the CD spectrum of nucleohistone can be interpreted as being due to mixed A,B and C forms of the helix, Hanlon et al. 1972).

The free energy of supercoiling

An important measurement for our understanding of DNA conformation is the free energy required to distort a linear helix into a supercoil. One approach to determining this value was followed by Bauer and Vinograd (1970) who measured the free energy available from superhelix unwinding for the binding of ethidium to closed circular SV40 DNA at buoyant equilibrium in CsCl.

Their calculation showed that in its native supercoiled state this DNA has a free energy excess of 18 cal/mole of nucleotide over the nicked molecule.

The free energy change $d\mathcal{J}G$ that occurs upon binding of dv molecules of an intercalative dye per phosphate residue to a closed circular DNA can be written as the sum of two terms $d\mathcal{J}G/dv = d\mathcal{J}G_l/dv + d\mathcal{J}G_s/dv$. The first $(d\mathcal{J}G_l/dv)$ represents the change in intrinsic binding constant of the dye to a linear DNA. Ideally this term should be 0, but because of site heterogeneity, and possibly because of interaction between binding sites a small change in the binding constant is observed with increasing v (see figure 27). The second term $(d\mathcal{J}G_s/dv)$ is some function of θ $[f(\theta)]$, the angular distortion that occurs in the primary helix during binding of the dye. If a constant unwinding angle $\mathbb{D}$ per molecule of bound ethidium is assumed $f(\theta) = f(av)$ where a is a proportionality constant depending upon $\mathbb{D}$. Since

$\Delta G^0 = -RT \ln K_b$, where K_b is the binding constant of ethidium to DNA $d\Delta G/dv = -RT\, d(\ln K_b)/dv$ and $d\Delta G_s/dv = -RT\, d(\ln K_b)/dv - d\Delta G_l/dv$. $d\Delta G_l/dv$ and $d(\ln K_b)/dv$ can both be determined experimentally by plotting $\ln K_b$ vs. v for linear and superhelical DNA's respectively.

Plots of $\ln K_b$ vs. v proved to be linear to within experimental error for M13 and PhiX174 RFI's and for PM2 DNA therefore $d\Delta G_s/dv$ can be expressed as $-k(v - v_c)$ where k is the difference between the slopes $d(\ln K_l)/dv$ and $d(\ln K_s)/dv$ and v_c is the molar ratio of dye to phosphate for which $K_s = K_l$, integrating between $v = 0$ and $v = v_c$ we obtain $\Delta G_s = -kv^2/2 + kvv_c]_0^{v_c} = kv_c^2/2$, which is the free energy of supercoiling available per mole of phosphate in the absence of bound dye, double this value gives the free energy of supercoiling per base pair.

A simple method for obtaining the binding constant K_b is to measure the relative fluorescence enhancement (X) of a solution containing a known concentration of ethidium binding sites, against that of two other solutions containing the same concentration of ethidium bromide, one containing no DNA (fluorimeter scale set at 0) and the other containing a large excess of linear DNA (fluorimeter scale set at 100). In solutions of low optical density the normal choice of exciting wavelength is 525 nm since this is the

optimum for bound ethidium, however the optical density of the solution in these experiments is significant and because the absorbtion maxima of bound and free ethidium differ colour quenching becomes a serious problem. The difficulty is avoided by exciting the complex at 510 nm, the isosbestic point in the spectra of the bound and free dye (Waring 1965). Quenching of fluorescence of bound ethidium by free dye through excitation transfer does not appear to be a significant since the binding constant of ethidium to linear DNA, calculated from the results of these experiments, is approximately constant over the range of ethidium concentrations used in these experiments, and the results are reproducible over a range of DNA concentration.

Knowledge of the fraction of ethidium in the solution bound to DNA (X/100) permits direct calculation of the concentrations of bound and free dye (C_b and C_f respectively). The concentration of free binding sites is the difference between the total concentration of binding sites ($C_s = C_p/5$, see LePecq and Paoletti 1967) and the concentration of bound dye (C_b). The binding constant K_b is

$$K_b = C_b/(C_f \times (C_s - C_b))$$

Plots of log K_b against C_b/C_s (= 5 x v) proved to be linear within experimental error, (Figure 27).

The values of the free energy of supercoiling were caculated to be 45.2 cal/base pair for PM2 DNA and 14.6 cal per base pair for PhiX174 and M13 RFI's. The latter value is slightly lower than that calculated for SV40 DNA (18 cals per base pair) by Bauer and Vinograd 1970. This DNA has a similar superhelix density to M13 and PhiX174 RFI's. The difference between the values obtained from these experiments and that of Bauer and Vinograd is not surprizing in view of the large difference between the condition of the experiments (0.05 and 0.1 M NaCl, against 5.8 M CsCl).

The results of fluorimetric titration plotted in this way provide a method for determining the superhelix density of an unknown closed circular DNA, since the graph of log K crosses that for linear DNA at the point where all the superhelical turns have been removed. Using an unwinding angle for ethidium of 30° the calculated superhelix densities of PhiX174 RFI and PM2 DNA are 0.067 and 0.12 respectively. After making a correction for the difference in salt concentration between these experiments and that used in sedimentation velocity experiments the values are 0.10 and 0.15, which are very close to those calculated by Wang (1969b) and Gray et al. (1972) after applying a factor

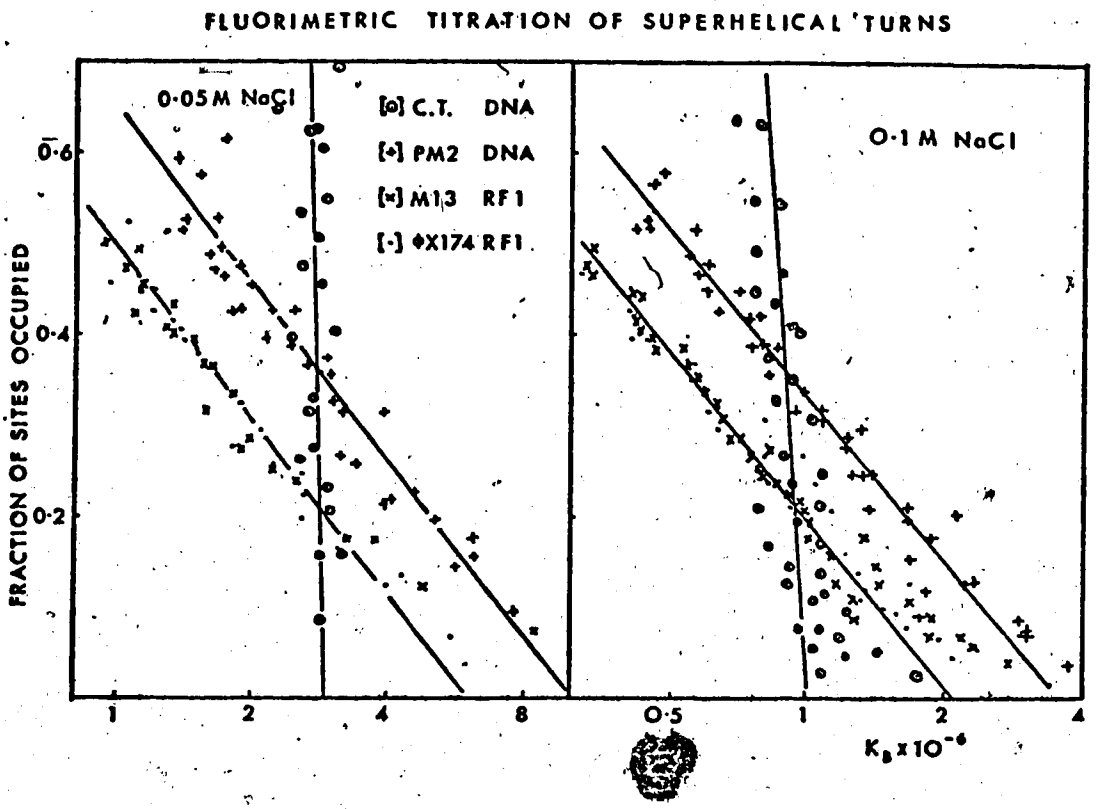


Figure 27. Semi-log plot of K_s against the fraction of sites occupied for linear and closed circular DNA's. K_s was calculated from the bound fraction of ethidium in a series of solutions containing differing concentrations of DNA, as described in the text. Concentrations of DNA used in 0.05 M NaCl were: PM2 DNA, 0.84, 2.1, and 4.1; M13 RFI 1.65, and 2.94; PhiX174 RFI 1.11 and 2.91; 1.24 and 3.08 micromolar in phosphate. Concentrations used in 0.1 M NaCl were: PM2 DNA 2.09, 4.18 and 6.25; M13 RFI 1.77 and 3.54; PhiX174 RFI 1.84 and 3.69; Calf thymus DNA 2.49, 4.98, 7.49 and 15.32 micromolar in phosphate. Solutions contained 1 mM Tris buffer pH 8, 0.1 mM EDTA. A single line is plotted for M13 and PhiX174 RFI's since these appear to have the same superhelix density. The scatter observed in these plots is due to a noisy fluorimeter.

for the difference in the assumed unwinding angle due to ethidium (0.0925 and 0.145 respectively). This method supplements a number of others already in the literature. However a simpler method of using fluorimetry is now described.

If two closed circular DNA molecules of differing, known superhelix density are available the fluorimetric determination of the superhelix density of a third closed circular molecule is an especially simple task. PM2 DNA and its relaxed counterpart (prepared by treatment of the native molecule with eukaryotic w protein) are suitable standards because they encompass the known range of natural superhelix density.

For a superhelical DNA $dG/dv = -k(v - v_c)$, therefore $G = \text{INTEGRAL}-k(v - v_c)dv = -kv^2 + kvv_c + C = -RT\ln K_b$ where C is a constant of integration equal to the intrinsic free energy of binding of ethidium to a linear DNA. Putting $B = -C + RT\ln C_f$, where C_f is the concentration of free dye (which may be taken as constant if $C_f >> C_b$), and $A = -RT/k$, and rearranging we obtain the expression $v_c = v/2 + (A/v)\ln[v/(n-v)] + B/v$ where n is the number of binding sites per mole of phosphate (0.2). If the values of v_c are known for the two standard closed circular DNA's, and the values of v for the two DNA's under a standard set

of conditions are also known the equation may be solved for the two constant terms A and B. Knowing v for any third DNA under the same set of conditions permits the equation to be solved for v_c and hence the superhelix density. In actual practice the absolute value of v for any given DNA is difficult to determine. Under the alkaline assay conditions discussed on p. 57 linear DNA is nearly saturated with ethidium. Since PM2 DNA gives an approximately 30% increase in fluorescence when it is nicked a reasonable value for v would be 0.15 (v at saturation = n, 0.2). A 30% decrease in fluorescence accompanies relaxation of the superhelical turns in PM2 DNA by w giving a value of v for this DNA of 0.1. Using these values in the equation given above the relative fluorescences (which are directly proportional to v) of closed circular DNA's of intermediate superhelix density fall on the curve plotted in figure 28. The form of this curve is remarkably independent of the value of v for the superhelical DNA over the range $0.08<v<0.18$ providing the ratio of the fluorescence of the superhelical to relaxed PM2 DNA's remains constant at 3/2 (see Fig. 28). These curves are sufficiently close to a linear interpolation of superhelix density against v that the latter may be assumed to hold to within experimental error under the alkaline conditions that are usually employed. These conditions are

particularly convenient since contributions to the fluorescence by nicked circular, and linear DNA can be eliminated by the heat step.

The Purification of W

Assay of the relative fluorescence enhancement due to a constant amount of PM2 DNA is the basis for a rapid and inexpensive flucrimetric assay for w factors (see p 66). Here the removal of superhelical turns from a closed circular duplex is detected by a decrease in fluorescence enhancement with time. Since the relative fluorescence is linearly proportional to the superhelix density the assay is linear with respect to the number of superhelical turns removed. The assay can be applied to the removal of positive superhelical turns by treating relaxed PM2 DNA with w in the presence of a low concentration of ethidium bromide.

The validity of this assay was checked by measuring the sedimentation coefficient of fully relaxed PM2 DNA with and without sufficient ethidium bromide to remove the superhelical turns formed in relaxed DNA by the increased salt concentration of the sedimentation conditions. In the latter case relaxed PM2 DNA has the same sedimentation coefficient as the unrelaxed molecule, (27.8s) while in the former the relaxed molecule has the sedimentation coefficient of a nicked molecule 21 s, (see Figures 22 and 29).

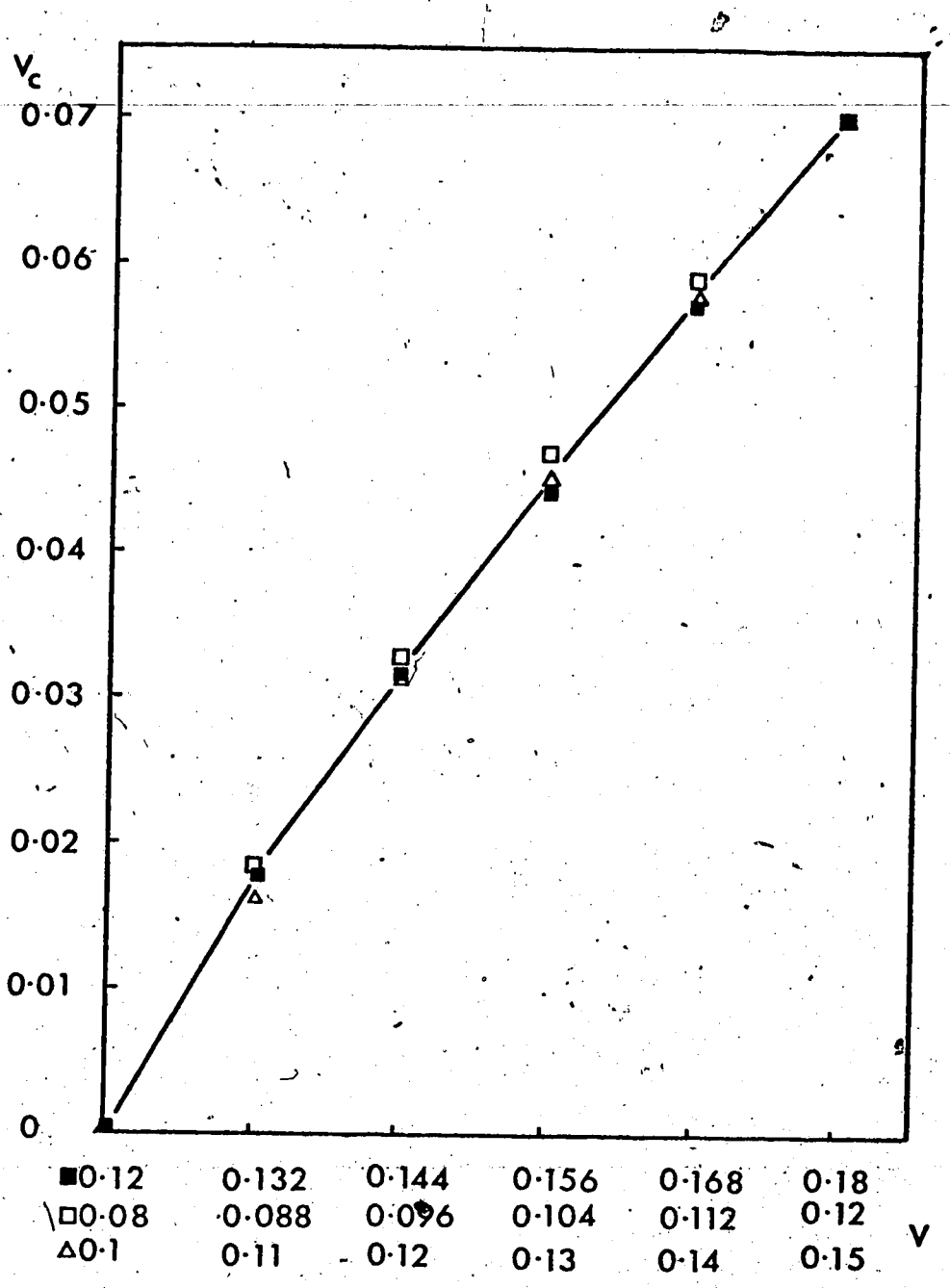
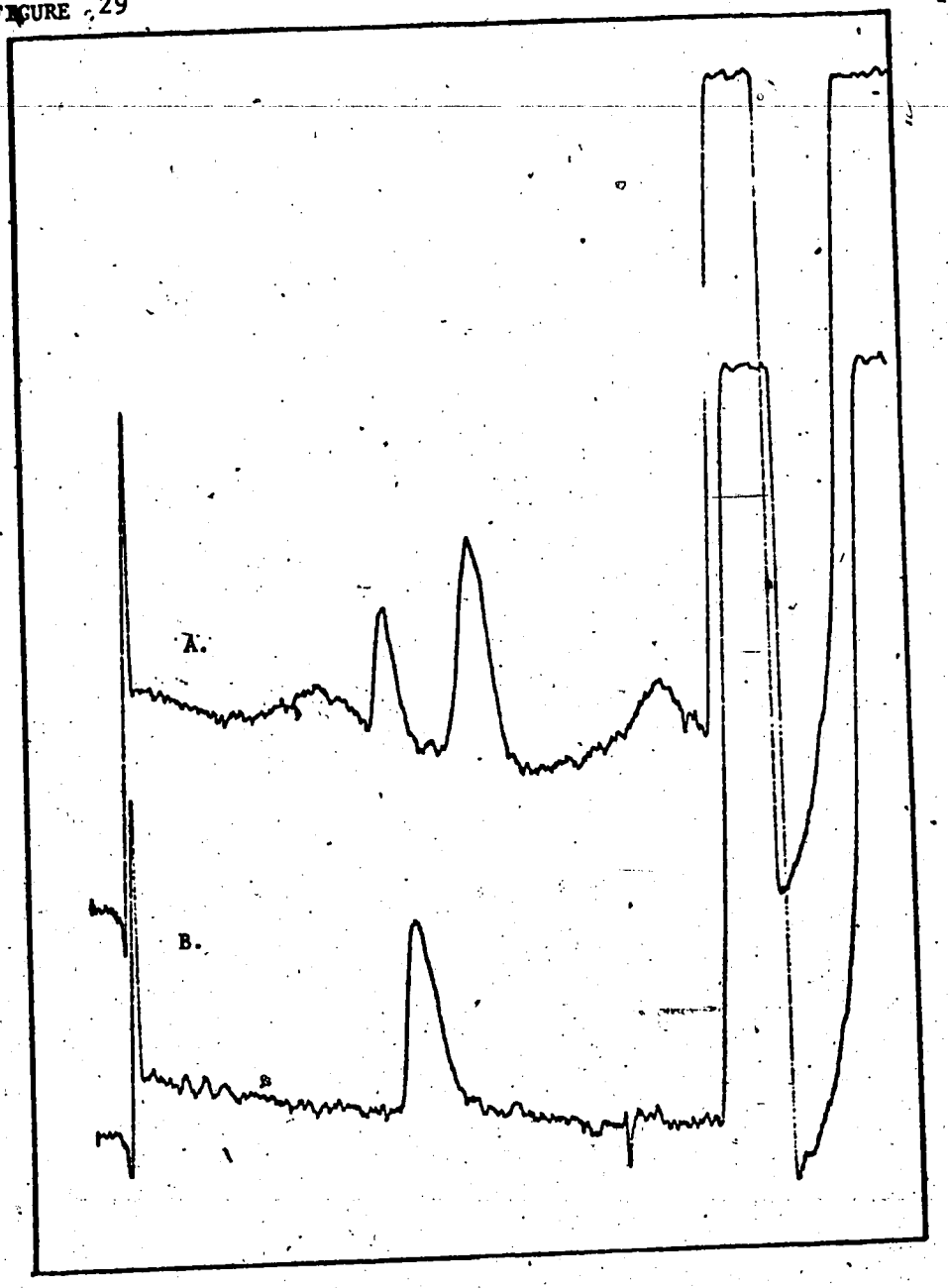


FIGURE 28. Relationship between v and v_c calculated assuming three different values for v of superhelical DNA. All curves were calculated assuming the ratio of v for fully superhelical PM2 DNA to that for the fully relaxed molecule is 3/2.

FIGURE 29



Comparison between Sedimentation profiles of a mixture of PM2 open circular and nicked circular DNA's:
A. Before treatment with w.
B. After treatment with w.
Sedimentation from left to right in 2.83 M CsCl, 1.5microgram of ethidium bromide per ml, 10 mM Tris Cl pH 8.0 44,000 rpm. Photographs are approximately 40 min after the start of the run.

Champoux and Dulbecco prepared their extracts by sonication of nuclei from resting secondary mouse embryo cells. Initial experiments (Morgan unpublished) indicated that the activity is of widespread occurence in eukaryotic cells. Calf thymus is a metabolically active tissue which is readily available, and is an abundant source of nuclei, making it a suitable starting material for the isolation of of w protein.

A small amount of enzyme can be detected in crude supernatants from calf thymus prepared by homogenization of the tissue in the presence of 2 mM calcium chloride and centrifugation to remove nuclei. This enzyme could be cytoplasmic or may result from the disruption of a small fraction of the nuclei during the initial extraction. It can be precipitated between 50 and 67 % saturation with ammonium sulphate, but with a large loss of activity. No further attempt was made to purify the enzyme from this source after it was recognized that the chromatin pellet obtained by centrifugation of the homogenate contains much larger quantities of the enzyme. The activity cannot be released by rehomogenization of the pellet, but is released upon extraction of chromatin with concentrated salt solution, along with histones and some acidic proteins. The DNA from such an extract can be removed either by high speed centrifugation of the unsheared viscous suspension, or by the addition of polyethyleneglycol (10% w/v) to a sheared solution of chromatin. The latter procedure has the

advantage of being rapid and convenient, and of allowing more than one extraction of a single chromatin preparation at differing salt concentrations. W appears to be eluted from the chromatin in two distinct steps. The first activity (w^1) is eluted from the chromatin by 0.7 M NaCl, the second (w^2) by 1.7 M NaCl. Re-extraction of the chromatin precipitated from 0.7 M NaCl with 0.7 M NaCl gives an insignificant improvement in yield of w^1 implying that the appearance of a second activity in 1.7 M NaCl is not a consequence of incomplete elution of w^1. The impression was confirmed when of the two activities prepared at 0.8 M NaCl and 1.8 M NaCl were diluted and applied to phosphocellulose. Elution of the activities from the columns with linear salt gradients gave peaks of activity eluting in the ranges 0.5 to 0.6 M NaCl and 0.74 to 0.84 M NaCl respectively. In the case of w^1 the activity initially appeared to resolve into two separate activities which were correlated with optical density peaks. The main components of the peaks observed by gel electrophoresis by the method of Panyim and Chalkley (1972) appeared to be fragments of histone KAP formed by autolysis (Bartley and Chalkley 1972) see figure 30. W^2 did not correspond to an optical density peak (see Figure 31). The main components present in the mixture were histones LAK and KAS, and autolysis fragments. Methods were sought to overcome the problem of proteolysis. Sodium bisulphite, the most commonly used inhibitor of nuclear proteases is unsuitable for use in ion-exchange chromatography since it

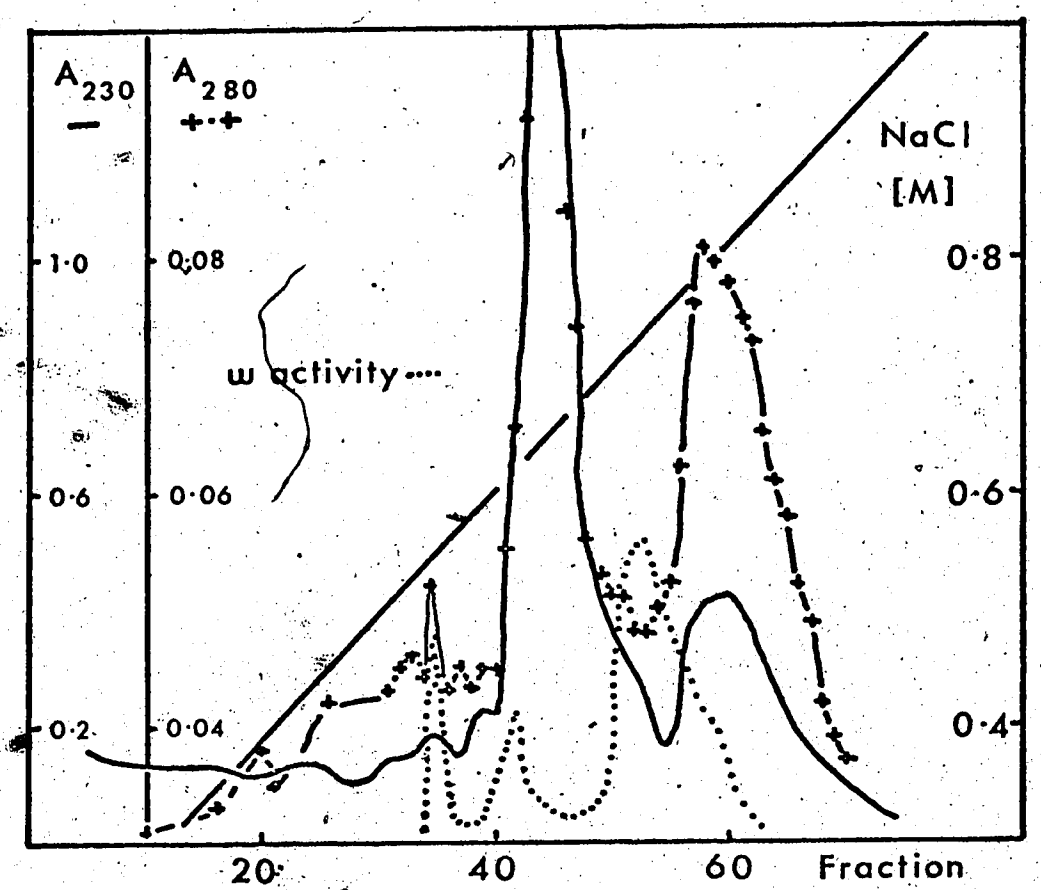


Figure 31. Elution profile of calf thymus w from phosphocellulose. The extract in this experiment was a cut from 0.5 to 1.2 M NaCl which was diluted and bulk adsorbed to phosphocellulose. Note the greater amount of the last w peak (w^2) compared to Figure 27.

oxidizes rapidly in air, causing the pH of the buffer solutions to decrease with time. Other protease inhibitors were examined for suitability using the solubilization assay of Panyim et al. (1968). Mercuric chloride was particularly effective in preventing the solubilization of chromatin, but its use leads to a complete loss of w activity. Benzyl sulphonyl fluoride and iodoacetimide are also quite effective and do not appear to inhibit the activity of either w as long as they remain bound to chromatin. However they partially inhibit isolated w^2. Treatment of the washed chromatin with a combination of the two inhibitors has been incorporated into the isolation procedure. On several occasions precipitates were observed when polyethyleneglycol extracts were diluted for application to phosphocellulose. It was found that this could be prevented if the prepared chromatin was dissolved in 1 M NaCl and then precipitated by dilution to 0.15 M final salt concentration. Under these conditions the w co-precipitates with nucleohistone, leaving much of the acidic chromatin protein in the supernatant. Chromatography of w^1 on phosphocellulose after these treatments gives a single peak of activity, not corresponding to the main protein peaks. At this stage in the purification the main component observed by gel electrophoresis is still histone KAP. Separation of the activity from the remaining histone KAP was achieved by concentration followed by chromatography on Sephadex G100 or G150 (fig. 14). The protein at this stage is still

relatively impure, the main components present have molecular weights of 53,000, 51,000 and 32,000 (see plate 6 and Figure 31). The molecular weights of the components were estimated by electrophoresis on SDS gels using the method of Lemmli (1967).

Each step in the purification results in a large loss of activity. The reason for this is unclear since the activity is stable in solution for several weeks at 4°C. It has been noted that simple concentration of a solution containing w_1 or w_2 by ultrafiltration or by dialysis against polyethyleneglycol can lead to a large loss of activity, in some cases visible precipitation occurred, suggesting that the problem is one of low solubility of the proteins in aqueous solution. Pretreatment of the phosphocellulose with histone before use in the purification of w appeared to improve the yield of protein from the columns, and may have given a small improvement in the yield of enzyme, suggesting that another problem is irreversible adsorption of enzyme to the resin.

The catalytic properties of the two w's appear to be quite similar, they are both effective in releasing both positive and negative supercoils, and are most active in 0.2 M salt. They are completely inhibited by 0.4 M salt (Table 2). Two activities are also found in trout testis (Dr B. Eskin unpublished observation). However these differ from the calf thymus w's in that they are active in 0.6 M salt.

Plate 6.

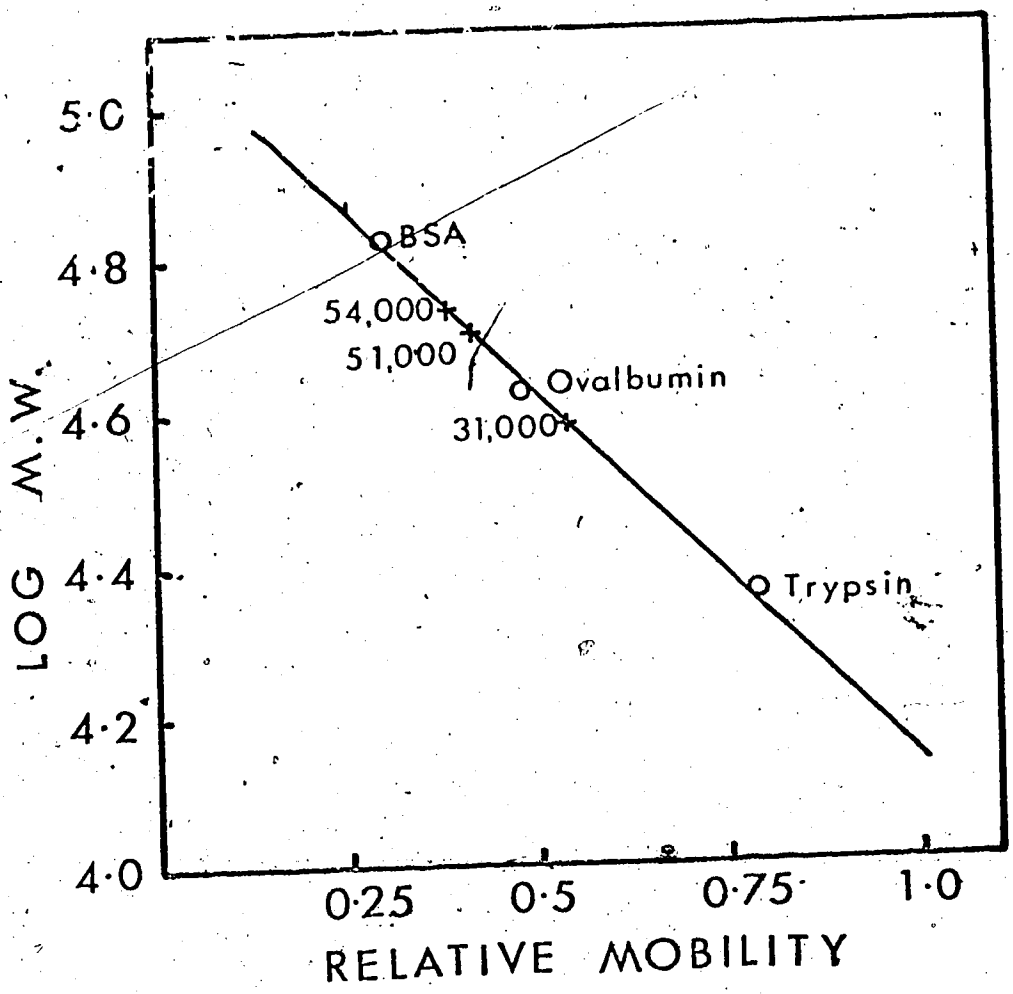


Figure 32. SDS gel electrophoresis on 10% polyacrylamide gels of the proteins present in the active fractions after chromatography on Sephadex G100 (see Figure 14).

It is possible that both enzymes from one source are in fact identical, but as isolated are bound to different histone

TABLE 2

[NaCl]	w^1	w^2
0.2	66	78
0.3	77	94
0.4	102	105
0.6	100	100
1.0	100	100

Relative fluorescence of 50 microlitres of PM2 DNA solution (O.D.260 = 0.8) treated for 15 minutes with a constant amount of enzyme (approx. 2 units w^1 or 1 unit w^2) with varying concentrations of NaCl in 0.01 M Tris, 0.1 mM EDTA pH 8.

fractions. This possibility was suggested by the strong specific interactions that occur between the histone fractions, and some acidic chromatin proteins (Hnilica 1972).

Little evidence is available on the mechanism of w action. Wang (1971) showed that the E. Coli enzyme does not act by a one hit mechanism. This has also been demonstrated for the eukaryotic enzyme (Champoux and Dulbecco 1972). These observations rule out a simple nuclease followed by ligase mode of action. Our own observations have shown that the kinetics are neither linear nor exponential (see Figures 15 and 33). In the simplest hypothetical model, the enzyme is capable of covalent reversible binding to DNA, with breakage of one of the backbone phosphodiester bonds. The protein is then free to rotate about the opposite strand of

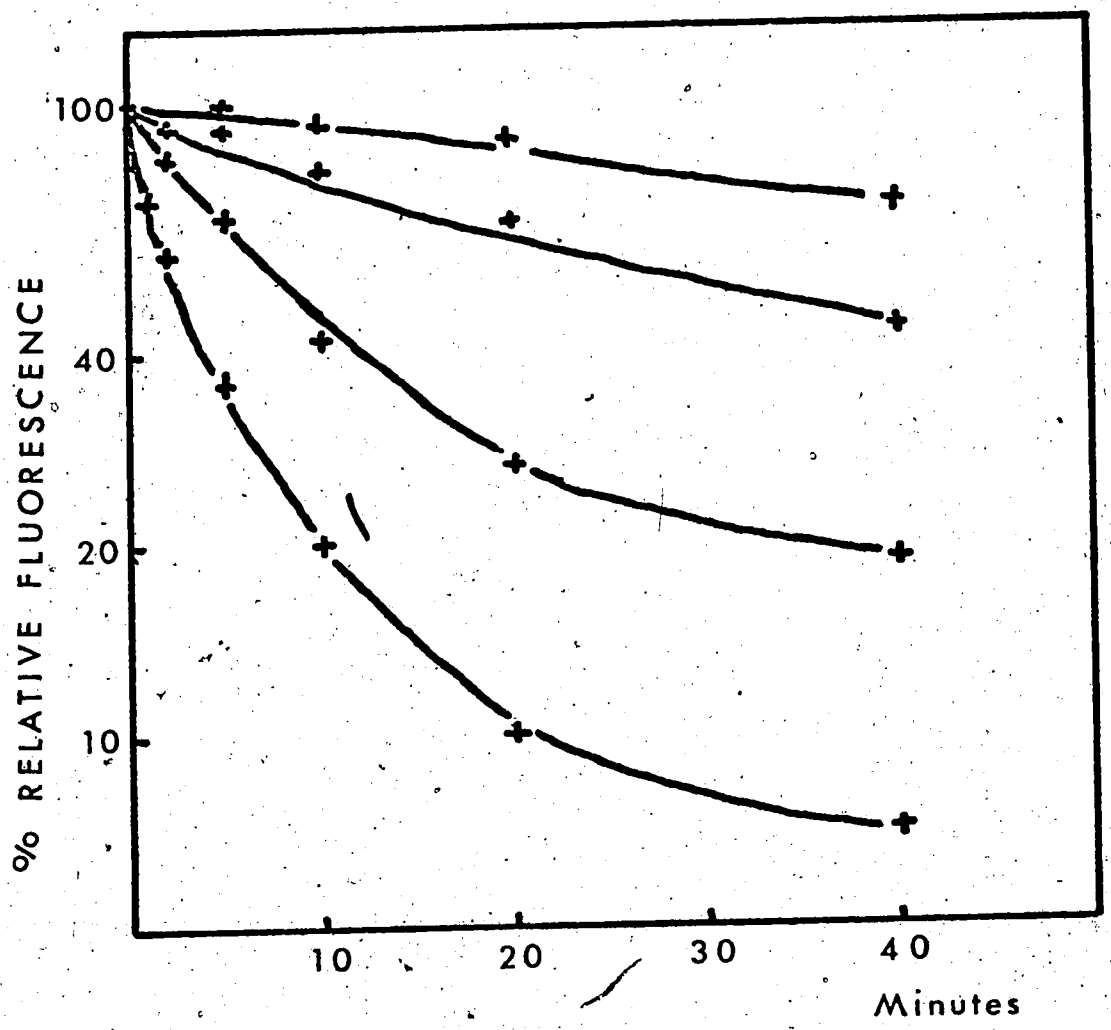


Figure 33. Semi-log plot of the data shown in figure 15 b, demonstrating the non-exponential nature of the w reaction.

the duplex and reforms the backbone phosphodiester bond after a statistically determined number of revolutions (Figure 34). In the case of the eukaryotic w the enzyme acts equally effectively against superhelical turns of either sense, therefore it is improbable that the superhelix free energy would contribute to the initial covalent binding reaction, or the resealing reaction, although the excess free energy could affect the number of rotations about the helix axis that occur between opening and closing reactions. This assumption implies that the superhelix density will not affect the binding constant of the enzyme to the DNA. As a direct consequence the reaction should be competitively inhibited by linear DNA, which is not the case (Dr. A. R. Morgan unpublished observation on w prepared by sonication of I cell nuclei). In the general case the kinetics of reaction by this mechanism would be expected to be exponential, but if the equilibrium in the reaction strongly favoured the covalently bound form of the enzyme one hit kinetics would be observed.

A more probable model for the reaction requires that the enzyme reform the backbone phosphodiester bond after each rotation. In this case the conformational state of the enzyme substrate complex during bond breakage would have to differ from that present at the time of resealing. Reversal of the reaction (i.e. the release of positive supercoils) could be accomplished if the two conformational states of the enzyme are at equilibrium in solution. The cycle of the

ONE STATE MODEL OF SUPERHELIX

RELAXATION BY ω

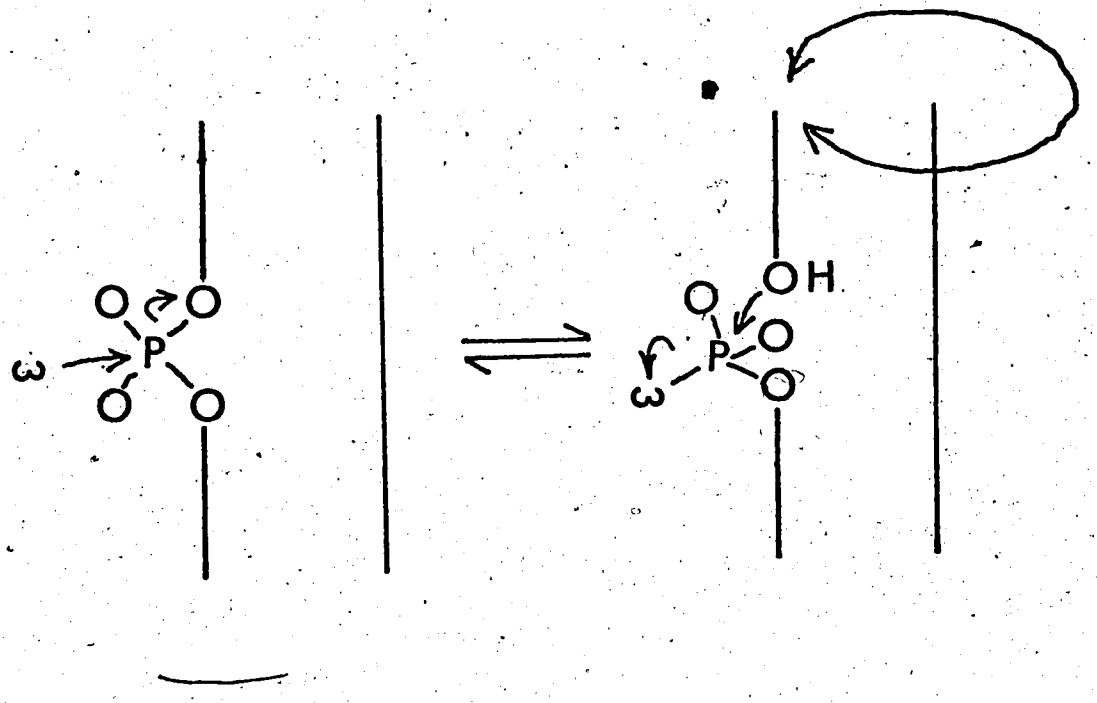


Figure 34. Proposed one state model for the action of ω. Since the stereochemistry of the phosphodiester bond breakage reaction is identical to that of the ring closure reaction more than one right or left handed rotation can occur between ring opening and closure.

the enzyme are at equilibrium in solution. The cycle of the reaction would then be: 1, covalent binding of the enzyme in one conformational state with breakage of a backbone phosphodiester bond 2, rotation of the strands about each other accompanied by a change in the conformation of the enzyme 3, reformation of the phosphodiester bond and finally 4, the release of the enzyme in the altered conformational state, which would then be free to revert to the initial form of the enzyme (5). The sequence of reactions is illustrated in Figure 35. It is convenient to consider this mechanism in terms of the Ahrennius activation energy of the phosphodiester bond breakage and resealing reactions. The reaction profile would probably have a form like that shown in Figure 36, in the presence of superhelical turns the activation energy of the reaction will be altered by the free energy of supercoiling. The activation energy in the direction of release of supercoiling would be reduced, whilst that in the opposite direction would be increased. The rate constant of the foreward reaction can be expressed as $k_1 = A\exp-(E_a - \not{E}_a)/RT$ and in the reverse direction as $k_2 = A\exp-(E_a + \not{E}_a)/RT$ where $\not{E}_a$ is the activation energy available from supercoiling. The interconversion between the forms of w, w+ /=======/ w- must also be considered when formulating an overall rate expression. This is too cumbersome to be of use in deciding between the proposed models.

TWO STATE MODEL OF SUPERHELIX RELAXATION BY ω

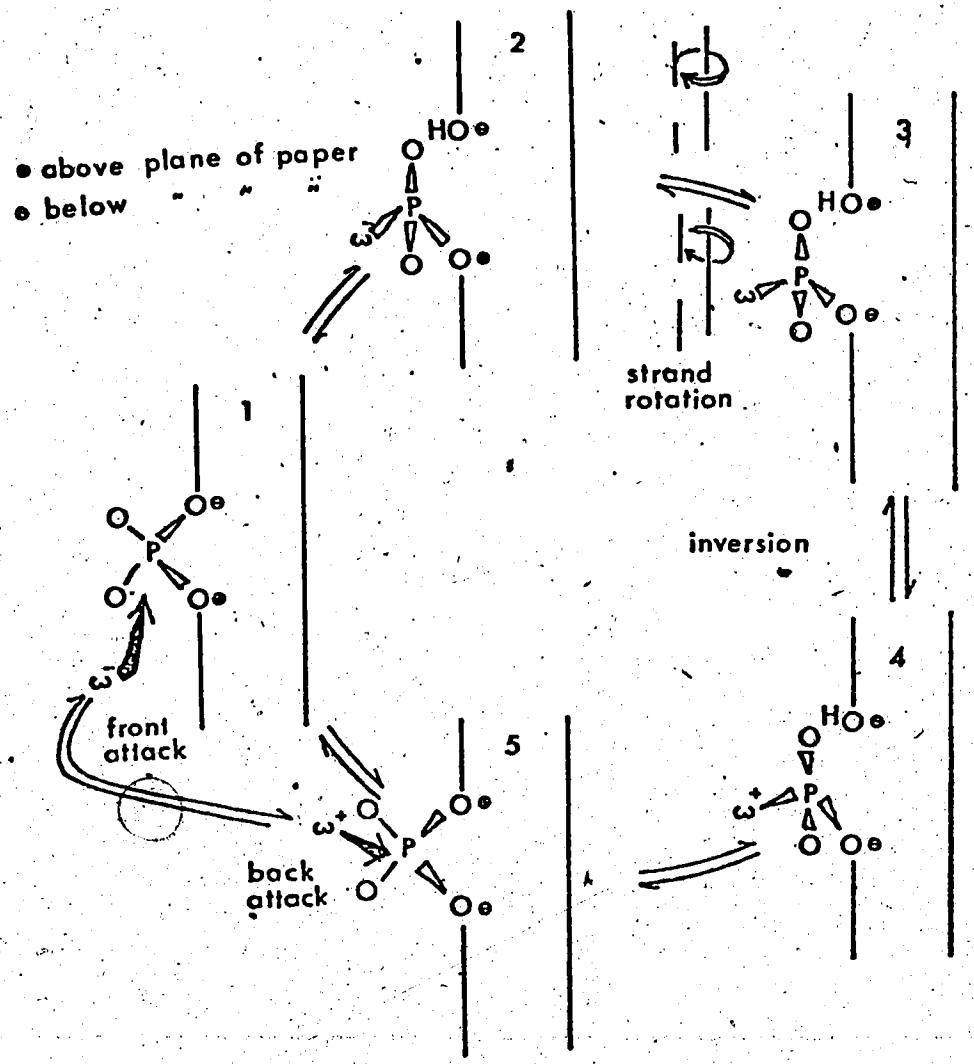


Figure 35. Two state model for the action of ω. In this model backbone phosphodiester bond breakage does not have the same stereochemistry as the ring closure reaction, permitting the relaxation of only one superhelical turn per reaction cycle. The relaxation of positive superhelical turns is a reversal of the reaction leading to the relaxation of negative superhelical turns.

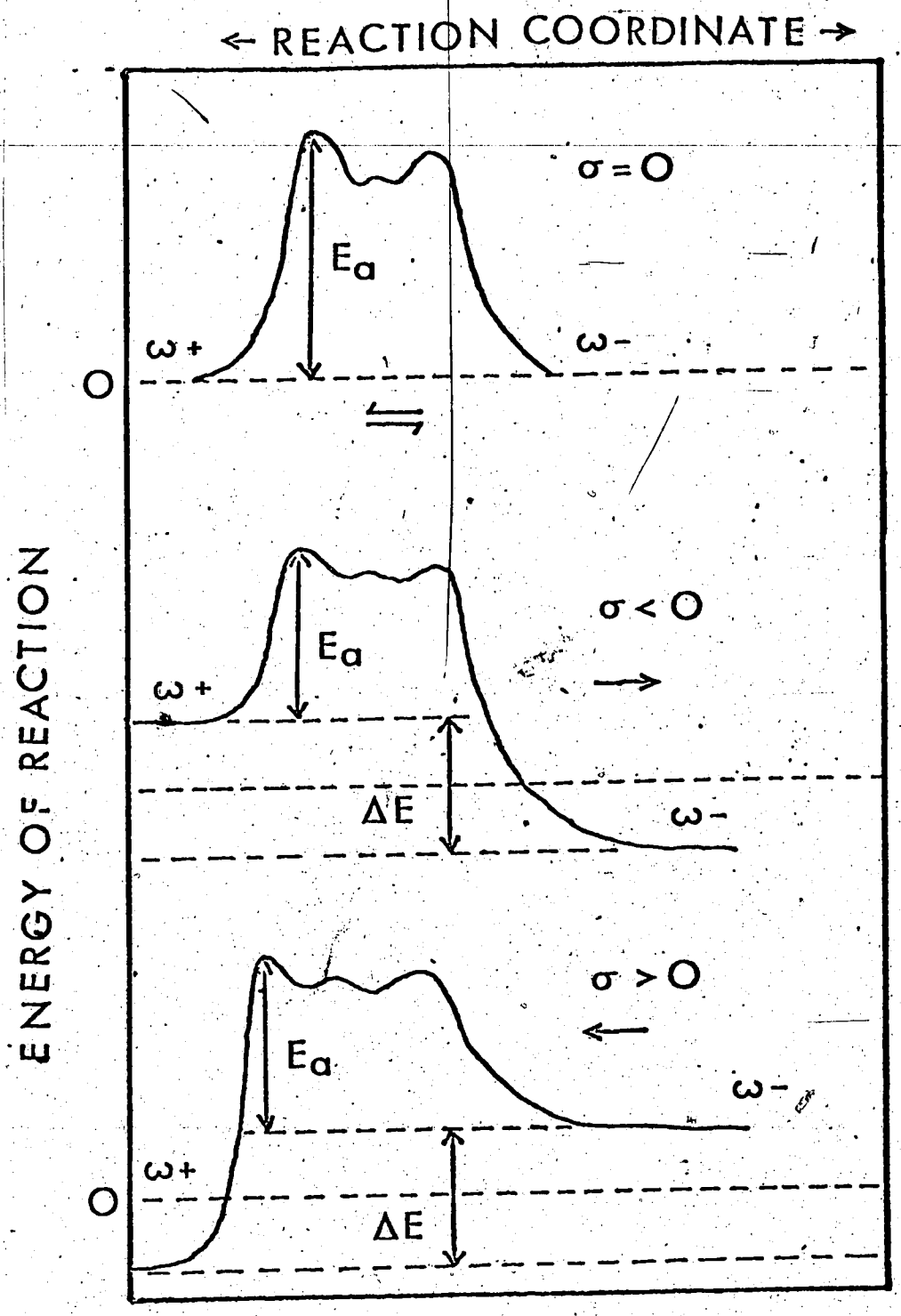


Figure 36. Illustration of the contribution of the superhelix free energy to the activation energy in the two state model of supertwist relaxation by w. In the upper panel w interacts with linear DNA. The centre and lower panels illustrate the reaction of w with DNA under torsional strain due to negative and positive supercoils respectively.

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APPENDIX

Programma 101 program for determining binding constants for Ethidium to DNA. Before use the concentration of the added Ethidium solution must be entered into register /D.

```
AV
S                    Enter initial Volume
/B↑
E*
S                    Enter Binding site Concentration (init)
/C↑
AW
S                    Enter volume addition
B↑
M↓
/B↓
/E↑
M↓
/B↑
M÷
/CX
M↓
/C↑
/D↓
BX
/E↓
EX
/E↓
/B÷
E↓
S                    Enter Fraction bound
M↓
EX
/E↑
/E↓
/C÷
A*                   Prints fraction of sites occupied
M↓
/E−
M↓
M÷
/E↓
E−
A↓
A*                   Prints free Ethidium concentration
M↓
/E↓
M÷
A*                   Prints Binding Constant
MW
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