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Topologically Non-linked Circular Duplex DNA

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The discovery of circular DNA, over 30 years ago, introduced an element of uneasiness in what had been, up to that point, the almost picture-perfect story of the elucidation of the molecular biology of heredity. If DNA indeed has the Watson–Crick right-handed helical secondary structure, then in circular DNA, thousands, or perhaps even millions of twists must be removed in each generation, and re-wound in the next generation.

Although enzyme systems adequate for this task have long since been found and characterized, there have nevertheless arisen a number of proposals for alternative DNA structures in which the strands are topologically non-linked, so that they might separate during replication without having to be unwound. These structures have generally been put forth as theory only, and have been largely unaccompanied by experimental evidence to support their applicability to native DNA from living systems.

Recently, however, a report has emerged suggesting that it might be possible to separate, intact, the individual single-stranded circular half-chromosomes which constitute the double-stranded circular chromosomes of certain plasmids. This would not be possible unless the chromosomes had one of the alternative, topologically non-linked structures.

It is widely believed that after a half-century of worldwide DNA research, any significant change to the Watson–Crick structure is unlikely to stand up to scrutiny. Nevertheless, the present author has found that in many instances in which the behavior of circular duplex DNA is considered to be explicable only in terms of the topologically linked helical model, it is also possible to explain that same behavior in terms of a topologically non-linked model. It is necessary, in these instances, to make certain logical assumptions which cannot be conclusively proven at the present time.

The author herein offers an example of one such instance, namely an examination of the behavior of circular duplex DNA in an alkaline titration experiment, where conformational changes in DNA are deduced from changes in its buoyant density at pH's between 7 and 14. These data have been explained in terms of topological linkage between the DNA strands, but they can also be explained without invoking any such topological linkage, provided that the above-mentioned logical assumptions can be accepted.

The principles which emerge from this are applicable to other settings in which knowledge of the topology of DNA is critical to the understanding of observed phenomena.

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INTRODUCTION

Wu and Wu (1996) reported, in these pages, the separation of the intact circular single strands comprising the circular duplex chromosomes of two different plasmids. These authors have either made a grand error, or else have discovered a system within which DNA does not have the right-handed helical structure, the strands of which, when circularized, would not be separable unless at least one of them was broken open.

These authors believe that circular DNA is not, in general, topologically helical, but rather has a structure not unlike those proposed by Rodley *et al*. (1976) and Sasisekharan *et al*. (1978), in which the two individual single-stranded circular half-chromosomes twist about each other alternately to the right and left, giving rise ultimately to a structure whose strands are topologically non-linked. Since the structures proposed by each of the above authors differ somewhat, I shall refer to them collectively by the initials TN DNA, for topologically non-linked DNA (Fig. 1).

It has been known for decades that the individual single strands which comprise the double-stranded chromosomes of most species of circular DNA do not separate under conditions commonly observed to cause strand separation in either linear DNA, or in circular duplex DNA which has had one or both strands nicked (Vinograd *et al*., 1965; Rush and Warner, 1970). Although the secondary structure of fully intact circular duplex DNA is indeed disrupted by denaturation, the strands nevertheless remain associated with each other, as if they were physically locked together. It is not at all surprising that this observation has discouraged many from seriously considering any proposals for alternative structures for DNA in which the strands are not topologically linked.

In order to attain the separation of the intact individual circular single strands from duplex circular DNA, Wu and Wu (1996) employed the ingenious subterfuge of growing plasmids in stationary phase cells, where there is little DNA replication (and therefore few replicative intermediates to confuse things), but ongoing transcription. Since the RNA is transcribed only from the sense strand of DNA, and since DNA–RNA hybrids on gel electrophoresis have been found to be more stable than DNA–DNA hybrids (Casey and Davidson, 1977), Wu and Wu deduced that the electrophoretic mobility of the sense and ‘nonsense’ strands were not the same, and that they could be separated by gel electrophoresis under the proper conditions. The data they have presented appears to confirm their prediction, at least in the system they have employed.

It is not the purpose of this paper to take any position on the structure of DNA in general, which will ultimately be determined by research and observation. Rather, this manuscript will focus on a single hypothetical question. We shall assume that Wu and Wu (1996) are correct, and that circular DNA, at least in the systems they have reported on, has a TN structure. The question is this: if any DNA has the structure Wu and Wu propose, how can we account for the failure of the strands to

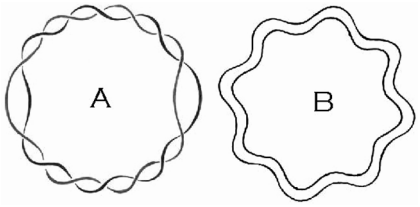

Figure 1. Two models of TN DNA. (a) *Schematic representation of TN DNA as being helical*, with three complete (i.e., 360°) right-handed helical turns on the top of the chromosome, and three left-handed turns on the bottom of the chromosome (a conformation admittedly unlikely to be found in nature). The net number of helical turns is zero. The strands of such a chromosome could separate during replication without being nicked and re-sealed. A model of this chromosome can be made by holding two rubber bands together in a circle, and twisting them. This will automatically introduce equal numbers of right-handed and left-handed helical turns. This, of course, is a *non*-topological linkage, and if the rubber bands are pulled apart, they will readily separate. (b) *A highly schematic representation of the structure proposed by Rodley et al. (1976).* This is, in effect, a double-helix with alternating right- and left-handed regions, but in which the length of each region is less than one full helical turn. Thus, when constrained to lie in a plane, the strands never cross one another, but undulate side-to-side without ever making a full helical turn.

separate under more usual conditions of denaturation, such as alkali denaturation, where decades of observations have confirmed repeatedly that the strands do not separate?

When one considers the question carefully, one's thoughts are compelled in particular directions by the body of data which exists concerning the behavior of circular DNA in various settings. We find that we must make certain assumptions. Each of these assumptions can be readily challenged, and we cannot assert that they are correct, but only that they find some support in existing evidence. I therefore present them as theory, in the spirit of the statement made by Crick *et al*. (1979): 'DNA is such an important molecule that it is almost impossible to learn too much about it'.

NECESSARY ASSUMPTIONS

If DNA has any of the TN structures which have been proposed to date, then it would be perfectly logical to expect that the strands would separate upon denaturation. They do not. I shall first present, without comment, the assumptions necessary to explain this. Then I will review some experimental evidence suggesting that these assumptions are at least possible. Finally, I will show how they can be applied to answer the question we have raised.

1. It must be assumed that DNA generally has the propensity to exist as either a right-handed helix or a left-handed helix, depending upon the prevailing conditions.
2. It must be assumed that conditions favoring denaturation will generally bring about a transition from the right-handed to the left-handed secondary configuration. For brevity, I shall refer to this as an R $\rightarrow$ L transition.

3. It must be assumed that the duplex product resulting from denaturation of circular DNA has an ordered structure requiring the participation of both strands, by means of which we may account for the failure of the strands to separate even though they are not topologically locked together.

Experimental Data which Support the Plausibility of the Above Assumptions

Left-handed DNA has been known for many years (Mitsui *et al*., 1970; Ikehara *et al*., 1972; Pohl and Jovin, 1972; Pohl, 1976; Wang *et al*., 1979; Nordheim *et al*., 1981). It was first observed in synthetic copolymers. It is not considered to be the structure of purified DNA in solution for any DNA obtained from natural sources, so that the presumption that DNA from natural sources can exist in the left-handed configuration is unproven. Nevertheless, we need to make this presumption for the sake of the argument which follows later.

There has existed evidence, for many years, that ordinary DNA might undergo an R → L transition under conditions which promote unwinding. Travers *et al*. (1970) found that the optical rotatory dispersion (ORD) spectrum of purified DNA inverted in aqueous methanol solutions. They suggested that this was best explained as an R → L transition, although there are other possible explanations. Similar inversions were demonstrated in the circular dichroism (CD) spectra of DNA at high salt concentration (Zimmer and Luck, 1974), and following complexing with mitomycin C (Mercado and Tomasz, 1977).

In the case of certain synthetic polynucleotides, the nature of these spectral inversions was further studied by x-ray crystallography (Mitsui *et al*., 1970; Wang *et al*., 1979). These authors concluded that the polynucleotides were left-handed.

Since methanol, high salt, and Mitomycin C have little in common chemically, I will propose that anything which unwinds DNA may, under the right conditions, bring about an R → L transition. This is obviously a sweeping assumption, but it is necessary for the argument we shall present.

The assumption is not without logical support. Such can be found in the work of Wang *et al*. (1979) on the pitch of the left-handed helical DNA fragment d(CpGpCpGpCpG); whose structure they referred to as ‘Z-DNA’. This helix has a rise per residue of 3.7 Å, which is considerably larger than the 3.4 Å spacing between the bases of the ‘normal’ right-handed DNA helix. With 12 residues per helical turn in Z-DNA (compared with 10 for right-handed DNA), it has a pitch of 45 angstroms (compared with 34 Å for right-handed DNA).

In other words, Z-DNA appears to be a *more loosely wound helix* than right-handed DNA. If this turns out to be generally true of left-handed DNA, then it follows logically that any substance which tends to *unwind* right-handed DNA might, if added in increasing quantity, cause the left-handed form of DNA to eventually become the favored form.

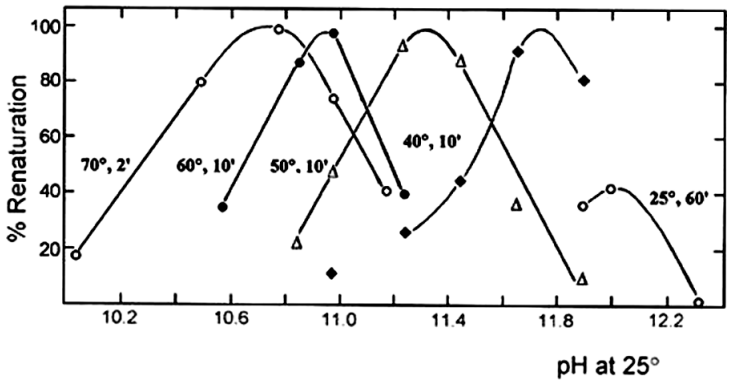

Figure 2. *Renaturation of 'irreversibly denatured' DNA (Form IV). Percent renaturation of Form IV as a function of pH*. Data is from William Strider's PhD Thesis (1971). Samples of ϕX174 replicative form DNA (Form I) were subjected to alkali denaturation, yielding Form IV. These samples were neutralized and then incubated at the temperatures and pHs shown, for the periods of time indicated. Conditions of incubation were 1.0 M NaCl, 0.1 M phosphate, 3 mM EDTA. The products of incubation were subjected to analytical ultracentrifugation, and the percentage renaturation was calculated accordingly. Note that for any given temperature of incubation, renaturation was rapid and nearly complete at an optimum pH, but that relatively small changes in the pH resulted in a dramatic *decrease* in the rate of renaturation. Thus, for example, at 70°, renaturation of Form IV to Form I was essentially complete in 2 min at pH 10.8, but incubation for that same period of time at pH 10.1 resulted in only 20% renaturation. Extrapolation of the data for 70° suggests that at pHs below 10, or above 11.5, there would be essentially *no* renaturation observed under the conditions of this experiment.

As for our final assumption, the existence of a non-topologically linked ordered duplex structure for denatured circular DNA, there is little which can be said with certainty. In days gone by I can still recall hearing molecular biologists describing the structure of denatured circular DNA as 'a tangled mess', as if the single strands had no interaction with one another at all, other than the physical constraint imposed by the presumed topological linkage. Early attempts to renature it failed, and the term 'irreversibly denatured' was employed at first. The studies of Robert Warner (Strider, 1971; Strider and Warner, 1971; Strider *et al*., 1981), however, showed that denatured circular DNA can indeed be renatured, but only under very narrow conditions of pH, temperature and ionic strength (Fig. 2). These data certainly do not suggest anything 'random' about the structure of denatured circular DNA, but rather a highly ordered structure; one which responds in a very exact way to even minute changes in the environment. But whether that structure is topologically linked or not cannot be determined from these data.

TERMINOLOGY

I shall adhere to the original Roman Numeral system for describing circular DNA and its cleavage products.

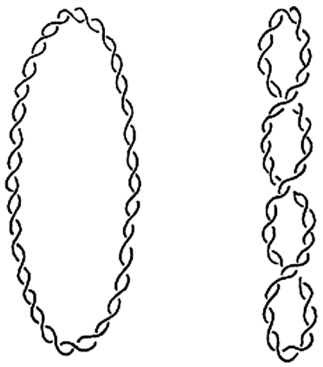

Figure 3. *'Traditional' models of circular DNA.* Circular duplex DNA with the Watson–Crick double helical structure, when constrained to lie in a plane, may be depicted as in the drawing on the left. It is hopefully apparent from the drawing that the two strands are *topologically linked* together. If the drawing does not make this clear, then think of the links of a common ordinary metal chain: each link of the chain is created by passing a length of metal through the neighboring link, winding it around *one time*, and then welding it shut. In Watson–Crick circular DNA, one strand is wound around the other not merely once, but *thousands* of times, and then 'welded' shut by covalent closure. If the concept of topological linkage is still not clear, make a circular DNA model from two pieces of string, to demonstrate to yourself that the strands cannot be separated unless at least one of them is cut open. The drawing on the right shows the appearance of native circular DNA when viewed through the electron microscope. It is a *superhelix*, where the secondarily wound chromosome is subjected to a higher-order, or *tertiary* winding. In 'traditional' theory, the explanation for this is that DNA, when created, is *under*wound. An underwound helix, like an unwound metal spring, will be under strain. In 'traditional' DNA structural theory, this strain can be relieved by taking on some right-handed teriary turns. The mathematical relationship between secondary and tertiary windings is explained in Fig. 5 and the accompanying text. The present manuscript explains the superhelicity of native circular DNA differently.

Form I: The replicative form of most small circular DNA. Form I is a covalently closed, circular duplex chromosome. The electron micrographic appearance of this species is rarely an open circle, however, because it is usually isolated in a form bearing tertiary superhelical twists (Fig. 3).

Form II: The duplex product resulting from the introduction of one or more single-stranded nicks into Form I. Even a single nick into either strand of Form I causes the tertiary superhelical twists to unwind. The chromosome is then relaxed, appearing in electron micrographs as an open circle.

Form III: The linear duplex product arising from the full duplex cleavage of Form I.

Form IV: The product resulting from the alkali denaturation of Form I. It is an extremely dense duplex of uncertain structure.

ALKALI DENATURATION OF SMALL CIRCULAR DNA

We are now in a position to apply the above assumptions to explain the failure of the strands of Form I to separate during ordinary denaturation, without invoking

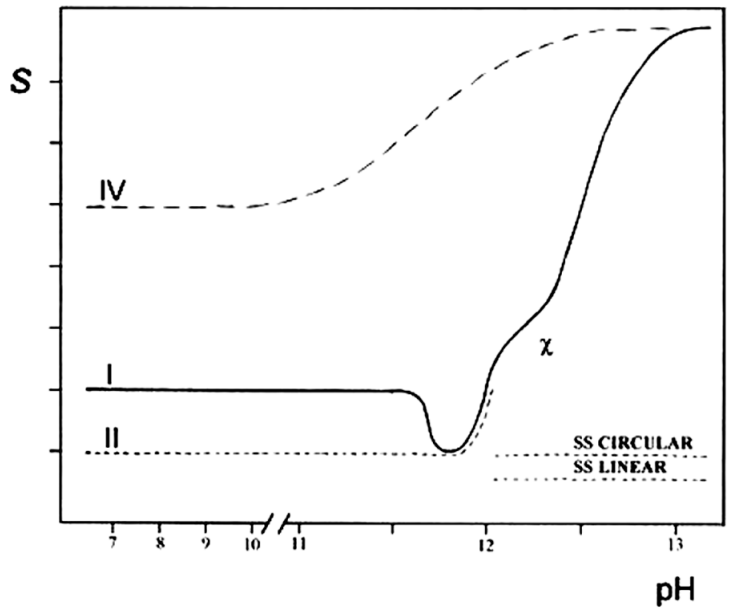

Figure 4. *pH vs. sedimentation coefficient curve for ϕX174 DNA* (based on data from Rush and Warner, 1970). For the replicative form (Form I), *s* is relatively constant between pHs 7 and 11.5. Above pH 11.5, *s* begins to dip, dropping to the same value as Form II at about pH 11.8. At higher pHs, *s* increases. There is a shoulder in the curve at about pH 12.2–12.3 (identified by χ), after which *s* increases steadily up to pH 13. At higher pHs, *s* remains constant. This product of alkali denaturation is called 'Form IV'. It is known to be duplex. When this denatured duplex product, Form IV, is titrated back to neutral pH, the upper curve is obtained. Although *s* for renatured Form IV shows some dependence on pH, it never drops back to the value of Form I. When this was first observed, it lead (erroneously, as it turned out) to the designation 'permanently denatured' DNA as a description of Form IV. For DNA with one or more nicks (Form II), the lowest curve is obtained. The behavior of Form II is, for the most part, reminiscent of that of linear duplex DNA. Its sedimentation coefficient (*s*) is constant up to the pH of the nadir of Form I (i.e., around pH 11.8). At a pH slightly above 12, it breaks up into single-stranded circular DNA and single-stranded linear DNA, each of which have sedimentation coefficients which remain constant with further changes in pH. There is one manner in which the behavior of Form II differs from that of duplex linear DNA. Starting around pH 11.8, Form II increases in *s*, paralleling the increase seen in Form I. This behavior is explained in the text.

topological linkage. Because it was hoped, at one time, that alkali denaturation might prove to be a useful tool for purification of Form I DNA on a large scale, it was studied very closely. We shall therefore take advantage of those abundant data to examine our model.

Figure 4 shows a typical *pH* vs. *sedimentation coefficient* curve for a small circular DNA (that of the bacteriophage ϕX174). This curve has been observed for a variety of small circular DNA's (Vinograd *et al.*, 1965; Rush and Warner, 1970), and it seems to be characteristic of naturally occurring small circular DNA's of average base composition. It has a number of important aspects, each of which

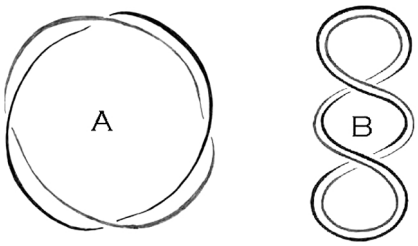

Figure 5. *Topological relationship between secondary helical turns and tertiary superhelical turns.* (a) A schematic representation of a 'conventional' Watson–Crick Form I molecule with exactly two right-handed helical turns. A secondary helical turn is defined as a 360° twist in the helix. (b) The same molecule shown in Fig. 5(a) can be converted into a duplex with *no* secondary helical turns at all by the introduction of two left-handed *super*helical turns. A superhelical turn is defined as a 180° twist in the superhelix (Glaubiger and Hearst, 1967). Note that when such a superhelix is constrained to lie in a plane, as in this figure, the strands of the duplex do not cross one another at all. The secondary helical structure has been completely unwound by the creation of a superhelix. These topological facts are difficult to grasp from a two-dimensional drawing. The reader who harbors any doubt that tertiary superhelical turns can unwind secondary helical turns, is advised to construct this model from string or rope.

we shall presently have need to consider. For the moment, however, it is sufficient to note that at high pH, the two strands of double-stranded *nicked* circular DNA (i.e., Form II) *separate* into single-stranded circles and linear forms, whereas the strands of intact duplex circular DNA (Form I) *do not*. The latter molecule, rather, remains double-stranded even at pH's greatly in excess of those required for denaturation.This denatured duplex product is the form known as 'Form IV'.

The traditional explanation for these observations depends upon the presumption that the strands of Form I DNA are *topologically linked.* As one can readily persuade oneself by working with circular chromosome models constructed from bits of string, two circular structures which are twisted together *before* being sealed shut into a circle are, in fact, physically *locked* together. This is referred to as 'topological linkage', and is treated as a quantitative property of circular DNA, having its own numerical parameter *alpha* (α), the 'topological winding number' (Glaubiger and Hearst, 1967), (or, alternatively, 'Lk', the 'linking number') (Crick *et al*., 1979).

Figure 3 depicts an all-right-handed Watson–Crick circular double helix. The reader who cannot clearly envision the topological linkage between the two strands is strongly urged to make a model from two pieces of string. Without a clear picture of topological linkage, the remaining arguments to be presented will be impossible to grasp.

I will give a brief description of the traditional explanation for the data in Fig. 4, then move on to the explanation in terms of the TN model. The traditional explanation is as follows: at neutral pH, the sedimentation coefficient of Form I is greater than that of Form II because Form I is superhelical. The superhelices are presumed to be the result of the chromosome having been sealed shut in an underwound

state, meaning that although it has essentially the Watson–Crick right-handed helical structure, it has, for some reason, a deficiency of right-handed turns. These are compensated for, one-for-one, by the appearance of right-handed *superhelical* turns (for reasons which will be explained presently). As the pH increases past 11.5, the DNA begins to denature ('melt'), and the chromosome starts to unwind, with the superhelical turns coming out first. As they do so, the chromosome proceeds to relax, until, at a pH just below 12, it becomes an open circle, with the same sedimentation coefficient as Form II. At higher pH's, the secondary structure 'tries' to unwind, but cannot, due to the presumed topological linkage. Therefore, it assumes an ever-increasing number of left-handed superhelical turns (each of which causes the unwinding of a right-handed secondary helical turn; see below), until, at some pH in the range 12–13, it reaches the point of 'irreversible' denaturation into Form IV. The sedimentation coefficient of Form IV is very high, indicating a dense, compact structure. If solutions of Form IV are neutralized (the dashed line at the top of Fig. 4), they become somewhat less dense, but they remain much denser than the Form I from which they arose.

Explanation of the Alkali Denaturation Curve in Terms of the TN Model

In order to proceed, we will need a way to visualize TN DNA. Figure 1(a) shows one such way. In this picture, TN DNA is depicted as consisting of a single long right-handed segment and a single equally long left-handed segment.

This 'model' emphasizes a critically important topological feature of TN DNA, namely that it is topologically 50% right-handed and 50% left-handed. But it certainly seems unlikely that such a structure will be observed in nature.

A more plausible structure would be the 'side-by-side' structure of Rodley *et al*. (1976), who proposed *no* complete helical turns at all, but rather a quasi-helical structure which alternately winds a bit to the left, then to the right, without ever making a full helical turn [Fig. 1(b)].

It should be specifically noted that the Rodley side-by-side structure is topologically 50% right-handed and 50% left-handed, only the length of each of the many alternating helical regions is less than one full turn, so that the strands, when constrained to lie in a plane, never cross one another. DNA with such a structure could replicate without the requirement of a mechanism for the unwinding of secondary twists.

Since x-ray crystallographic studies have shown that purified crystals of *linear* DNA molecules from natural sources are right-handed helices, we may assume that the common DNA's will generally 'prefer' to be right-handed, insofar as it is topologically possible for them to be so.

But in TN DNA, it is *not* topologically possible. Rather, exactly and precisely half the molecule *must* be left-handed, if the other half is to be right-handed (as

can be seen in Fig. 1, or more readily by constructing string models). The TN chromosome is therefore under *strain* at neutral pH, since it 'wants' to unwind its left-handed helical portions, but cannot.

In solutions of TN DNA, however, it *would be* possible for the individual molecules to form *superhelices* (see Fig. 5). The relationship between right-handed helical turns and superhelical turns in Form I DNA is fixed by the laws of topology. The introduction of a single 180° tertiary, or *superhelical* turn into circular duplex DNA causes the *unwinding* of a single 360° secondary *helical* turn of the opposite sense (Glaubiger and Hearst, 1967). In Fig. 5(a) and 5(b), for example, the introduction of two such left-handed tertiary superhelical turns causes the unwinding of two right-handed secondary helical turns. Again, the reader who cannot accept these topological facts from the figure alone is urged to verify them by constructing a string model.

To state these facts anthropomorphically, under conditions in which DNA 'wants' to unwind its secondary left-handed helical turns, it can accomplish this by assuming *super*helical turns in the opposite sense.

Once these topological facts are appreciated, it can immediately be seen why TN DNA must be *superhelical* at physiological pH: it 'prefers' the right-handed helical conformation, but must, by reason of topological restraint, be at all times exactly and precisely 50% left-handed, *when constrained to lie in a plane*. In order to *maximize* its right-handedness, it therefore refuses to lie in a plane. Rather, it takes on as many right-handed *superhelical* turns as possible, each of which causes the *unwinding* of 'unwanted' left-handed secondary helical turns.

We have therefore provided an explanation for the known fact that native Form I DNA is isolated as a superhelix (Glaubiger and Hearst, 1967; Vinograd *et al*., 1968; Shure and Vinograd, 1976), *without* presupposing any topological linkages between the strands. We have also correctly predicted the *direction* of Form I superhelical winding, which is, in fact, known to be *right* handed.

Therefore, the well-documented fact that Form I DNA is superhelical, and hence more dense, with a higher sedimentation coefficient (s) than its nicked Form II 'cousin' (Fig. 4), is not necessarily incompatible with the theory that Form I has the TN structure.

Let us now return to Fig. 4 and consider the next step in denaturation of Form I DNA, which occurs between pH 11.5–12.0. In this region of the curve *s decreases* to a value similar to that of Form II nicked, relaxed DNA. As in 'traditional' theory, we shall presume that this occurs because of the *unwinding* of superhelical turns, yielding a less compact molecule. According to our theory of TN DNA, this is consistent with the expected behavior of the molecule, since we have assumed that *conditions tending toward denaturation favor an* $R \rightarrow L$ *helical transition.*

In other words, as the pH increases, right-handed helical DNA starts to unwind, causing the left-handed helical form to become increasingly favorable until, at some pH between 11.5 and 12.0, the right-handed and left-handed forms become energetically equal. At that point, Form I DNA no longer 'cares' which way it

twists. So it relaxes, the superhelices unwind, and the molecule appears indistinguishable at that moment from Form II relaxed DNA.

We have therefore again accounted for the behavior of Form I DNA in Fig. 4 *without* assuming any topological linkages between the strands.

We now arrive at the most critical part of the curve. As the pH approaches 12, *s* begins to increase for both Forms I *and* II. For Form I this is readily explained. But why should this occur with Form II? The 'traditional' theory of DNA as a right-handed helix offers no explanation. But the theory of TN DNA, which incorporates the observation of the tendency of DNA to convert to the left-handed helical form under conditions tending toward denaturation, states simply that for *both* Forms, I *and* II, the DNA begins to express a 'wish' to be left-handed, which translates into left-handed *superhelix* formation (since left-handed superhelical turns unwind right-handed secondary helical turns—see Fig. 5(a) and 5(b)).

Next, at pH's above 12, Form II splits into separate strands of linear and circular single-stranded DNA, and its sedimentation coefficient, *s*, returns to that of relaxed DNA (Fig. 4).

But Form I DNA behaves differently. No denaturation into single-strands is observed. Why not?

Explanation for the Peculiar Differences Between Form I and Form II at High pH

The first reason: topological barrier to rapid unwinding of Form I. It is possible to identify at least *two* reasons why Forms I and II DNA might behave differently at pH 12. In order to understand them, we must do a 'thought experiment', mentally shrinking ourselves to the size of a denaturing TN DNA molecule, and asking the question 'What would we actually see?'.

The first thing we would see is that Form I, topologically speaking, is 50% left-handed at all times. Mathematically, this may be expressed by the statement

$$\alpha = 0$$

. . . alpha being the 'topological winding number' mentioned earlier. This means that if, in our minds, we constrain the molecule to lie in a plane (without superhelical twists), the number of right-handed and left-handed helical turns must always be seen to be exactly the same, and that the *net* number of secondary helical turns in the entire molecule must *always add up to zero.*

This means that there is a topological *constraint* on the molecule, such that every *right*-handed helical turn which is to be unwound *must* be accompanied by the compensatory unwinding of a *left*-handed helical turn also (or, if the molecule is not constrained to lie in a plane, the compensatory *winding in* of a left-handed *superhelical* turn).

These are not biological facts, but mathematical truths, fixed by the laws of topology. Again, see Fig. 5, or simply hold two rubber bands together and twist them.

It is entirely different with Form II, which has no topological constraints. Thus, our theory states that at pH 12, Form II, after briefly taking on some left-handed supertwists (note again the increase in *s* in Fig. 4 at pH 12), 'decides' to convert itself quantitatively into an all-left-handed helix, and there is no topological barrier to it doing so. So it tries to twist to the left. But what happens when it tries to do so?

One can readily envision Form II, at this pH, spinning rapidly about its long axis, like an electric drill bit. This process, occurring at high pH where base-pairing interactions are vastly weakened, must surely be a *disruptive* process; one which encourages denaturation by the sheer centrifugal force of the cooperative R → L transition. In fact, Form II, at this pH, does indeed denature, yielding single-strands.

I am suggesting, of necessity, that Form II denatures at a pH where base-pairing, although weakened, is not gone entirely, and that the event which triggers denaturation is therefore not the loss of base-pairing, but rather the rapid rotation of Form II about its long axis, as it tries to convert itself into a left-handed helix.

In Form I DNA, on the other hand, the above-mentioned topological constraint bars the possibility of a wholesale 'drill-bit-like' R → L conversion. Why? because, as we have noted (or as may be seen by twisting-untwisting a pair of rubber bands), every right-handed helical turn which is removed must be accompanied by the removal of a left-handed helical turn. But the left-handed turns do not 'want' to be removed at this pH; on the contrary, they want to *increase.* Thus, they will *resist* being unwound. Clearly, therefore, there will be no rapid R → L transition in Form I at pH 12.

The 'desire' to become left-handed at high pH, however, *can* be satisfied, at least in part, by *superhelix formation*. Thus, the molecule will twist itself into a left-handed superhelix, which will bring about the *removal* of 'unwanted' right-handed secondary turns *without* the necessity of simultaneously removing the now-desirable left-handed secondary turns.

Therefore, at pH's around 12, the ultimate effect of increasing the pH of solutions of Form II DNA is rapid and abrupt cooperative unwinding leading to strand separation, but the effect for Form I DNA is merely the gradual formation of ever-increasing numbers of left-handed *superhelical* turns.

Thus, without pre-supposing topological linkages between the strands of Form I, we have explained the alkali denaturation curve of Form I up to pH's of around 12. We still have not, however, explained why the strands of Form I do not separate at pHs *above* 12.

The second reason: cooperative protection against denaturation. In our 'thought experiment', we may make the following further observation about Form I DNA: it has no *free end.*

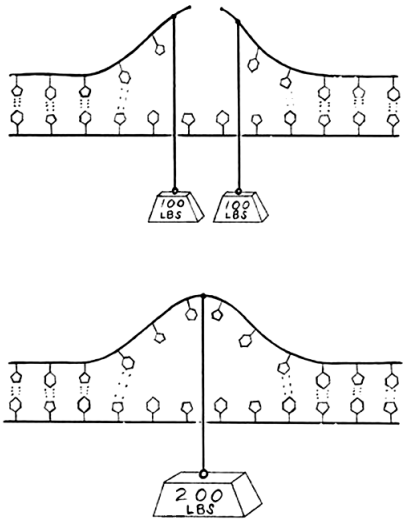

Figure 6. *Force required for denaturation of nicked vs. intact duplex DNA*. If an imaginary 'miniature strong-man' entered into a DNA duplex with a single-stranded nick (upper drawing), and if he found that pushing apart the strands on either side of the nick required a force which felt, to him, like '100 pounds', then it stands to reason that the same 'miniature strong-man' would have to exert 200 pounds of force to separate the strands of fully intact duplex DNA (lower drawing).

Denaturation of linear DNA has always been thought of as being initiated at a free end. As Fig. 6 shows (in highly schematic form), it will, as a first approximation, require fully *twice* as much disruptive energy to initiate denaturation in a covalently closed-circular molecule as in a nicked one which has a free end.

The degree of protection against strand separation in Form I can be roughly estimated. In Fig. 6 we have represented the divisive forces of denaturation as if they were the forces of a miniature weight-lifter lifting physical weights. It may readily be seen that it will, as a first approximation, require *twice* as much force to initiate denaturation in an intact duplex circle with no free end. But in reality, it is not the lifting of weights, of course, but the *hydroxide ion concentration* which constitutes the divisive force. Therefore, a doubling of this force may, as a first approximation, be thought of as a doubling of the hydroxide ion concentration, corresponding to a pH increment of about 0.3.

As a matter of fact, there is a *shoulder* on the pH vs. *s* curve of Form I at about 0.3 pH units above the point where its Form II 'cousin' dissociates into single-strands. This shoulder is labeled 'χ' in Fig. 4. In our TN theory, this corresponds to the point at which the above-mentioned cooperative protection against denaturation expires, and TN DNA finally denatures, converting from superhelical Form I into the mysterious 'Form IV'. All theory aside, the fact that this is indeed the true point of irreversible conversion to Form IV has been confirmed by the painstaking work of Strider *et al*. (1981).

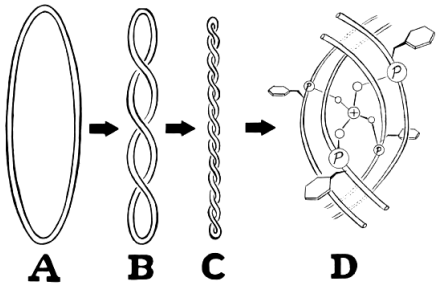

Figure 7. *Proposed structure for Form IV*. (a)–(c). Graphic representation of the behavior of Form I DNA in the approximate pH range 11.8–12.3. In this range of pH, Form I DNA is known to become a left-handed superhelix (see Fig. 4). As the pH increases, *four* strands of DNA (two from each side of the superhelix) get drawn closer and closer together, as the superhelix gets more and more tightly wound. Water will be 'squeezed' out of the core. The forcing together of four perfectly aligned strands of DNA is something which never occurs with linear or nicked circular DNA. (d) Artists representation of a four-stranded DNA structure with the phosphate groups on the *inside,* and the bases on the *outside.*

Why then does TN Form I not denature into single-stranded DNA? The only possible answer is that the formation of a tightly wound superhelix brings about a condition conducive to the formation of a new tertiary structure; one which is stable at high pH. What might it be? Obviously, it cannot be said with certainty. Based upon what is known about multi-chain DNA structure, both theoretically and empirically, two broad categories of structure suggest themselves: multi-chain structures with the phosphate groups on the inside, and multi-chain base-paired structures.

STRUCTURE OF FORM IV

What is the structure of Form IV? The question, in this direct form, cannot be answered.

If we alter the question slightly, however, we can do better. *Why* might the strands of denaturing Form I DNA be predisposed to forming multi-chain structures, whereas in other forms of DNA the strands fall apart?

The question, in this form, can be answered. Returning to our 'thought experiment', we note that in Form I DNA at pHs above 12, there are *four* DNA strands lying close together [Fig. 7(a)–7(c)]. The molecule is becoming increasingly superhelical at these pH's; much more so than is ever seen at physiological pH, where the superhelix is relatively *loosely wound*. According to what criterion can we say this? According to the criterion that at pHs around 12.3, *s* for Form I is nearly twice its value at pH 7 (see Fig. 4), which suggests that the superhelix surely *was* loosely wound at physiological pH, and has now become dramatically more *tightly wound* at pH 12.3. Consider what this means: the two sides of the double-helix have been

progressively twisted together, with water being 'squeezed' out of the core, so that there are literally *four* DNA strands lying closely juxtaposed [Fig. 7(a)–7(c)]. *This is a circumstance which never occurs with nicked DNA* (it begins just below pH 12, but is interrupted by total denaturation and strand separation).

It is not at all difficult to imagine this forced proximity of four DNA strands giving rise to structures which might not form spontaneously in other settings. But what might these structures be? We shall consider two.

THE LINUS PAULING MODEL OF MULTI-STRANDED DNA

In 1953, just before publication of the Watson–Crick–Wilkins–Franklin model of right-handed double-helical DNA, Linus Pauling (Pauling and Corey, 1953) published a paper suggesting a three- or four-stranded structure held together by phosphate salt bridges. Pauling, a double Nobel-laureate, was, according to James Watson's popular book *The Double Helix*, the world's leading authority on the chemical bond, and the scientist considered most likely to win the race to discover the structure of DNA.

Lacking knowledge about genetically specific base-pairing, Pauling found that the best way to construct a helix from DNA, from the purely chemical point of view, was to put the phosphate groups in the inside. His structure, a three-stranded helix with the bases on the *outside*, had the phosphates on the *inside*, close-packed into tetrahedral formations, giving rise to a helix with the required pitch of 34 Å. He chose a three-stranded structure because it was more readily made to accommodate itself to the existing x-ray crystallographs of DNA, but it was pointed out that it was easier still to pack the phosphates into a *four*-stranded structure (Pauling and Corey, 1953).

It has been known for many years that there actually are examples in nature of viral DNA helical chromosomes with the phosphate groups on the inside. Loren Day, of the Public Health Research Institute of New York City has identified two such viruses (Day *et al*., 1979; Liu and Day, 1994).

One possible structure for Form IV, therefore, would be a four-stranded helix stabilized by phosphate salt bridges [Fig. 7(d)], not unlike the structure originally proposed by Pauling as the structure for all DNA.

THE RICH MODEL OF MULTI-STRANDED BASE-PAIRED DNA

More recently, Alexander Rich and his co-workers have identified four-stranded DNA structures which are base-paired (Kang *et al*., 1994, 1995). These are described as consisting of two pairs of duplexes, each of which consist of two parallel strands held together by atypical hydrogen bonds (cytosine-protonated cytosine, or $C–C^+$ base pairs). The two base-paired duplexes are then intercalated into each other in opposite orientations.

These structures were identified in cytosine-rich DNA's, namely the copolymer d(C_3T) and the telomeric cytosine-rich repeating sequence d(TAACCC).

If DNA has a TN structure, then in the four-stranded configuration which exists just before the complete alkali denaturation of Form I [Fig. 7(c)], the possibility exists for two pairs of parallel strands to intercalate into each other in opposite orientations. Whether atypical base-pairing, however, will be seen in more typical species of DNA which are not necessarily cytosine-rich, I cannot say.

In any event, since the existence of any significant base-pairing at high pH seems unlikely, we would have to presume that such structures at high pH were stabilized solely by base stacking interactions.

All these things considered, I favor the Pauling model of DNA for the structure of Form IV.

NEUTRALIZATION OF FORM IV

Whatever the structure of Form IV, it is not based on genetically specific hydrogen bonding, so the complementary bases do not remain in register.

I would suggest, based upon what little we know about the circumstances of its formation (pretty much all of which is shown in Fig. 7), that Form IV is a quadruple helix of some type. If so, then we can say also that the tightness of the quadruple helical winding can be varied by varying the pH (see the upper curve in Fig. 4), but that it remains a non-base-paired quadruple structure under all circumstances. All, that is, except those narrow sets of circumstances described by Warner and his coworkers (Fig. 2), under which renaturation to Form I takes place.

DISCUSSION

The explanation for the behavior of Form I DNA in terms of the theory of topologically non-linked structure may be reduced to five basic principles:

1. The individual strands of circular duplex DNA in nature are presumed to be topologically non-linked, giving rise to a duplex chromosome having a structure such as has been proposed by several authors. Topologically, these structures are 50% right-handed and 50% left-handed, arranged as short, regularly alternating helical regions each less than one full turn in length.
2. Purified solutions of topologically unrestrained DNA (i.e., linear DNA or Form II nicked DNA) are presumed to have the 'traditional' right-handed helical structure at neutral pH, and the left-handed helical structure under conditions which promote unwinding.
3. At neutral pH, purified solutions of Form I TN DNA have the right-handed *super*helical tertiary structure, because this structure maximizes the right-handedness of the secondary winding. This it does by converting 'unwanted'

left-handed secondary helical turns into right-handed tertiary superhelical turns.

4. At higher pH (i.e., about pH 12), purified solutions of Form I DNA have the *left*-handed superhelical tertiary structure, because this structure maximizes the *left*-handedness of the secondary winding. This it does by converting 'unwanted' right-handed secondary helical turns into left-handed tertiary superhelical turns.

5. At pH's above 12.3, the left-handed superhelical winding is so tight that four DNA strands are forced into perfect alignment, overcoming the activation energy for the formation of a new structure: a four-stranded helix, most likely one with the phosphate groups in the core, stabilized by salt bridges.

These five principles may be employed to explain other topological phenomena reported for Form I DNA, including the electron microscopic appearance of replicative intermediates (Jaenisch *et al*., 1971; Sebring *et al*., 1971), the appearance and distribution of discreet bands in topoisomerase experiments (Crick *et al*., 1979), and the observation of greater stability of DNA–RNA hybrids than DNA–DNA hybrids during gel electrophoresis (Casey and Davidson, 1977). Space limitations preclude detailed discussions of these phenomena.

Stettler *et al*. (1979) set out to disprove all TN hypotheses by showing that the reannealing of separated single-stranded circles of complementary DNA did *not* produce Form I, as would have been required by any TN theory. In the place of Form I, these authors reported the appearance of an anomalous duplex structure they dubbed 'Form V'. Their report ought not to be accepted uncritically, since their experiment was essentially uncontrolled (the control experiment employed a different DNA, in a different solvent, at a different temperature than the real experiment) (see also Fig. 8 in the present manuscript). Furthermore, although most of the Form V structure was alleged to be in the double-helical base-paired conformation, the thermal denaturation profile showed no cooperativity at all (see Stettler *et al*.'s Fig. 13).

They had a 'competitor' in this work, Dr Robert W. Chambers, at that time acting chairman of the Biochemistry Department at the New York University School of Medicine, where I was a graduate student. Professor Chambers had also set out to prove that one cannot make Form I from complementary single-stranded circles, but wound up proving just the opposite! After becoming aware of the publication of the Stettler paper, Chambers retired his painstakingly isolated preparation of complementary single-stranded circular DNA to the refrigerator. Three months later, a significant portion of it had turned into Form I. Chambers, a staunch 'traditionalist', was unwilling to challenge the Watson–Crick theory, and, perhaps because he was unable to provide a satisfactory explanation for his discovery in terms of 'traditional' theory, he chose not to publish it (R. W. Chambers, personal communication, 1978).

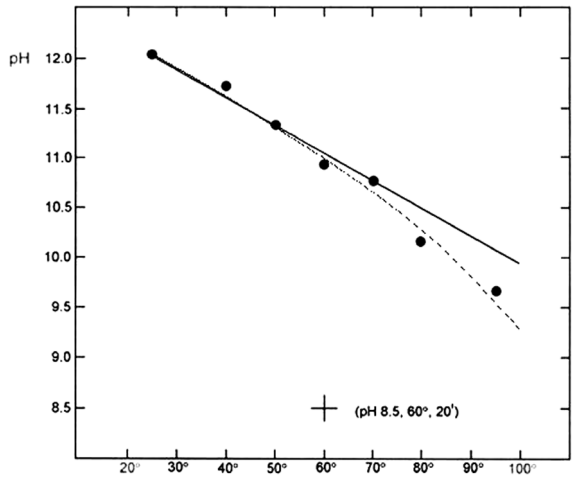

Figure 8. *pH optima for renaturation of Form IV*. The straight line is a graph of the pH optima from Fig. 2. The dashed line takes into consideration two additional data points added by subsequent studies (Strider *et al*., 1981). Extrapolation of these data suggest that at 100°, the boiling point of water, the pH optimum for renaturation of Form IV would be somewhere between pH 9–10. These data might be employed to find optimum conditions for re-annealing the strands of separated complementary single-stranded circular DNA, or conversely for the splitting of Form I into its component single-stranded circles, as explained in the text. The '+' sign at the bottom of the figure represents the conditions under which Stettler *et al*. (1979) incubated single-stranded circular DNA to produce 'Form V'. Note how far these conditions are from Strider's pH optima. If DNA has the TN structure, then reannealing of separated single strands should have pH optima similar if not identical to reannealing of Form IV, as explained in the text. If so, then Stettler *et al*. could not possibly have produced any measurable amount of Form I by incubating DNA under the conditions they arbitrarily chose for their experiment. They were simply too far from the optima.

I believe that the best way to formulate a strategy for definitively testing the TN hypothesis is to start by noting that Form IV, in the final analysis, is a *type* of separated complementary single-stranded circular DNA. It may therefore be that the problem of getting the single strands of Form IV to re-anneal may not be so different than the analogous re-annealing problem when the starting material is separated complementary single-stranded circles floating freely in solution.

Under the conditions of optimum temperature and pH shown in Fig. 2, denatured circular DNA is induced to enter into a conformation from which it readily passes into Form I.

If, as the TN theory states, the individual strands of Form IV are topologically non-linked, then separated complementary single-stranded circles, floating freely in solution, ought to associate with one another in much the same way that aggregated complementary single strands associate in Form IV, under the conditions of temperature–pH optima depicted in Fig. 2.

Therefore, incubation of separated complementary single-stranded circles under these conditions—and these conditions only—ought to give rise rapidly and quan-

titatively to Form I DNA. Incubation under any other conditions should be avoided, since, as Fig. 2 suggests, small alterations of pH and temperature might have profound effects on the outcome of such an experiment.

Figure 8 is a plot of the temperature-pH optima from Fig. 2. Incubation of separated complementary single-stranded circles under any of these conditions ought to yield rapid and quantitative conversion of the single-stranded circles back to normal Form I duplex circular DNA.

In any laboratory possessing complementary single-stranded circular DNA, this experiment can be done in a single day.

Another way to test the TN hypotheses would be to take advantage of the data in Fig. 8 to try to separate the strands of Form I by boiling. Under physiological conditions of pH and ionic strength, Form I does not denature when boiled. But if the data in Fig. 8 were extrapolated to the temperature of boiling water, Form I might behave as linear DNA does, and separate into single strands. This experiment is not 'clean', however, because boiling causes scission of DNA strands. Also, relative to the stringent temperature–pH requirements made evident in Fig. 2, it may be that the data in Fig. 8 are not precise enough to determine the conditions of this experiment with certainty.

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