Reconstitution of the circular double-stranded plasmid chromosome by re-annealing of its separated single-strands

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The theory that DNA has a net helical twist depends, more than anything else, upon the observation that the individual circular single strands of a small circular chromosome are resistant to separation. This suggests the Watson-Crick plectonemic winding, which would impart a topological linkage between the strands, making them impossible to separate unless one or both were broken open.

In a remarkable 1966 publication, Tai Te Wu proved that the strands of a circular chromosome could, after all, be separated, suggesting that native DNA has no helical twist. But the experiment was technically challenging, time consuming, and expensive, making it difficult to repeat. Moreover it was published in an obscure journal which few scientists read.

Years earlier, in 1979, Robert W. Chambers, a highly-respected scientist, had accidentally discovered that the fully-intact, separated single-strands of a circular chromosome, upon reannealing, gave rise to a duplex form which turned out to have all the physical properties of native circular DNA. Since the Watson-Crick plectonemic twist cannot have been restored in this experiment (which would have required, at the very least, that one strand be broken open, wound around the other, then re-sealed), this discovery, in agreement with the later Wu study, proved that native DNA has no helical twist.

Incredibly, this experiment was never published. It needs to be repeated and publicized. In 1979, it was difficult and time-consuming. Using current technology, however, the experiment can now be completed in 1-3 days, for a cost of under $1000.

Experimental Overview

The theory we shall test is that the duplex circular DNA chromosome, prepared by re-annealing its component complementary single-stranded circular halves, has the same electrophoretic properties as the native chromosome. "Traditional" Watson-Crick theory states that this is categorically impossible, so there is no possibility of ambiguity in the outcome.

The experiment is presented graphically in the top row of Fig. 1 below. Please note that we have omitted all tertiary supertwists from the figure, for graphic simplicity. (Note also that this is an actual photograph of rope models, which is why the two "strands" are different sizes)**:**

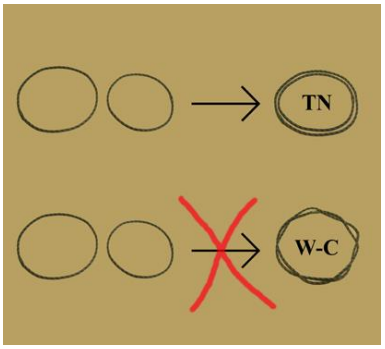


Fig. 1. Rope models of the reannealing experiment proposed herein. The top row shows the two single-stranded complementary half-chromosomes reannealing to a topologically non-helical ("TN") duplex. The bottom row illustrates an impossible outcome, namely the creation, by simple reannealing, of a topologically-twisted Watson-Crick duplex. Such could not possibly happen unless one of the two strands was broken open, wrapped around the other, then covalently re-sealed. Since we intend to employ no enzymes capable of doing any of those things, the twisted structure *cannot* arise in this experiment.

In the top row we see two single-stranded, complementary half-chromosomes, re-annealing to give the complete duplex chromosome on the right. The label "TN" stands for "topologically non-helical", since, as the figure legend states, no net helical twist can possibly be introduced in this experiment. The TN duplex is therefore drawn as having no Watson-Crick twists. We believe that, topologically-speaking, this is the structure of most, or all DNA, *in living systems.*

"Traditional" Watson-Crick theory predicts that such a reconstituted duplex structure, if indeed it can even be made to appear, will have grossly abnormal properties compared with native DNA, because the reconstituted structure cannot have the supposedly-"required" helical twists.

Please refer back to the figure above. The "traditional" reasoning is shown in the bottom row. If, as almost all scientists believe, DNA really had the Watson-Crick structure, then the re-annealing depicted in the top row couldn't possibly be seen in the experiment depicted in the bottom row, because the strands of a W-C duplex are bound together by topologically-locked-in twists. Watson-Crick twists can neither be created nor destroyed, unless one or both strands are broken open, re-wound, then covalently re-sealed. Thus, such twists cannot be reconstituted by merely re-annealing the separated strands (hence the red ***X*** in the bottom row). Therefore, according to "traditional" theory, the product depicted in the top row, if indeed it can be created at all, will *not* have the properties of twisted, *i.e.*, supposedly-"normal" DNA.

This experiment therefore distinguishes sharply, and definitively, between our theory of topologically-non-helical (TN) DNA, and "traditional" Watson-Crick DNA.

If we can isolate the complementary single-stranded components of a plasmid chromosome, and if we can re-anneal them back to a complementary base-paired duplex state, then the physical properties of the re-annealed product will answer the question. If its properties are bizarre or unusual, then the "helicists" will have the last laugh, and we

are thoroughly discredited. If, on the other hand, the properties are those expected for native DNA, then native DNA is not a helix, and the Watson-Crick structure may be retired forthwith; a retirement that is over 60 years overdue.

In the present experimental proposal, we shall outline a method for the final adjudication of this matter. The method we shall employ will involve enzymes which were not available until relatively recently; enzymes which will make it possible to reproduce the Chambers experiment — which took months in 1979 — in a matter of days now. The experiment will be rapid, inexpensive, and readily reproducible in other laboratories. Furthermore, it will distinguish *sharply and unequivocally* between the diametrically opposite results predicted by the TN and "traditional W-C" theories.

Experimental protocol

1. Preparation of a reconstituted duplex chromosome from its separated single strands.

We propose to take advantage of the pairs of semi-synthetic "nicking" enzymes now available (originally called "site-specific, strand-specific nucleases"). These enzymes introduce a nick to a duplex circular plasmid chromosome at a single site, and on one strand only. This strand, once nicked, can then be digested by single-strand-specific exonucleases such as exonuclease III, yielding a pure population of single-stranded, fully-intact-circular DNA from one strand of the chromosome only, which we may arbitrarily designate the "+" strand. The process can then be repeated with the sister nicking enzyme, yielding a pure population of "–" strands.

(In case it's not perfectly clear, "+" and "–" correspond to the "sense" and "antisense" strands of the chromosome. In recent years the terminology "+" and "–" strands has been largely replaced by the new terminology "**top**" and "**bottom**" strands.)

The diagram below depicts the plasmid pBR322, a plasmid which has a single recognition site for each of the "sister" nicking enzymes N**t**.BbvCI and N**b**.BbvCI. The second letters of these otherwise-meaningless-looking enzyme names identify the strands upon which the sister enzymes act: N**t**-(etc.) cleaves the "**t**op" strand, and N**b**-(etc.) the "**b**ottom" strand. In the drawing below, we have distinguished between the two strands by coloring the recognition sites for the enzymes blue and red, respectively. The action of the single-stranded nuclease, exonuclease III, is represented by a little "PacMan" figure:

(Over)

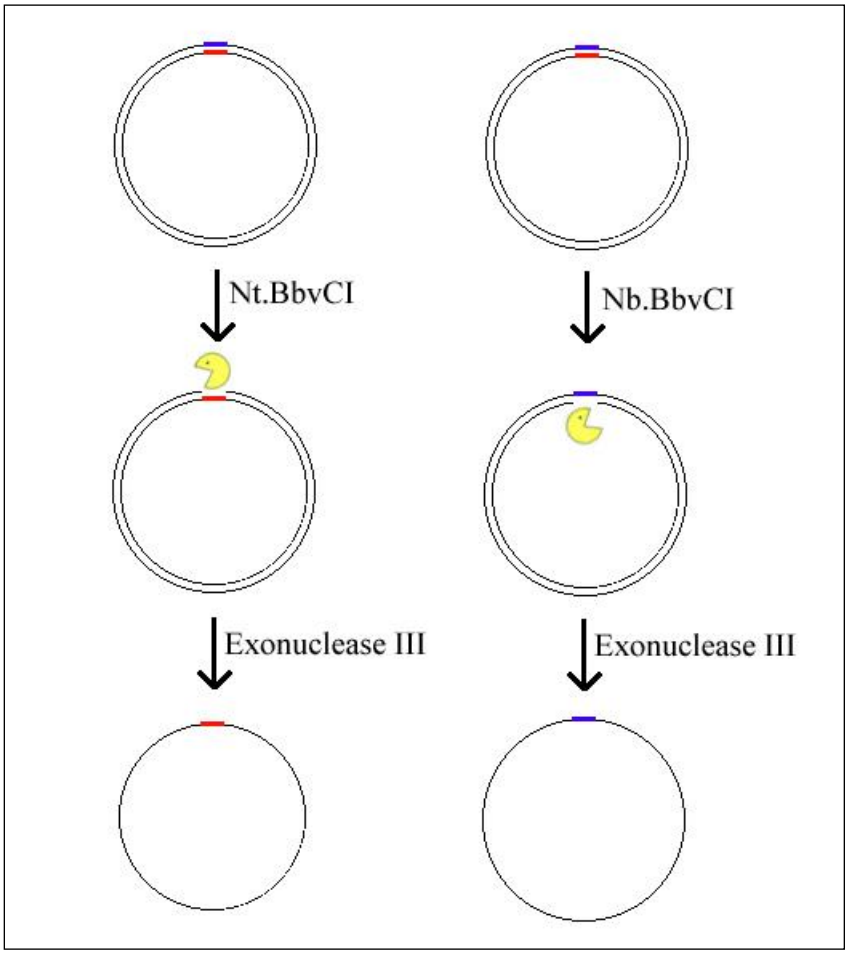


Figure 2. Creation of pure populations of single-stranded half-chromosomes by the use of nicking enzymes and exonuclease iii.

There are hundreds, perhaps thousands of laboratories with access to these enzymes, and you might be wondering why someone hasn't intentionally, or even accidentally re-annealed these strands already. The answer is that re-annealing of denatured circular DNA is an exceedingly exact science, requiring that the concentration, pH, ionic strength and temperature all be minutely-adjusted to optimum values for each of those parameters. It is well-established, for example, that in typical re-annealing experiments involving circular DNA, changes as seemingly-slight as a pH error of only 0.5 unit will destroy 80% of re-annealing. Increasing the error to 1 pH unit eliminates re-annealing altogether, even with prolonged periods of incubation at high temperatures. (Under the conditions of ionic strength and temperature employed by Charles Weissmann, in his undoubtedly well-intentioned but ultimately bumbling 1979 experiment [Stettler *et al*, 1979, J Mol Biol, 131:21-40] -- alluded to at several places in this web site -- the pH was 2 entire units below the zero-reannealing point!).

In other words, this re-annealing experiment is not something that is going to happen by accident. The re-annealing conditions we shall employ were painstakingly determined by a decade of research by dedicated scientists.

Once the DNA is re-annealed, it shall be subjected to agarose gel electrophoresis in the presence of various concentrations of ethidium bromide, with native DNA as a control.

2. Electrophoresis of the reconstituted duplex circular chromosome — "ethidium bromide fingerprint".

Ethidium bromide (EtBr) is an intercalating agent, and has a marked effect on the electrophoretic mobility of native DNA. Native DNA is often referred to as "Form I". We may thus say that EtBr *changes the superhelical winding of Form I* in a very precise and predictable way, as depicted in Fig. 3 below:

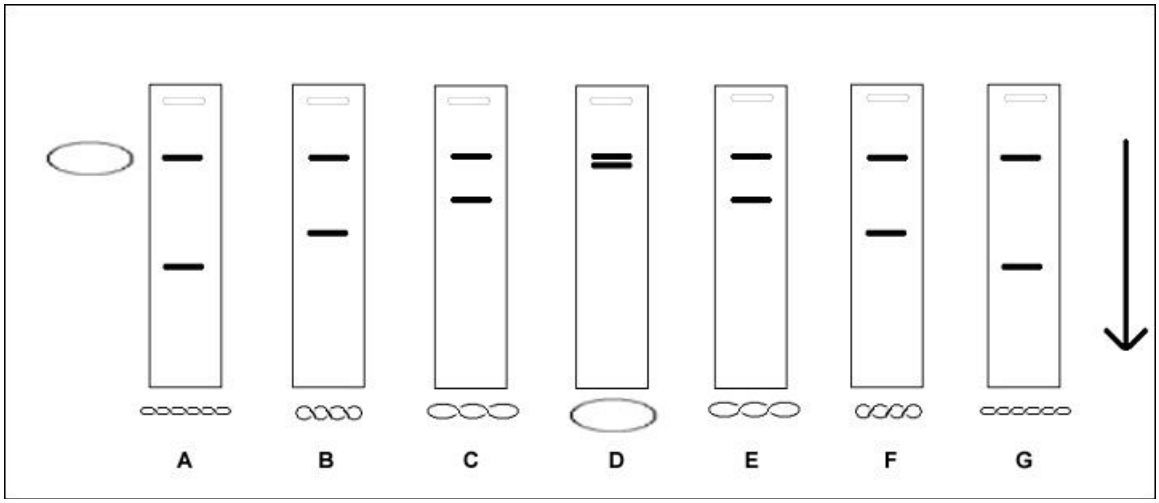


Figure 3. Agarose gel electrophoresis of Form I (superhelical) and Form II ("relaxed") duplex circular DNA in the presence of increasing concentrations of the intercalating agent, ethidium bromide (EtBr) in the agarose gel. The large arrow depicts the direction of migration in the electric field. The EtBr concentration increases as you move to the right.

Each of the gels, A-G, contain two bands of DNA, corresponding to Forms I & II respectively. The Form I band is always faster.

The position of the slower Form II band is not affected by EtBr. The little oval, on the extreme left of the drawing, schematically represents the simple tertiary structure of Form II.

Form I, on the other hand, is markedly affected by EtBr. The little drawings on the bottom of the figure schematically represent the tertiary structure of Form I.

In the absence of EtBr (lane A), Form I is a relatively tightly-wound, right-handed (or "negative") superhelix. Because of its compactness, it migrates significantly faster than its relaxed Form II "cousin".

As the EtBr concentration is increased (lanes B-D), the supertwists are progressively unwound, with a concomitant decrease in the electrophoretic mobility, until the Form I chromosome relaxes entirely (D). At that point, which typically occurs at EtBr concentrations around 0.2 μg/ml, the tertiary conformation of Form I, and

hence its electrophoretic mobility, are the same as that of Form II (for graphic clarity, a small space has been left between the Form I and Form II bands).

At higher concentrations of EtBr (E-G), supertwists in the opposite direction (*i.e.*, left-handed or "positive" supertwists) progressively appear in Form I, with concomitant *increasing* of the electrophoretic mobility. This increase continues up to EtBr concentrations of about 1 μg/ml (lane G), at which point the Form I chromosome exhibits about the same compactness (and hence electrophoretic mobility) as the native structure in the absence of EtBr (lane A). Further increases in the EtBr concentration (not shown) have little or no effect on either Form I or Form II.

(Adapted from electrophoresis data in Stettler *et al,* J Mol Biol 131, 21-40, 1979, Fig. 12, and from comparable data in CsCl velocity gradients, from Bauer & Vinograd, J Mol Biol 33:141-171, 1968, Fig. 8.)

Fig. 3 above depicts a hypothetical set of agarose gels, with ethidium bromide concentrations ranging from none (lane A) to about 1.0 μg/ml (lane G). Each gel has a pair of bands: a more-rapidly migrating Form I band, and a slower-moving band of "Form II" (the form assumed by circular DNA after being nicked, also referred to as "relaxed" DNA).

Form II is not significantly affected by EtBr. Form I, on the other hand, is markedly affected. Form I is isolated in nature as a compact, right-handed, interwound superhelix. In the language of DNA topology, a right-handed superhelix is described as having a "negative" tertiary winding. The superhelical winding confers upon it a relatively rapid electrophoretic mobility, compared to its relaxed Form II "cousin". As depicted schematically in lanes B-D, however, progressive increase in the EtBr concentration causes a progressive relaxation of the Form I chromosome, as the supertwists are progressively unwound.

In lane D, typically representing an EtBr concentration of about 0.2 μg/ml, all supertwists are gone, and Forms I & II co-migrate in the gel (the little space between the Form I and Form II bands is included only for graphic clarity).

At higher concentrations of EtBr (lanes E-G), Form I once again becomes supertwisted, but now in the opposite, or left-handed ("positive") sense, with eventual complete restoration of the original compactness and fast electrophoretic mobility (lane G). Although not shown in the figure, further increases in EtBr, above 1 μg/ml, do *not* bring about further increases in the electrophoretic mobility of Form I. (The fact that the electrophoretic mobility is the same at the beginning and end of this titration is important, but a discussion of this phenomenon is beyond the scope of this "Overview"; see, at https://NotAHelix.net, the PowerPoint presentation *Form IV: the Final Puzzle Piece,* slides 126-144).

I shall refer to this pattern of changing electrophoretic mobility, in the presence of varying concentrations of EtBr, as an "ethidium bromide fingerprint", since, like a real fingerprint, it uniquely identifies a species of DNA as being Form I.

Our experimental protocol shall therefore be to recreate duplex circular DNA from its separated single strands, and to study its properties. Our control shall be native DNA, sham-treated with everything except the enzymes. We shall then subject both species to agarose gel electrophoresis at varying concentrations of EtBr, to obtain an "ethidium bromide fingerprint" for each.

What will the outcome of this experiment be?

According to "traditional" Watson-Crick theory, the EtBr should have the expected effect on the native DNA, as depicted in Fig. 2 above, but either ***no effect at all***, or, in any event, a markedly ***different*** effect on our experimentally re-constituted duplex DNA.

There is no earthly reason, in the entire realm of Watson-Crick theory and dogma, for our reconstituted circular chromosome — whose strands have *no topological linkage* — to have the same ethidium bromide fingerprint as a native chromosome consisting of a pair of plectonemically-intertwined strands.

According to our own "heretical" predictions, however, the two species should have exactly *the same* ethidium bromide fingerprint, *co-migrating* at all EtBr concentrations, because the native and re-constituted DNA are the same thing.

It is virtually inconceivable that any substance — other than the native plasmid itself, of course — will co-migrate with the native plasmid in each of the gels A-G, just as it is virtually impossible for two human beings to have the same fingerprints.

There is thus no significant possibility of ambiguity in the outcome. This experiment will definitively answer the question, once and for all.

3. Final experiment: alkali denaturation - renaturation

Let us now play "the devil's advocate". Suppose that the experiment goes entirely as we predict, with native and re-constituted DNA giving the exact same ethidium bromide "fingerprint". Would that end the matter?

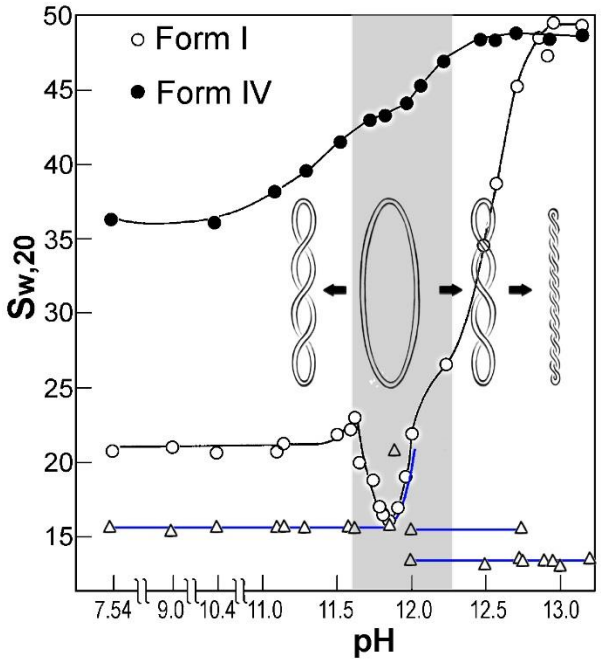
Probably not.

It is anticipated that many hard-line "helicists" would not be persuaded even by this, but would instead hypothesize that, for reasons which would defy explanation (which they would not be able to provide in any event), the re-constituted DNA might, in spite of its abnormal structure, nevertheless co-migrate with native DNA at all concentrations of ethidium bromide. What else could we offer such skeptics?

The answer lies in the ***pièce de résistance***, namely the alkali denaturation experiment. Under certain circumstances, the density of circular duplex DNA increases nearly threefold at high pH. If the density of our experimentally-reconstituted circular DNA also increases threefold at high pH, then only a demagogue, hell-bent on mischief, would continue to insist that native DNA has a helical twist.

This part of the experiment is based upon a fascinating, but little-known property of circular DNA — a property which was at one time closely-studied, but which is now long-forgotten: Duplex circular DNA undergoes a transformation into an astonishingly dense form when subjected to alkali denaturation at pH 13. This form is known as "Form IV". (See again the above-referenced PowerPoint presentation *Form IV: the Final Puzzle Piece*). In the figure below we illustrate the behavior of Form IV by means of a typical sedimentation rate vs. pH curve, in this case for the duplex circular chromosome of the replicative form of the virus φX174 (re-drawn from Rush & Warner, J Biol Chem 245:2704-2708, 1970).

There are three curves, closed circles (●), open circles (○) and triangles (△). Please look first at the open circles (○), representing the behavior of native plasmid or viral DNA at different pHs:



This curve (○) gives the ***s*** (Svedberg) values, or *sedimentation coefficients*, for Form I DNA at the indicated pHs. (The sedimentation coefficient reflects the rate at which a substance moves during high-speed, or *ultra*centrifugation, and may be thought of as a measure of compactness, or density). Note that at physiological pH, the ***s*** value for this

particular species of DNA is around 21 ***s***. At pH 13, however, the value shoots up to about 50! That's a nearly 3-fold increase in density!

At pH 13, the Form I is "permanently denatured" (but see below), having become transformed into so-called "Form IV", indicated by the filled circles (●). "Permanently denatured" means that when the pH is lowered, Form IV does *not* return along the path of the open circles (○), but rather follows the upper curve (●). An examination of this upper curve shows that Form IV, although less dense at physiological pH than at pH 13, nevertheless remains far denser than Form I at every point in the pH scale.

What is Form IV? In the molecular biological "establishment", no one knows, and no one cares! (But we care — for those who are interested, the likely structure of Form IV is discussed extensively at the above-referenced *Form IV: the Final Puzzle Piece,* at NotAHelix.net).

Note that at pH 7, the ***s*** value of Form IV is nearly 40, almost twice that of the native structure! These enormous density differences, as we shall see, will be of surpassing importance in establishing the non-helicity of native DNA.

Look now at the bottom of the figure. The triangles (△) and the blue lines connecting them show the ***s*** values for nicked duplex DNA ("Form II", or relaxed DNA), and of single-stranded circular and linear DNA. Note that none of these are importantly affected by pH.

(The dip in the Form I curve [○] at about pH 12, as well as the shoulders seen there in the Form I and Form IV curves, are beyond the scope of this discussion – see the PowerPoint presentation *The Science and History of Topologically Non-Linked DNA,* at NotAHelix.net, for further information.)

With these facts in mind, let us consider the expected results of *alkali denaturation* of our re-constituted experimental DNA, followed by gel electrophoresis. "Traditional" theory states that the strands of our re-constituted DNA will have nothing binding them together except perhaps a few weak, poorly-aligned base-pairs, all of which must disappear at pH 13. *"Traditional" theory therefore requires that at pH 13, our structure would behave exactly as linear duplex DNA behaves, splitting instantly into its component single strands,* which would migrate relatively slowly in an agarose gel (see the triangles [△] at the bottom of the figure above).

In sharp contrast, however, our *TN theory predicts that at pH 13, both the native and re-constituted DNA will co-migrate with "lightning speed",* since they would both be converted into Form IV, which has 2-3 times the density of Form I.

There is simply no possibility of ambiguity here. It is inconceivable that our experimentally-reconstituted DNA, if it has any structure other than the native one, would do anything other than split apart into single strands at pH 13. If, instead, it remains duplex, doubles or triples in density, and co-migrates with Form I, then it ***must be*** Form I.

With these considerations in mind, let us now consider our last, and most critically-important experiment. It has been known for years that Form IV, which used to be called "irreversibly denatured" DNA, can in fact be re-natured, but only if the conditions of pH, temperature and ionic strength are properly controlled. This subject, alluded to above, is beyond the scope of this brief protocol (see "The Science and History of Topologically Non-Linked DNA", *ibid.*, for details). Suffice it to say that the fastidiousness of the requirements for exact and precise conditions of pH, temperature and ionic strength, for the renaturation of Form IV, are so extreme that they provide a final opportunity to prove, beyond the slightest remaining shadow of a doubt, that DNA in real life is non-helical.

Execution: The last experiment is conceptually simple: First, we shall subject our experimental and control DNA to alkali denaturation, demonstrating that they both increase in electrophoretic mobility by a factor of at least 2. (NOTE: The anticipated increase in electrophoretic mobility will *not* be seen unless the salt concentration of the agarose is appropriately adjusted. See the Detailed Experimental Protocol for details).

Second, we shall, by careful control of pH, temperature and ionic strength, convert them both back to Form I, then go on to prove that the native structure, with the native electrophoretic mobility, is once again seen.

Even the most hardened, profoundly skeptical "helicist" will be compelled to admit, at such a point, that the Watson-Crick structure is not a good choice for the structure of DNA within living systems.