The Double Non-Helix

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Transcript of Soundtrack

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Slides 1-5 (Titles)

Slide 6 (History)

Slide 7

This is the world-famous Watson-Crick structure. As much, or more so than any other discovery ever made, it has changed the course of human history, opening the doors to the entire world of molecular genetics. Its structural features are, in general, proven

way beyond any shadow of a doubt, but, startlingly, one aspect of the structure, namely the helical twist, not only remains unproven, but in fact is contrary to all reason, and also is controverted by the weight of physical evidence.

Before proceeding, a word is in order about the rotating virtual models you will be seeing throughout this presentation. First of all, I must thank Mercury Computer Systems for making their AmiraMol program available for these studies, since these movies are all AmiraMol exports.

Secondly, I must point out that in general, and I think perhaps invariably, the direction of rotation in each AmiraMol export is to the right, *i.e.,* the direction defined by the right-handed rule of electromagnetism. You may think, however, that in some instances the molecule seems very definitely to be rotating to the left, or even changing direction every so often. This is an illusion which comes about because there is no perspective in these models, and the size of the atoms and bonds does NOT decrease when they move into the background.

Slide 8

The situation is quite analogous to the old perspective problem with the 2-dimensional representation of a cube. I'm sure you've all seen this picture before — if you stare at it long enough, the letter "A" will seem to move back and forth from the front of the picture to the back. There is no "correct" interpretation — it all depends on your perspective.

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Now take a look at the Watson-Crick structure again. If it seems to be rotating to the right, then all is well. If not, don't drive yourself crazy trying to force your eyes to see differently, because in most cases, the point being made is not affected by the perceived direction of motion.

Slide 10

The history of the Watson-Crick structure was picture-perfect for about 10 years. Then, in 1963, a monkey wrench was thrown in.

Here's a photo many of you may remember. It changed the history of molecular biology forever. It's Australian researcher John Cairn's world-famous autoradiograph of the E. coli chromosome, revealing the structure of that chromosome to be a *closed circle.* In this instance, we've caught the circle in the act of replicating, which will give rise to *two* daughter chromosomes, both circular.

It is now known that all bacterial chromosomes, all plasmid chromosomes, and most virus chromosomes are also circular. The viruses whose capsids contain chromosomes which are NOT double-stranded circles have an intracellular replicative form which IS, so the circular structure appears to be ubiquitous in nature.

Why is this a problem?

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Here's a picture from my "NotAHelix" web site. The caption reads: "The strands of a W-C double helix are locked together. If you cannot appreciate this from the drawing, make a model from string".

I'm going to save you the trouble of doing that. The problem, which is minimized by the "helicists", is actually one of enormous magnitude, and I'm therefore going to illustrate it extensively, so that you realize that the Watson-Crick structure, pretty though it may look, is not a good choice for DNA in actual living, replicating systems.

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The linkage between circular DNA strands which are locked together by a helical twist is like the linkage between the links of a chain. Here's how you make a chain. First, you start with a closed metal loop.

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Then you pass another length of metal through the loop.

Slide 14

Then you seal it.

If this chain was actually made of metal, you'd need a strong bolt-cutter to get the links apart.

Slide 15

The situation with DNA is far worse, with each strand mutually wrapped around the other not merely once, like a metal chain, but thousands and thousands of times, in some cases perhaps even *millions* of times.

Slide 16

Here's a rope model of a circular chromosome with the W-C structure. When you try to separate the strands, you just get a terrible knot.

Without a doubt, the strands of a circular chromosome with a helical twist are extensively linked together, a very awkward situation for a molecule whose main purpose in life is replication.

Slide 17

When I first realized these things, in my senior year at Cornell in 1972, I immediately concluded that DNA probably didn't have a helical twist inside cells. As the caption says, "Wouldn't this make more sense"? — that is, wouldn't logic dictate that the single, most-probable solution to the problem would be a non-helical structure, whereby the strands could separate relatively effortlessly during cell replication?

Slide 18

Here are two schematic representations of what non-helical DNA might look like. In "A", you have large regions of right-handed helicity alternating with equal lengths of left-handed helicity. This is obviously not a structure very likely to be found in nature. "B" shows a more likely alternative, where the chromosome alternatively twists a bit to the right, then to the left, repeatedly throughout its length. A number of proposals based upon this concept have been put forth, as we shall see.

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These, and other models not shown yet, all have one thing in common: The strands of DNA in these models are Topologically Non-linked, and will separate readily when the chromosome replicates. I refer to all such models, regardless of their molecular specifics, as "TN" models.

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"Although by 1963 it was established, to the satisfaction of nearly all, that DNA was a right-handed helix when stripped of all proteins, dried, and crystallized, this hardly proved that it would have the same structure in the cell nucleus."

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"The nucleus, after all, contains not only DNA, but also an approximately equal weight of highly-positively charged basic protein, which would surely exert some sort of effect on the conformation of the DNA."

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"What did Cairns think?"

Slide 23

We now come to what I am calling "A Turning Point In History", and I mean that quite literally, because I believe that the words of this article steered an entire generation of molecular biologists down the wrong path. The setting was the Cold Spring Harbor Laboratory in Long Island, without a doubt the "Mecca of Molecular Biology". A little under a year after the publication of his landmark autoradiographs of E. coli, Cairns had been invited to participate in a Symposium on DNA structure and replication.

He had had nearly a year to think about it. In order to understand what happened there on that day, you must keep your historical perspective: First of all, the fact that the strands of circular DNA resist separation when denatured, was NOT YET KNOWN. None of the enzymes currently believed to be necessary for the un-winding and re-winding of circular DNA during replication had yet been discovered. All that was known was that chromosomes were circular, presumably with a massive degree of topological linkage, and that they were yet able to separate during replication. HOW WOULD CAIRNS, THE WORLD'S RANKING AUTHORITY, EXPLAIN THIS?

In *my opinion,* for what it's worth, the best explanation was that the E. coli chromosome simply did not have the literal Watson-Crick structure, and that the strands were, in fact, Topologically Non-linked. The only obvious alternative was that all the twists were painstakingly unwound in each generation, and re-wound in the daughter cells**;** surely an awkward proposal.

WHAT WOULD CAIRNS THINK??

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Well, here's what he thought. Let us read: "To make the diagram in a sense complete, some provision must be made for free rotation of the unduplicated part of the circle with respect to the rest, so that the parental double helix can unwind as it is

duplicated; this provision, which we may noncommittally refer to as a "SWIVEL", has been placed at the junction of starting and finishing point…"

Slide 25

Hey! There's no mention at all, either here or anywhere else in this entire Cold Spring Symposium volume, of the possibility that maybe the strands simply AREN'T twisted together, and therefore DON'T need to be unwound. How did Cairns rule that out? Who told him that DNA HAD TO HAVE a helical twist? Did he even think the problem through?

Slide 26

Those of you who are familiar with the theory of discontinuous DNA replication must keep in mind that it was not known back then. What, in terms of the level of knowledge which existed in 1963, can we say about the *implications* of the "swivel" theory?

In particular, if all of the E. coli purported Watson-Crick twists need to be removed, HOW FAST IS THE CHROMOSOME SPINNING?

Slide 27

In order to answer this, we need some basic statistics about E. coli. And here they are.

Slide 28

The cell is about 2 microns long.

Slide 29

But the single, circular chromosome is 1.35 mm long!!

Slide 30

Why, that's *700 times* as long as the entire cell! That means the DNA is REALLY packed in TIGHT!

Slide 31

Here's the crux of the matter: There are 4 MILLION base-pairs in E. coli.

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Armed with this information, we can now answer our question: HOW FAST IS THE E. COLI CHROMOSOME SPINNING?

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In "log phase", E. coli replicates in 20 minutes. Under optimal conditions, the daughter cells begin to divide before the parent cells have fully separated!

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If the E. coli chromosome has the Watson-Crick double-helical structure, then, with 4 million base pairs, there would have to be 400,000 Watson-Crick twists. Every one of these twists would have to be un-wound and re-wound in the space of 20 minutes.

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Conclusion: The chromosome, in log phase, MUST be spinning at 400,000/20 = ***20,000*** rpm!

Slide 36

This is my Black & Decker drill. It'll tear through this piece of wood in a matter of seconds. How fast do you think this thing turns? -- According to the manual, it's spinning at only 1,000 rpm!

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So the concept Cairns sold the molecular biological world in 1963 was that the chromosome of E. coli was spinning 20x faster than an electric drill, *i.e.,* the speed of an ultracentrifuge.

Slide 38

Do you really believe that this chromosome, or even part of this chromosome, is spinning at 20,000? It seems unlikely, does it not?

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Are all the processes of life, including transcription, recombination and DNA repair, taking place as the chromosome spins at 20,000 rpm? Not very likely.

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If we attempt to evaluate Cairns' view of circular DNA replication by examining his published writings from 1963-64, we are compelled to conclude that he did not think the problem through.

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How did he reach the conclusion that DNA replicated by virtue of a "swivel"? No such structure was known at the time. Why did he make no mention of the severe rotational problem involved in replicating a circular W-C structure?

Slide 42

And, most of all, why did he not even mention in passing the seemingly-obvious possibility that DNA might be non-helical in cells?

Slide 43

Although it didn't take long to realize that entire chromosomes don't spin at 20,000 rpm, there's never been any doubt that small molecules can, and do spin that fast and faster. Therefore, the concept quietly arose that DNA replication was *discontinuous.*

If so, then only a part of the chromosome would have to be spinning at 20,000 rpm at any given moment, as depicted in the following somewhat crude movie:

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It's true that our knowledge of DNA replication has advanced astonishingly, and that the enzymes *topoisomerase* and *gyrase* theoretically allow unwinding of DNA, as the DNA replication fork advances along the chromosome. But if you look at this lovely drawing from Germany, you'll note that the incredibly complex and minutely-coordinated activities of the various replicative enzymes take place within a flattened-out; that is, *non*-helical region of activity in the replicating fork, with the topoisomerase placed at the entrance like some sort of guard.

Slide 45

The roles of the enzymes *within* the fork are apparently well-characterized, and complicated in the extreme. Lehninger refers to these events as "elegant enzymatic choreography", and that description is, if anything, an *understatement*. What we're dealing with here is a miraculous sequence of closely-linked processes which proceed with blinding speed, and unfathomable accuracy. *However,* the supposed "winding and unwinding" events, at the ends of the replicating fork, are a much more murky affair.

There is no demonstrated temporal relationship between the well-documented "choreography" of the enzymes within the replicating fork, and the murky activities of the topoisomerases and gyrases without. The presumption that DNA must be unwound and re-wound during replication remains in the realm of theory, known as the "two domain hypothesis".

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This hypothesis is based upon the fixed, immovable belief that DNA has the Watson-Crick structure. If so, then it is necessary to accept, and believe that the DNA in front of a replicating fork, represented here by an orange arrow, will become "overwound" as the fork moves, and that likewise the DNA behind the fork will be stretched out, becoming "underwound". These abnormal windings, it is said, must be removed by topoisomerase and gyrase, respectively.

But is this true?

Most of the topoisomerase and gyrase research is based upon *in vitro* studies, where the native structure of DNA is destroyed. As soon as you remove DNA from its natural protein environment, it's going to wind itself up into a helix, and everything you discover subsequently is at risk of being laboratory artifact. Even if these enzymes do what they are claimed to do *in vitro,* isn't it possible that in the living cell, where the DNA structure may be non-helical, that their true roles may be in other processes, such as DNA repair? There's no way to know by direct evidence.

It would be consistent with my admittedly argumentative nature to try to find fault in the 2-domain theory, but the literature on the subject is so confusing, and the publications so mutually inconsistent, that it's hard to find a well-enough established fact to merit a critical analysis. The typical 2-domain experiment involves either enzyme mutants, or administration of enzyme poisons, neither of which necessarily even stops DNA replication, but may merely slow it down. The products generated may or may not

be supercoiled in various senses, according to 2-dimensional electrophoresis gels which are exceedingly difficult to interpret. Virtually any result reported in one lab has been contradicted by a different result elsewhere.

In the presence of such uncertainty, it would be foolish to draw too many conclusions from this line of research.

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Besides, none of those enzymes were known in 1963.

Therefore, we still have no explanation for the curious fact that Cairns insisted that DNA replication had to be by means of a "swivel".

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I can think of 3 explanations.

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1. Perhaps Cairns was not intelligent enough to figure out that DNA was not necessarily helical in cells (a very unlikely explanation).

Slide 50

2. Perhaps experimental evidence existed which proved, or strongly suggested, that DNA was indeed helical; not only in synthetic laboratory crystals, but in the nuclei of living cells as well. Such evidence was, in fact, starting to emerge. But there's no evidence that Cairns, or any other speaker at Cold Spring Harbor at that time, knew about it. They certainly made no mention of it.

Slide 51

3. Perhaps it had already become "politically incorrect", as early as 1963, to speak publicly of any DNA structure other than the "double helix", because DNA had already risen to the level of a quasi-religious icon in the minds of most scientists.

Slide 52

And the correct answer is…

Slide 53

#3: It had already become "politically incorrect", even as early as 1963, to speak publicly of any DNA structure other than the "double helix", because DNA had already risen to the level of a quasi-religious icon in the minds of most scientists.

Slide 54

You can say that this picture is a gratituitous insult, or a cheap shot, but it is neither. It is a high-order and well-intentioned satire, to dramatize a serious problem which arose in 1963, and persists, almost full-force, to this very day. Whether you're talking about viral, bacterial, or human chromosomes, or the structure of DNA in the nucleosome, or the structure of DNA when complexed to the lac repressor or any other modulator of DNA activity, you find, with *not a single exception I know of,* that the

concepts listed at the top of this slide are required, as if by statute: "DNA is a Helix", "No other structure is possible", and "No other structure can even be discussed".

So, we're going to have to do something about that.

Slide 55

A LOGICAL QUESTION ARISES:

Why on earth should DNA, in the presence of the strongly-charged basic proteins of the cell nucleus, have the same structure as DNA in the laboratory, where all those charged proteins have been artifactually removed?

Is there any reason to even expect that?

Slide 56

Now, at the risk of being accused of "saturation advertising", I'm going to belabor this issue — a lot. Those of you who are practicing nucleic acid/protein chemists will surely deny believing this, but you're just fooling yourselves, and you know better. Without a doubt, the presumption that DNA has the same structure whether or not nuclear proteins are present is so ubiquitous, that it might almost be described as "universal".

So I *am* going to belabor this issue, until the point gets across. Let's start with a rosebush. Is the structure of a rosebush *with* a trellis…

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The same as the structure of a rosebush *without* a trellis?

Slide 58

I think not.

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How about a man with a cane? Is the structure of a man with a cane the same as the structure of a man…

Slide 60

…*without* a cane?

Slide 61-62

Well, certainly not in this case.

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Now, those of you who insist that DNA must have the same structure whether or not nuclear proteins are present might also think that a bow and arrow has the same structure whether or not…

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… the attractive young woman is present.

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In this case, we can do a little experiment. You can stand in front of the bow and arrow, and then we can remove the woman.

Slide 66

Oops! That could be a problem.

Slide 67

Well, no harm done. ***This*** time!

Slide 68

You could say that all this has nothing to do with chemistry, but what if I told you that some influential group of senior scientists had promulgated a decree which stated that *all enzymes must have the same structure whether or not substrate is present?* You'd laugh in my face, wouldn't you?

Slide 69-70

If that was the case, after all, then half of all biochemistry would be null and void.

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Of course it's ridiculous. But then why do you insist that, alone among all the chemicals in creation, ONE only, namely DNA, MUST have the double-helix structure in every setting in which it's found?

Slide 72

Okay, I admit I've been doing my very best to present belief in the double helix in the most ridiculous light I could conjure up. That's because the notion should have been questioned by biochemistry's elite in 1963, and it wasn't. But to be fair and even-handed, I must now admit that the story is more complicated than that. Biochemists unwilling to consider non-helical structures got a *reprieve* in the early 1960's, because at about the same time that Cairns was doing autoradiographs, a discovery by Jerome Vinograd and his associates demonstrated something that seemed to prove that blind faith in the Watson-Crick helix was justified, after all.

That discovery…was that the chromosomes of some viruses were, like the chromosome of E. coli, closed circular duplexes. Additionally, however, they were *small.* Whereas it remains true to the present day that because of its large size, it's almost impossible to manipulate a bacterial chromosome without breaking it into a shower of fragments, the small circular chromosomes of viruses are quite stable to ordinary manipulation. They can be shaken, stirred, frozen and thawed, without breaking. So they are very amenable to various physical studies.

Slide 73

Among the first of these studies were denaturation studies. These, to no one's surprise, showed that:

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The strands of circular DNA do not separate when boiled.

Slide 75

The strands of circular DNA do not separate at alkaline pH.

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In the latter instance, it was demonstrated, through studies such as the measurement of light absorption at 260nm, that at high pH there was a complete breakdown of the base-paired structure. Yet, all attempts to separate the strands physically failed, and it became very clear that they were somehow linked together, even when denatured.

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This was considered to constitute totally satisfactory evidence that small viral DNA had the Watson-Crick structure where, as we have seen, the strands would be locked together, and no separation would be expected, even upon denaturation.

Thus, the unwillingness of molecular biologists to consider non-helical structures for circular DNA seemed to have Mother Nature's "seal of approval". BUT...

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...Shouldn't someone at least have asked the following question: "Is it possible that there's something about circularity which imparts unexpected characteristics on DNA, so that under conditions where the strands would separate, if linear, they do not separate when circular?"

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The answer is emphatically “yes”. Circular DNA turns out to have properties entirely different than those of its linear cousins.

Slide 80

The first studies in this area were done by Jerome Vinograd and Rene Dulbecco, both of whom were major figures in American science.

Working with the chromosome of Polyoma, a small mammalian oncogenic virus, they found what at first seemed to be a very peculiar behavior of the DNA as the pH was increased. At first, as the pH approached 12, the DNA clearly and significantly *decreased* in density. Thereafter, it increased relentlessly until pH 13, at which point it leveled off and became constant at higher pH’s.

A painfully detailed study of these phenomena was carried out by Robert Warner, a colleague of Vinograd’s. Most of the work was actually done by Mark Rush, a post-doc in Vinograd’s lab at Cal Tech, and Bill Strider, a doctoral student at NYU School of Medicine. I knew them both very well. Mark joined the faculty of NYU, where he was my thesis advisor, and Bill Strider was a post-doc in the same department for most of the 5 years I spent there.

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Shown here is a preliminary view of Rush’s data, utilizing not polyoma, but the replicative form, or “RF”, of the chromosome of phi-x174, a virus about the same size as polyoma.You are going to have to pay strict attention to this data if you wish to

understand circular DNA, because the entire argument which has been swallowed whole by the molecular biological establishment WRT the necessity of believing in the Watson-Crick structure is based upon this work. If you don't take the trouble to understand this alkali denaturation data, as I walk you through it, you'll never understand the structure or topology of circular DNA.

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We shall have need to understand everything in this slide, but for now I would like to draw your attention to the lower curve, that is, the white circles, which show the sedimentation coefficient of the ϕx174 chromosome. The sedimentation coefficient, ***s***, is the rate at which it drops in a cesium chloride density gradient ultracentrifugation study. If you're not familiar with the procedure, which was one of the backbones of mid-20th century biochemistry, just think of ***s*** as being a measure of density, although in this case it is not only the primary chemical structure which determines density, but also the secondary and tertiary conformational changes which take place as the pH changes.

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The drawings above the graphs illustrate the sorts of tertiary structures which give rise to the changes in ***s***. You will note that in one of the drawings, DNA is depicted as an open circle (or oval, to be exact). If you look closely, you'll see that I have neglected to indicate any W-C helical twisting, because the nature and extent of helical twisting is the very issue which forms the substance of this slide presentation, and I am making no presumptions about it. Yet.

The other 3 drawings depict the DNA as having a higher-order twisting, known as *supertwisting*. It's primarily the state of supertwisting that determines the density, and hence the ***s*** value.

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The situation is entirely analogous to sky diving. If a sky diver has his limbs spread out, he drops relatively slowly, with a terminal velocity of only 90 mph. But if he goes into dive position, he minimized air drag, and his terminal velocity more than doubles, to 190 mph. The DNA sketches below show the analogy. When DNA appears as an open circle, it has a relatively low sedimentation coefficient in CsCl velocity gradient ultracentrifugation, and a relatively slow rate of migration in an electrophoretic field. But when it is supertwisted, friction is reduced, and it will move more rapidly during either centrifugation or electrophoresis.

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Back to our graph. Note that at a pH of about 11.8, ***s*** drops to a minimum. Then it increases to a maximum at a pH of about 13.

Slides 86-88 (no audio)

Slide 89-91

If the DNA is neutralized in stages, we get the upper curve, *i.e.,* the black circles.

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Note that ***s*** NEVER returns fully to its pre-titration value, but remains permanently elevated. How can we explain this?

The so-called "standard" explanation, concisely illustrated by the drawings which appear in the graph, starts with yet another unfounded assumption, namely that DNA is, for some strange reason, synthesized "underwound", which causes it to have superhelical twists at physiological pH. If you're not familiar with the topology of circular DNA, don't worry; that's what this slide show is going to be all about. For now, just accept that circular DNA, as usually isolated in nature, is in fact found to be *superhelical* in addition to whatever secondary helical winding it does—or does not— have. Here's the picture again, without the skydivers this time:

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On the left side of this slide we see a drawing of a DNA "classic" Watson-Crick double helix, which has been circularized. This open circular form is NOT the form in which most naturally-occurring circular DNA is isolated, however. In general, circular DNA in nature is SUPERHELICAL, as illustrated on the right. When the circular helical duplex is further twisted, to impart a higher-order twist, it is generally referred to as a "tertiary" twist. The Watson-Crick helical twist, on the other hand, is referred to as a "secondary" twist.

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Note the directions of helical twisting, which are correctly depicted here. The Watson-Crick structure, on the left of the picture, has a RIGHT-HANDED twist. This means that as you follow it up, you find yourself moving to the right. This is true regardless of which way the drawing faces – if you turn it upside down, it remains right-handed.

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The direction of winding of the SUPERHELICAL turns, on the right side of the picture, is also RIGHT-HANDED. This too is correctly depicted, since this is the direction of superhelical twisting ordinarily found in nature. DNA topologists generally refer to this NOT as "right-handed", but rather as "negative" supertwisting, for reasons I'll explain shortly. For now, however, I'm going to assume that most viewers of this slide presentation are not DNA topologists, and it's far less confusing, WRT the concepts we shall discuss, to use right/left terminology for both secondary and tertiary twists, for the time being.

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Let us now turn to the basic rules of DNA topology. I'm going to state them succinctly first, then illustrate them. So if they don't seem clear at first, hang on, because the concepts are not really all that difficult.

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The first rule is this: **The number of secondary helical turns in fully-intact circular DNA, when it is constrained to lie flat, *i.e.*, in a plane, is absolutely fixed at**

the time the rings are closed. In our so-called "heretical", non-helical model, to be presented shortly, that number is *zero*. In the "classic" Watson-Crick crystal structure for linear DNA, the number is one complete right-handed helical turn per 34 Å of length.

In circular DNA, as isolated in nature, if you believe in the Watson-Crick structure, then the number actually turns out to be 5-7% fewer turns than found in corresponding linear DNA, as we shall explain shortly.

Whatever the number is, it cannot be changed unless one of the strands is broken open.

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In spite of the fact that I'm about to propose a non-helical structure for DNA in living systems, I nevertheless present the following 2nd rule of DNA topology:

Under physiological conditions of pH, temperature and ionic strength, the only known fully-relaxed, unstrained structure for DNA of average base composition is the Watson-Crick structure, with one full secondary helical turn per 34 Å's of length.

A circular duplex chromosome which, at the moment of covalent closure, has either a *greater* of *lesser* number of secondary helical turns per unit length will be under *strain.*

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The situation is really quite analogous to an ordinary metal spring. If I was to stretch this spring, altering its pitch, then, when I let go, it would immediately snap back to its normal length. The same if I compress it.

Like this spring, DNA "wants", so-to-speak, to have a certain pitch, which, in the absence of complicating factors, is 34 A per helical turn. If you either overwind or underwind it, it gets "upset", just like a metal spring does.

Slide 100

Now here's the THIRD PRINCIPLE of DNA topology:

Tertiary, *i.e., superhelical* turns, *increase* the tightness of the secondary winding of a helix whose twist is in the same direction, and *decrease* the tightness of the winding of a helix whose twist is in the opposite direction.

If you don't understand what I mean, relax, because I'm going to show you some models. This is the sort of thing which is very easy to see and grasp, but very difficult to explain in words.

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Let's look at some pictures. The left side of this slide shows a Watson-Crick circular double helix with exactly and precisely two right-handed twists.

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Note that each strand crosses the other exactly 2 times.

Slide 103

In real DNA, these crossings are very intimate, and occur within the constraints of the exact dimensions of the double-helix. They cannot be removed without affecting the geometry of the entire helical structure.

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Now what if the chromosome didn't "like" the way it was twisted? That is, suppose there were too many, or too few helical turns, or even left-handed turns? What could the chromosome do to "cure" the defect?

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Look again at the picture. The cure is to take on *superhelical* turns in the opposite sense. Look at the right side of the picture. This is the structure which results from the introduction of 2 *left-handed* SUPERHELICAL TWISTS into the chromosome. Please look at the structures on both sides of the drawing, and verify that the directions of helical and superhelical winding are opposite, namely right-handed in the original chromosome and left-handed in the superhelical structure.

Note also that in the superhelical structure, neither strand ever crosses the other. The introduction of 2 left-handed superhelical twists has totally unwound the secondary helical twists in the original structure, replacing then with a new structure which has only superhelical crossings.

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NOW, you may be wondering what benefit there is in removing secondary twists, if we're just going to add tertiary superhelical twists. That's a good question. In order to see the benefit, let's look at a real model made from rope.

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This is the rope model which was the basis of the drawing above. This model is the ultimate in simplicity, having only *one, single right-handed "Watson-Crick" twist,* whose secondary crossover is indicated by the arrow.

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When a single left-handed superhelical twist is added, in the direction indicated…

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…the single secondary strand cross-over is eliminated, just as we saw in our previous 2-twist drawing.

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If we follow this structure around, we see that once again the strands never cross in the secondary structure, but…

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…they cross in the tertiary sense.

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Now let's see what the benefit is to the DNA. And here it is. If we look in 3 dimensions, we see that the strands, which seem to be quite intimately close as they cross superhelically in the flat picture, may actually be quite far apart. Note that there's room to put a ruler between the strands, so that the superhelical crossing actually has no steric effect on the structure of the chromosome.

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Bearing in mind what I said about the chromosome not being "happy" when either under- *or* over-wound, we can now see the "secret" of reducing helical strain by the winding in (or out) of superhelical twists in the opposite sense; namely that a small number of superhelical twists in the opposite sense do in fact have the ability to significantly reduce secondary helical strain, and add no additional element of steric hindrance to the molecule.

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The process of removing secondary helical strain due to over- or under-winding through the formation of superhelical twists in the opposite sense cannot, however, continue indefinitely. There does come a point where an excessive number of superhelical twists will cause the DNA strands to be brought so close together that the negatively-charged phosphate groups will begin to repel each other, discouraging further superhelical twisting. This is illustrated in the following drawing:

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This drawing, like some of the previous drawings, makes no attempt to indicate the direction of helical twisting, but illustrates only superhelical twisting. It attempts to make the point that the addition of a small number of superhelical turns (B) to an open-circular duplex (A) does not force the strands on the opposite sides of the duplex to lie too close together, but continuing to add more superhelical turns will eventually do so (C).

There will therefore be an equilibrium number of superhelical twists, which will come about as a result of a balance between the *benefit* of removing (or adding) superhelical twists to "neutralize" unwanted secondary twists, and the *drawback* of adding steric strain due to DNA backbone phosphates being forced too close together.

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To summarize, we now show DNA in three stages of winding, "normal" on the left, "underwound" in the middle, and "overwound" on the right. In each instance I've inserted a scale calibrated in units of 34 angstroms of length, that is, each numbered unit on the Y-axis corresponds to a distance of 34 angstroms, the length of a single turn of W-C DNA under "normal" conditions.

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Thus, on the left, the 10 units of length correspond to exactly 10 turns of DNA.

Slide 118

If this DNA was circularized, it would be in a relaxed, open-circular conformation, as depicted in the drawing below.

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In the middle, however, the DNA has been partly unwound, that is, it has become "underwound", so that there are LESS THAN 10 turns of DNA along the same 10 unit length; in this instance the number being between 9 and 10. This DNA is under strain, and, if circularized, it will attempt to return to its normal winding.

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In order to accomplish this, it takes on some right-handed superhelical turns, as depicted in the drawing below.

Slide 121

Conversely, on the right, the DNA has somehow been "overwound", and now has *more* than 10 helical turns per 10 length units. It "wants" to unwind, and in order to do so…

Slide 122

…it takes on some LEFT-handed superhelical turns, as in the drawing below. Hopefully, these drawings and photographs make these principles clear.

Slide 123

We're now ready to examine and understand the alkali denaturation curve of ϕx174 DNA. The data are taken mainly from the publication on the subject by Rush and Warner. We'll start with the traditional "helicist" explanation for these data.

Slide 124

At physiological pH's,

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… the chromosome has a middling sedimentation rate of about 21***s***, significantly higher than that of a relaxed chromosome, because, we are told, ϕx174 DNA is UNDERWOUND at the time of its creation,…

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…and therefore takes on RH'd tertiary supertwists to increase the right-handedness of the secondary helical winding, as depicted in the over-simplified drawing above the curve. The proposed underwinding in traditional theory is 5-7%, which, in a chromosome the size of ϕx174 — if it's presumed to have the Watson-Crick structure — would amount to about 550 secondary RH's W-C helical twists, and about 25-30 right-handed superhelical twists.

Slide 127

I've always found it odd that people are so ready to believe that chromosomes are routinely *underwound.* Exactly *why* should we believe this? There's really no logical reason for it. It's merely another assumption the "helicists" must make in order to force the data to accommodate to the Watson-Crick helical structure. The closest thing to a rational explanation offered by the "helicists" is that the 25-30 supertwists somehow facilitate packing of the DNA in the virion — a rather *arbitrary* proposal; one which hardly scratches the surface of the almost-incomprehensible problem of packing huge lengths of chromosomal DNA into the tiny spaces Nature provides for them. I have a much better explanation, but we're not up to that yet.

Slide 128

Back to our data, as the pH increases, the hydrogen bonds between the base pairs of duplex DNA are progressively weakened, and the DNA begins to unwind. In our traditional "helicist" explanation, this means that the superhelical turns, which were, after all, only there to compensate for the chromosome having been closed in the "underwound" state, will become progressively less necessary, since a denaturing, *i.e.* an *unwinding* duplex is perfectly "happy" to be underwound, and no longer needs superhelical twists to compensate.

Slide 129

At a pH of about 11.8, the superhelical twists have all been removed, and the chromosome appears as an open circle.

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Now, as the pH continues to increase, the chromosome would like to continue unwinding, but of course it can't, because it's a closed circle with the secondary windings locked in, so it now supertwists in the other direction, with each new LEFT-handed supertwist removing one of the now "unwanted" right-handed W-C twists. Each additional supertwist increases the compactness and density of the chromosome, resulting in a relentless increase in its ***s*** value.

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At the highest pH, around 13, the chromosome has become very dense, as reflected in the high sedimentation coefficient of nearly 50***s***. This form of DNA has been dubbed "Form IV". What is the structure of Form IV? No one knows, and, as far as I can tell, no one cares. In the 30+ years I've been studying this problem, I've yet to see a single paper on the subject. I asked Bill Strider for his opinion; Bill being the man who probably did more of the actual laboratory research on the denaturation and renaturation of small circular DNA than any other scientist living. Bill got an annoyed look on his face, and said "It's just a tangled mess!"

Slide 132

Here's an actual picture from an early publication on alkali denaturation of circular DNA, showing the author's conception of the "structure" of Form IV. To this

date, this remains the level of sophistication regarding this structure. It leaves much to be desired, does it not?

Slide 133
All of the behaviors we have discussed so far pertain to intact circular duplex DNA. What happens if a nick is introduced into a closed-circular chromosome?

Slide 134
To complete our study of the alkali titration curve for phi-x-174, we need to add the data for nicked DNA. The behavior of this form is indicated by the triangular data points and the blue lines.

Slide 135
At this juncture, I'd better introduce some terminology.

Slide 136
"Form I" is the name traditionally applied to covalently-closed-circular duplex DNA, such as is found in most viruses and plasmids. As we have already begun to see, the covalent closure, which locks in an unchangeable number of secondary helical twists, imparts striking topological properties to the molecule.

Slide 137
"Form II" is the form which appears if one of the two strands is nicked. Even a single break in the sugar-phosphate backbone allows the strands to rotate around one another, so that any deficiency or excess of secondary helical turns is immediately relieved. Therefore, in general, Form II appears as a relaxed, open circle.

Form II behaves like linear DNA, and has none of the striking topological properties we've seen for Form I.

Slide 138
"Form III" is the form which results from cleavage of BOTH strands, which linearizes the molecule. The terminology "Form III" is rarely seen, since linear DNA is usually just *called* "linear DNA". It is included here just for completeness.

Slide 139
"Form IV" is the extremely dense form which results from alkali denaturation of Form I. As we have already seen, its structure is totally unknown.

Slide 140
Although the data for Form II yield, for the most part, a straight line, there are some peculiarities in it. For now, we'll merely note that at pH's above 12, Form II splits into two components. The upper line represents single-stranded circular DNA,…

Slide 141
… and the lower line single-stranded linear DNA.

Slide 142

There are two additional things I would like to draw your attention to at this time. The first is that the sedimentation coefficient of Form II, between pH's 11.8-12, follows that of Form I. If you're paying attention, you'll notice that there is only one triangular data point in that pH range, and you might be tempted to conclude that I'm taking *inexcusable* liberties in the way I drew the line, but that is not the case. Alkali denaturation titration curves for other viral DNA's have emerged from the laboratories of Jerome Vinograd and other researchers, and ALL of them show a confluence between the Form I and Form II curves in this pH range. It's no mistake, and it's no "taking of liberties". It's real, and it needs to be explained.

This phenomenon, however, is NEVER discussed in papers on this subject. That's because it *can't be explained* by Watson-Crick helical theory. It just doesn't fit. But it can be readily explained in our non-helical theory, as I shall demonstrate shortly.

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The second thing I would like to point out is the shoulder on the Form I denaturation curve, labeled "kei", or, more accurately, "chi". This was eventually proven, in painstakingly detailed papers by Warner and his associates, to be the pH range within which Form I becomes reversibly denatured, with a breakdown of base-pairing. A few tenths of a pH unit higher, at pH 13, the denaturation becomes irreversible, giving rise to the upper Form IV curve, that is, the black circles at the top.

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Notice, by the way, that the upper Form IV curve ALSO has a shoulder in the same region. The vertical bar of gray shading encompasses a pH region within which it certainly seems that both Form I and Form IV are undergoing conformational changes. Interesting, no? What do you suppose it means? Once again, these shoulders are never discussed in published papers on these data. We, however, *shall*l be discussing them shortly.

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Let us now go through this alkali denaturation data, only this time without assuming that DNA has any net Watson-Crick twists. Can we explain the data with a topologically non-twisted model for DNA? I'll tell you the answer to that right now: We *can* explain it, and we can provide an explanation that is *better* than the one offered by the helicists.

I refer to all models of non-helical duplex circular DNA as "TN" models, where "TN" stands for "topologically non-linked", the state of the two strands. There are a surprisingly large number of models to choose from, so first, we are going to have to find a convenient way to visualize a TN chromosome.

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Here are two such ways. "A" is a model in which half the chromosome is right-handed and half left-handed. Such a model can be made from two rubber bands by merely twisting them together:

Slide 147

In addition to the benefits I've derived from working with an $80,000 molecular modeling software program, I've also learned a lot from the lowly rubber band. If you get two differently-colored rubber bands which are large enough, you can do some experiments.

Slide 148

Here are some experiments done with some *really* oversized rubber bands. Look please toward the left of the slide.

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In the top panel, labeled "A", our oversized rubber bands are twisted together. If you look closely at the resultant structure, you will see that it is RH'd on top, and LH'd on the bottom. Although my hands cover part of the structure, you can pretty much see that the number of RH'd twists on top is the same as the number of LH'd twists on the bottom, which, in this picture, appears to be 5 each (plus whatever lies beneath my fingers). This is no coincidence—when you twist rubber bands together in this way, every twisting motion of your hands automatically and simultaneously produces exactly as many RH'd as LH'd twists, and every *un*-twisting motion likewise removes an equal number of RH'd and LH'd twists.

The rubber bands remain, of course, topologically NON-linked throughout, and they only stay together because I'm holding them that way. If I was to simply drop them, which I can't show you without a movie, they would instantly unwind themselves and fall to the table as 2 separate rubber bands, which is, after all, what they are.

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If, however, instead of simply dropping them to the table, I release the tension in these rubber bands by slowly bringing my hands together, as shown in panel B, then something very interesting happens. Superhelices spontaneously form. Let's look more closely.

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The formation of these superhelical structures is in no way the result of the physical properties of the rubber from which the model is made. This phenomenon occurs whether one uses rubber bands, string, or sewing thread. The longer and thinner the material used; that is, the closer it is to the proportions of actual DNA; the more readily these superhelical structures form.

I would conclude that the formation of superhelical structures from circular DNA under strain is, in part at least, and maybe in total, a purely mechanical process; one which I wouldn't pretend to understand, but which can be clearly visualized in these models.

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Another thing which can be visualized in these models is a macroscopic demonstration of my so-called "third principle of DNA topology", which stated, in part, that "tertiary, *i.e., superhelical* turns, *decrease* the tightness of the winding of a helix

whose twist is in the opposite direction". If you look at this photo, you'll see the rule in action. As the right-handed secondary helical turns on top of the structure unwind…

Slide 153

…they turn into *left-handed* tertiary superhelical turns…

Slide 154

…whereas the left-handed helical turns on the bottom…

Slide 155

…unwind into *right-handed* superhelical turns.

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One final observation on these rubber-band models is that, whereas the twisted rubber bands in panel "A" are unstable, and will fall apart as soon as the experimenter lets go of them, the supertwisted structures in "B" are, for some reason, quite stable, and do not unwind when one lets go.

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This is illustrated in panel "C", which shows that the fully-superhelical structures no longer need to be held by the experimenter — they are completely stable.

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The panels on the right of the slide show that all these phenomena occur with smaller rubber bands in exactly the same way. I can't explain this, so I'll just post this picture for the benefit of those among you who know more about mechanical engineering than I do.

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Although the "A" model provides a convenient way to visualize and study the 50% RH/50% LH'd topological nature of TN DNA, it's obviously not a very likely candidate for DNA structure in real life. "B", however, is a much more realistic model, where short right-handed and left-handed segments regularly alternate, each segment being less than a single full twist in length.

This sort of structure was first proposed by the New Zealander Gordon Rodley in 1976, and, in a turn of events reminiscent of Alexander Graham Bell getting to the patent office first by 10 minutes, only a month later, the prominent Indian biochemist V. Sasisekharan published essentially the same structure.

These publications caused a small uproar in the DNA community, which Crick responded to in a manner we shall consider shortly.

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Rodley called his structure the "Side-By-Side" model. Everyone who has ever taken an interest in DNA topology has heard of it. But not too many people have seen it. Well, here it is, in 2 dimensions. Let's look around.

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Here it is in 3D. This model was made from Rodley's published coordinates. He never lived to see the sort of virtual modeling software we have these days, and I'm sure he would have gotten a big kick out of seeing this.

Crick thought that the "Side-By-Side" model was uglier than his double-helix, which I suppose is an aesthetic judgment call. What is true is that the model is entirely hypothetical, not based upon scientific observations, but constructed entirely by modifications of the Watson-Crick structure.

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Now here's another TN structure which most people do *not* know about. It's the work of my friend and colleague, Clive Delmonte, and it's called the "paranaemic model", to distinguish it from the "plectonemic", or coiled model of Watson and Crick. The paranaemic structure strongly resembles the Rodley structure, although it arises from an entirely different concept. Here the sugar-phosphate backbones are RH'd helices, but they never cross one another. The structure as a whole therefore has no full twists, undulating instead in a Rodley-like fashion, as I have endeavored to show in this drawing. Clive has a preliminary virtual model, but it's not ready for showing. You can see a photo of his physical model on my "Not A Helix.com" web site.

The paranaemic model is in no way an arbitrary exercise in alternative DNA structure, but rather an attempt to create an asymmetric model of DNA which is consistent with the results of 1953 measurements of DNA dimensions, taken in molecular monolayers in Langmuir troughs, by James and Mazia.

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Any one of these models would allow DNA to replicate without a swivel, and without the need to posit a rotational speed of 20,000 rpm at the replicative site.

I actually believe that DNA, in vivo, has a different structure entirely, namely the structure proposed by an ingenious fellow named Tai Te Wu. The Wu structure is taken up in Part 2 of this series, "The Probable Structure of the Protamine-DNA Complex".

For the remainder of the present discussion, I shall not assume anything in particular about the structure of circular DNA other than that the strands are topologically non-linked (TN).

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NOW WE COME TO THE CRUX OF THE MATTER. Let us assume, for the sake of argument, that circular duplex DNA has the TN structure. ***What can we predict about the topology of the chromosome, specifically with respect to tertiary superhelical winding?*** Would it be a relaxed circle? Or would it be a superhelix? Or is it just *impossible* to make any prediction at all?

What you're going to see is that the helicists, lead by Francis Crick, automatically and mindlessly do make assumptions about this, and their assumption is that if TN DNA existed, it would be a relaxed circle. Is this reasonable? Have they thought the matter through?

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To put this in perspective, let's do a review. On the top is what has now become the so-called "classical" view, namely that DNA is a RH'd double helix.

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We know that when isolated in nature, circular DNA is, in fact, a superhelix. Is this what you'd expect for a circularized Watson-Crick chromosome? Of course not. There's no earthly reason to pre-suppose that merely circularizing a W-C length of DNA would give rise to anything other than a an open circle. So you've got to add the *additional* arbitrary and irrational assumption that the DNA is *underwound,* as previous discussed. This is portrayed here by the thumbtacks, which indicate that circular DNA, as isolated in nature, will not lie in an open circular conformation unless restrained.

What about TN DNA? Suppose that you found a way to close a length of DNA into a circle WITHOUT any net helical twists. What would its structure be?

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Would it be an open circle, as portrayed on the left?

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Or would it be a superhelix, as portrayed on the right? You may think that this question requires years of experimentation, and reams of data to answer, or, in the absence of such, a near-Einsteinian intuition and intellect. Well, you don't need any of that.

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But you do need 3 things:

1. You need an IQ above 100. I'm sure everyone watching this presentation is at least as intelligent, and probably a lot *more* intelligent than me, so that shouldn't be a problem at all.
2. You need sufficient humility to admit that it's at least *possible* that there's something basic about DNA that you don't know, but—MOST IMPORTANT OF ALL,
3. You need to be sufficiently free of financial attachments to publicly-traded biotech companies to maintain the capacity for independent thought, as dramatized by the following VERY SCIENTIFIC GRAPH, WHICH SHOWS WHAT HAPPENS WHEN SCIENTISTS GET TOO RICH, AND TOO SUCCESSFUL:

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I've lived long enough to see science go from something resembling a temple of truth, albeit with many flaws, to a community of openly greedy, grasping entrepreneurs, for most of whom the truth is nothing but an inconvenience, standing in the way of more money. So I'm not 100% sure, at this point, that there's enough love of the truth remaining in organized science for anything which doesn't come with a financial reward attached to it. I guess we'll just have to wait and see….

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Well, the answer to the above question is in, and it's clear. And the answer is that if DNA had any of the TN structures, it would have to be isolated as a right-handed superhelix. Why?

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In order to say "why", we must decide how we're going to visualize TN DNA. For the purpose of answering this particular question, it's most convenient to visualize it as model "A", the half-right, half-left structure.

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Model "B" is topologically the same, only here the distribution of RH and LH turns is different. There is no reason to presume, however, that the energetics, wrt 3° winding, will be different simply because the RH and LH portions are not segregated into separate regions.

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Model "A", topologically speaking, is 50% left-handed, which *cannot change* unless a swivel is created by the rupturing of at least one covalent bond in the sugar-phosphate backbone.

What do we know about left-handed DNA?

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We know that the LH helix, or the "Z" form of DNA, does *not* normally occur in natural linear DNA of average base sequence, but only in certain synthetic copolymers. Therefore, there is no reason to doubt that DNA, under physiological conditions, will assume the Watson-Crick RH helical structure whenever possible. Here, however, it is NOT possible because the LH portion is locked in at the time of creation. **We must therefore conclude that a chromosome constrained to be 50% LH , in the absence of the nuclear proteins necessary to support this structure, will be topologically unstable, and will do whatever it can to convert to a 100% RH helix.**

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And what can it do? The left-handed secondary turns are locked in, and cannot be removed. Only one option remains:

It can take on RH *tertiary superhelical* turns, each of which unwinds one of the unwanted LH *secondary helical turns.*

In the one-thousand-fold over-simplified drawing shown here, the bottom of the chromosome has exactly 4 LH helical turns. If such a simple structure actually existed, then the mere introduction of 4 RH superhelical turns would totally untwist the bottom into a pair of parallel lines, and 4 more would twist it in the opposite direction, yielding a secondary structure which was an all-RH Watson-Crick helix! (I have not attempted to draw a picture of this).

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In practice, it will be impossible for the molecule to remove *all* its LH turns, because of the problem we discussed earlier, namely that of steric hindrance, which will

increase as the number of superhelical turns increases. Picture "C" depicts, schematically, the equilibrium state, beyond which there can be no further supertwisting.

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The point is, that if TN DNA existed, it would have to be a RH superhelix, and the extent of right-handed superhelicity would have to the maximum extent possible, established by an equilibrium between the push of the LH secondary helical winding as it tries to unwind, but balanced by the resistance of the structure to very high degrees of *super*helical twisting.

Slide 179-190 (NO AUDIO)

Slide 191

Complete Explanation of the Alkali Denaturation Titration Curve in terms of the TN (topologically non-linked) Model

Slide 192

Let us look once again at our alkali denaturation data, with all the labels, and with all the pictures of the various forms.

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We've added a tightly-wound superhelix to the picture portion of the data, now highlighted in purple, and we shall be explaining it shortly.

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Otherwise, the data is exactly the same data we employed previously, from the laboratories of Rush and Warner. Let us now see if we can explain these data without making the assumption that DNA has any net helical twist.

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As previously, we note that Form I sediments more rapidly than nicked Form II, indicating that Form I is superhelical, illustrated in the small drawing above the curve. As we pointed out before, "Traditional helicists" explain this by alleging that DNA, for some strange reason, must be created in an underwound state. TN theory, however, requires no strange explanation, simply noting that the chromosome is 50% RH and 50% LH, topologically speaking, and that some of the "unwanted" Z-type LH secondary helical turns can – *and will* – be converted into the more-desirable Watson-Crick RH helical turns, by the formation of RH *superhelical* tertiary twists.

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As explained previously, this process continues until steric hindrance brings it to a halt.

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Thus, we have accounted for the first portion of the curve without the need for presuming any topological linkage between the strands.

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Next we turn to the pH region of 11.6-12, where the *s* value dips to that of relaxed DNA, indicating that the superhelical turns are removed. Why?

It's really quite simple. All observers, both traditionalists and anti-helicists, agree that in this pH range, RH DNA becomes less favorable, and, in fact, begins to unwind. I don't imagine anyone will be terribly surprised to hear me say that. But now I'm going to show you something you may not have thought of: LH DNA, in this pH range, starts to become *more* favorable. I'll show you why momentarily. For now, I'll just say that as the pH increases, RH DNA becomes less favorable, and LH DNA becomes *more* favorable, until at pH 11.8, the stability of the two forms become equal, and all superhelical twists vanish.

Slide 199 (The R→L Transition)

Why do I say that at high pH LH DNA becomes more favorable?

Although the subject is rarely discussed, it's been known for many decades that the addition of denaturants to linear duplex DNA sometimes causes the direction of helical winding to reverse, that is, the RH helical winding converts to LH helical winding. I refer to this as an "R→L conversion".

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Although there is a rather large literature on this subject now, in 1974, when I began studying it, there were only a few articles.

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The earliest I found was by Travers *et al* (1970), who found an inversion of the ORD (or Optical Rotatory Dispersion) spectrum of DNA in methanol at low temperature. They interpreted this as indicating a reversal of helical winding direction, *i.e.,* an R→L conversion.

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The same thing was deduced from the inversion of the CD, or Circular Dicroism spectra of DNA in high salt,

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…and in the presence of the intercalating agent Mitomycin C.

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Here's some of the data from these authors. The blue arrows show the ORD/CD spectra of the native DNA's, and the orange arrows show the spectral inversions.

The significance of these spectral inversions, wrt the proposed R→L conversion, was, in those early days questioned by some, but, in the process of time,

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…subsequent publications provided further evidence that DNA under these conditions was indeed LH'd, culminating in Alexander Rich's x-ray crystallography studies which elucidated the so-called "Z-DNA" left-handed form.

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What do alcohol, salt and Mitomycin C have in common? Chemically, nothing. But each causes unwinding of DNA. It would appear, then, that *anything* which unwinds DNA might produce an R→L conversion. Can such a thing be explained?

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Again, the explanation is really very simple. It lies in the structure of Z-DNA. Let's look at it:

Look how pretty it is from the top!

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Now let's look at it in 3-D. By the way, this is one movie in which it matters which way your eye perceives the molecule to be spinning. You should see it spinning to the right, and the direction of helical winding will correctly appear to be left-handed. If, however, your eye is playing that perspective trick on you, then it will appear to be spinning to the left, and the direction of helical winding will seem to be right-handed, which is *backwards*. If this is what you see, just keep looking at it, and eventually it will change direction.

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Here's an accurate drawing of 12 base pairs each of "classic" RH'd B-DNA, alongside LH'd Z-DNA. Please note that Z-DNA is significantly LONGER. In other words, relative to the RH double helix, it's LESS TIGHTLY WOUND. This is critical to our understanding of the behavior of circular DNA, so let's look at the actual numbers:

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	Rise per residue	Residues per helical turn	Pitch
Z-DNA	3.7 Å	12	45 Å
B-DNA	3.4 Å	10	34 Å

The rise-per-residue for Z-DNA is 3.7 Å, whereas for classic B-DNA the rise-per-residue is only 3.4 Å. Z-DNA has more residues per helical turn, and a much larger pitch.

At the bottom of the slide, I've shown a comparison of the lengths of the two forms for a segment of 12 base pairs, which reveals that Z-DNA is 4 Å, or about 10% *longer.*

Looking at these numbers, we can see *immediately* why all those different agents cause an R→L conversion. Z-DNA is a *more loosely-wound* helix than RH'd DNA. The base pairs are stacked 10% farther apart, and the pitch is 45 Å, which is 34% longer than the pitch of RH'd DNA.

No one has ever seen a Watson-Crick ***RH***’d structure with such wide spacings, which presumably doesn’t exist. Therefore, we may be well-justified in assuming that anything which causes a 10% unwinding of DNA will precipitate an R→L conversion, since only the LH’d form is known under such conditions.

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Here's an animated drawing which dramatizes this concept. Note the length scale we used earlier, where each unit of length = 34 Å, the length of one W-C turn. Against this scale we can see immediately that the Z form of DNA is clearly stretched out, *i.e., more loosely wound.* One picture tells a thousand words. When B-DNA unwinds to the point of having the same length as Z-DNA…it BECOMES Z-DNA, because at that pitch, RH'd-DNA DOES NOT EXIST! That is, when the DNA has unwound to the point that the base pairs are 3.7 Å apart, where the only known structure is the LH'd Z-structure, the DNA will undergo an R→L conversion.

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Now we can return to the alkali denaturation titration curve. Backtracking for a moment, I will reiterate that at physiological pH, the native chromosome is more dense than nicked circular or linear DNA, because it takes on right-handed supertwists in order to unwind some of the unwanted left handed secondary twists.

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As the pH approaches 11.8, the relative stability of the right- and left-handed forms becomes equal, and all the supertwists come out. At this point, the *s* values for *all* forms of circular DNA, both intact, nicked, and even single-stranded circular DNA, are essentially the same, because they all sediment in relaxed form, as open circles.

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As the pH increases *beyond* 11.8, the RH'd twists start to unwind in earnest. At this point, the LH'd form actually becomes MORE stable than RH DNA, and we start to see supertwists in the opposite sense, namely LEFT-handed supertwists, each of which unwinds the now-undesirable RH helical twists. Note in the drawing above the curve that the direction of superhelical winding has now reversed.

The behavior of DNA in this pH range is non-controversial, and is explained in the same way by both traditional “helicists” and us trouble-making “anti-helicists”.

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This process of LH’d superhelical twisting continues up to the point marked "χ", around pH 12.2, at which pH it has been reported, by several authors, that the DNA finally denatures, with the loss of all hydrogen bonding.

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Let us pause for the moment, and look at nicked DNA, *i.e.,* Form II. This is the blue data at the bottom of the graph. We are now in a position to address one the rarely-discussed aspects of these data, namely that the Form II curve coincides with the Form I curve from pH 11.8 up to pH 12.

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At this latter pH, Form II (but not Form I) splits into its components, namely single-stranded circular DNA and single-stranded linear DNA. What's the meaning of this upward sweep of Form II, following Form I, when Form II is supposed to be free of topological constraints, being nicked? How can this be explained?

"Standard" Watson-Crick theory offers no explanation for this, and it is never discussed. We can explain it here, however. In TN theory, we note that as the pH rises above 11.8, the Form II chromosome begins to unwind. Remember that Form II is entirely RH'd, since the nick acts as a "swivel", allowing the chromosome to assume the most stable conformation, which is the standard W-C structure. As the pH increases, however, and the longitudinal base-pair spacing increases from 3.4 Å to 3.7 Å, the chromosome takes on more and more left-handed superhelical twists for the same reason Form I does so in the same pH range, namely because left handed supertwists convert the RH secondary twists, which have now become undesirable, into LH secondary twists.

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What happens at pH 12? Because no one in this field seems to care about this portion of the curve, we therefore lack data to answer the question with certainty. That doesn't mean, however, that we can't take a good educated guess.

It may be simply that at pH 12, base-pairing is so weakened that the duplex cannot survive, being held together, as it is normally, by hydrogen bonds. I would strongly opine, however, that it is more than that. The fact that Form I survives up to pH of about 12.3 indicates that base-pairing, *i.e.* hydrogen bonding, still exists in the pH range 12.0-12.3.

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Look at the purple arrows here. They show the range within which Form II no longer exists, but Form I persists. Clearly, there are still at least weak H-bonds in this pH range, or Form I would also denature.

With respect to Form II nicked DNA, I suspect that at pH 12, the beginning of this pH range, the advanced degree of unwinding causes the base-pairs to reach the critical 3.7 Å spacing, which is incompatible with the Watson-Crick structure. This gives rise to a sudden, cooperative R→L conversion. The chromosome, as a duplex structure, does not survive this maneuver, because base-pairing at pH 12 is so weak that the centrifugal force of the spinning chromosome drives the strands apart.

Slide 220

Here's an admittedly silly effort to portray this in a movie. I'm the so-called 'artist' for most of these slides, and I don't have the graphic ability to portray the duplex spinning, so in lieu of that I settled for just making it shake around. Keep in mind, however, that denaturing DNA *spins*, and that the force I'm trying to portray is centrifugal force:

Slide 221

All right. We've seen that the strands of Form II split apart into single stranded linear and circular forms at pH 12. If, as I've asserted, the strands of Form I are Topologically Non-Linked (TN), why don't they *also* split apart at pH 12?

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There are at least 2 reasons for this, one fairly obvious, and the other somewhat subtle.

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The obvious one is that Form I has *no free ends.* Denaturation of linear or nicked circular DNA is considered to be a cooperative process, which starts slowly with the separation of a critical number of base pairs at a free end, thereafter proceeding rapidly, with the entire duplex structure opening up like a zipper.

Form I has *no free end* to be the site of the initial strand separation event, and is therefore protected, to some extent, from denaturation. Can we estimate the magnitude of this protection?

Slide 224

The top of this slide depicts a DNA duplex with a single nick, and therefore two free ends. In order to initiate denaturation, a disruptive force must be applied, depicted here in caricature as a 100 lb weight which must be lifted up. This force must be applied to either one of the two free ends. Keep in mind that a "nucleus" of disrupted base pairs sufficient to initiate denaturation only need be obtained at one of the two free ends, since denaturation, once initiated, will spread rapidly along the entire length of the DNA.

Now look at the bottom of the slide. To initiate denaturation in the absence of a free end, a force of twice the magnitude must be applied, as the diagram should explain without the need for a thousand words. If the denaturing "force" is high pH, *i.e.,* hydroxide ions, then we may, as a first approximation at least, consider this to correspond to a doubling of the hydroxide ion concentration, or, on the logarithmic pH scale, an increase of 0.3 pH units.

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Looking back at our alkali denaturation curve, we see that the denaturation pH of Form I, known to be about 12.3, is in fact approximately 0.3 units *higher* than that of nicked Form II. Coincidence? I guess you'll have to decide for yourself.

As I said earlier, there is another, more subtle way to approach this same question. Since Form I is a base-paired structure, and since it survives up to pH 12.3, it follows that there is some hydrogen bonding, albeit weak, in the pH range of 12-12.3. It is therefore possible, in principle, for nicked Form II to also survive as a duplex up to pH 12.3, but it doesn't. This is why we have proposed that an R→L conversion at pH 12 provides sufficient *extra* disruptive energy to cause strand separation at the lower pH. The subtle question is, "Why don't the strands of Form I also undergo a rapid change in the direction of helical winding at pH 12, and separate at that lower pH as the strands of Form II do?"

Slide 226

The answer lies in topology. Let us look again at this not-very-likely, but very educational version of a TN chromosome. If you examine it and think about it, or, even better, simply make a model by twisting together 2 rubber bands, you will see immediately that when DNA has the TN structure, every addition of a RH'd turn *must* be accompanied by addition of a LH'd turn, and every removal of a RH'd turn *must* be accompanied by removal of a LH'D turn. Speaking anthropomorphically, at pH 12, even if Form I "wanted" to convert to the all-LH'd form, it couldn't, because every RH'd turn unwound would have to be accompanied by the unwinding of a LH'd turn. But in the pH range 12-12.3, LH'd DNA is not only possible, it is *favored,* and therefore the 50% of the chromosome which is topologically LH'd, desiring to increase, not decrease, will *oppose* any removal of helical turns at all.

Slide 227

In fact, if we look at the denaturation curve for Form I, now highlighted in purple, we can see that the only way Form I can remove RH'd twists without also removing LH'd twists is to become increasing superhelical in the LH'd sense. Note the tremendous increase in ***s*** which accompanies this, the density of the chromosome quite evidently increasing by 100% in the pH range of 12.3-13.

Slide 228

Now look at the pictures I've superimposed on the data. All writers on this subject agree with the first 3 pictures.

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But what about the 4th? What is the structure of DNA above pH 12.3, and why don't the strands separate?

Concerning the exact structure of denatured Form I, known as "Form IV", no one seems to care. I've never seen a word written on the subject. As for the question of why the strands don't separate, traditional "helicists" have their very strongest argument here; one which has, in all probability, been the backbone of ALL resistance to TN theory for over 30 years: They simply fall back on the obvious fact that in a W-C duplex which is covalently closed into a circle, the strands are topologically locked together. Why *should* they separate?

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Why indeed. But wait a minute – we still don't know the structure of Form IV. Is it possible that the failure of the strands to separate at high pH is due to some unforseen structural property of Form IV, having nothing to do with topological linkage?

Anyone who counts himself a champion of the W-C structure will say that my back is up against the wall in this portion of the curve, and he would be right. But guess what: I went through the seemingly hopeless task of considering the question, and not only is there an answer, but it's a darned good one. So good, in fact, that it's hard for me to believe that it isn't true.

Slide 231

STRUCTURE OF FORM IV

Slide 232

I may be the only scientist in the world who gives a damn about the structure of Form IV. Remember this slide, which I showed before? This is the establishment view of Form IV structure, described by Bill Strider, one of the world's leading experts on it, as "a tangled mess". That's almost like saying it has no structure at all! But its properties hardly support this view.

Slide 233

Look again at the curve for renaturation of Form IV, highlighted in purple. It forms at high pH, but when the pH is restored to normal, the original properties are NOT restored – with a sedm coeff of about 36s, it remains 50% *more* dense than the native Form I, which has a sedm coeff of only 21s. This behavior contrasts sharply with that of calf thymus or other linear DNA, which can be denatured by boiling, and re-annealed by slowly cooling. Intact circular chromosomes cannot be heat-denatured at all, and when alkali-denatured, as shown here, they do NOT re-anneal when the pH is normalized.

When this was first discovered in the 1960's, Form IV was dubbed "permanently denatured" DNA. Robert Warner, a highly-respected scientist who chaired the Biochemistry Department at UC Irvine for many years, got interested in Form IV as a vehicle for purifying viral DNA. Because of its extreme density, he reasoned that it would be a cinch to separate it from other proteins and nucleic acids. This, however, required that there be a way to convert the Form IV, once purified, back to native Form I. Most of the laboratory work on this project was done by the above-mentioned Bill Strider, who was a doctoral candidate at the time. Subsequently, Warner continued to refine the work, with an almost maddening precision, for another decade, but Strider's results continue to define the problem sufficiently for our purposes:

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Follow with me, if you will, some of these data. Form IV was prepared by alkali denaturation of the Replicative Form, or "RF", of the chromosome of the virus ϕx174. The DNA, in buffered 1M NaCl, was subjected to many different combinations of pH and temperature, as shown. The percent renaturation, *i.e.* back to native Form I, was measured. Note that at any temperature of incubation, there was only a single narrow range of pH within which renaturation was rapid and complete.

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For example, please look at the first curve, labeled 70°, 2 minutes.

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What these data mean is that at a pH of about 10.7, 100% renaturation occurred in only 2 minutes.

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But lowering the pH a mere 0.5 units, to 10.2, almost totally wiped out ALL renaturation. I think you can mentally extrapolate these data to the physiological pH range of about 7, and it's easy to see that at physiological pH, there would be NO measurable renaturation under these conditions. This is true for all six curves shown.

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And, if we lower the temperature to room temperature (*i.e.,* 25°), it was difficult to get any reannealing at ANY pH. Even at the elevated pH of 12.0, it took an hour to get less than 50% of the Form IV to re-anneal back to Form I at room temperature.

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At each temperature, there was only a single, narrow band of pH within which renaturation occurred rapidly and efficiently. Moving to the right in the slide, you can see that decreasing the temperature raised the pH optimum, but the fastidious requirement for exact adjustment of pH, temperature and ionic strength is evident under all circumstances.

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Does this strike you as the behavior expected for a random coil of structureless collapsed DNA; a "tangled mess" as it has been called, or, on the contrary, is this a sign of a very highly ordered structure? I would strongly suspect the latter.

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But what structure? Let's look back to the alkali titration curve, with the alkaline end of the Form I curve highlighted in purple, and see if there are any clues. Although there is a shoulder in the Form I curve at χ, the general shape of the curve might be construed to suggest that there is no enormous conformational change between pH 12.3 and 13. Other than the shoulder at χ, the curve just proceeds upward, in an uncomplicated, orderly, and nearly-linear fashion.

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It is not controverted by any writer on the subject that circular DNA becomes a left-handed *super*helix above pH 11.8, represented here by the highlighted picture behind the curve. All agree, as well, that the explanation is the unwinding of RH'd secondary helical turns, which forces the chromosome to assume the left-handed superhelical conformation.

Slide 243 (Highlights for 12.3 forms, with native highlight “flying” in at ~12 sec)

It is also not controverted that by pH 12.3 (that point labeled χ), the superhelical twisting has progressed to the point that the superhelix density is the same as that of the native chromosome, only with superhelical twisting now in the opposite direction.

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I would propose that as the pH increases past 12.3, the superhelical twisting simply continues to increase, as per the highlighted drawing, with water being

progressively squeezed out of the core, culminating in a highly-supertwisted, relatively anhydrous structure. Why do I say this?

We need to ask what sorts of structures are possible for the chromosome at these high pH's. Well, how *can* 2 strands of DNA interact? The possibilities are not unlimited.

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First of all, we must take into consideration that at high pH, the structure becomes, in effect, a FOUR-stranded structure. Look what happens as LH'd supertwisting progresses. Two duplex strands from each side of the formerly-circular chromosome get twisted closer and closer together, until 4 strands lie much closer together than ever occurs at neutral pH. What structures are possible here? Structures built around base-pairing can be excluded, because at highly-alkaline pH, the atoms involved in base-pairing will all be protonated. Base-stacking remains possible, but I know of no precedent for a 4-stranded structure at high pH involving what would have to be a virtually random pattern of stacked bases.

But there is a well-characterized structure which could pertain in this case, and I am going to propose that it appears at high pH, because I can't think of any reasonable alternative. It is the structure originally proposed by Linus Pauling about a month before Watson & Crick published their double-helical structure in 1953.

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Prior to Watson & Crick's 1953 publication, no one knew how the structure of DNA was going to reflect DNA's role as the bearer of genetic character for all life. Specifically, the structural counterpart, and genetic significance, of the molar equivalence between adenine and thymine, and between guanine and cytosine, was NOT known. Lacking this information, Pauling could only propose a structure which was consistent with the rules of chemistry, concerning which he was the acknowledged world master.

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Based upon the principles of chemistry, Pauling put the phosphate groups on the inside……and the bases on the outside.

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As the vast number of phosphate crystalline compounds in the world demonstrates, phosphate groups *like* to line up, bound together by salt bridges. If you're old enough to remember, you undoubtedly will not have forgotten Pauling's world-famous blunder, immortalized by Watson in his popular book "The Double Helix", of putting hydrogen-bonds in the inside instead of salt bridges. Of course Pauling really *meant* salt bridges; it was the scientific equivalent of a "slip of the tongue", but upon publication of the Watson-Crick structure in Nature, quite evidently embarrassed by the whole affair, he quit all work on DNA forever. That's too bad, because I think he was right. He *didn't* deduce the structure of DNA in the normal state, but it seems to me that he inadvertently discovered the most probable structure for DNA in the denatured state.

Although Pauling gave the world a 3-stranded structure, as illustrated in his drawings here, he stated in his writings that a 4-stranded structure was even easier to assemble. He chose the three-stranded model only because he was better able to make it

fit Wilkins' x-ray data. In other words, the Pauling structure for DNA, long-forgotten, is in fact the perfect structure for Form IV circular DNA, where the denaturation process brings 4 strands of DNA into unusually close approximation because of extreme supertwisting, under conditions of high pH where base-pairing is ruled out.

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I should note, for the benefit of those not familiar with the variety of structures found in viruses, that examples are known of viruses whose DNA consists of a helix with the phosphate groups on the inside. Loren Day, of the NYC Department of Public Health Labs, has discovered two such viruses, and provided a detailed molecular structure for one of them.

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Here's some pictures of what a small section of a Pauling Form IV quadruple helix might look like. This model was made by simply taking 4 DNA strands and spacing them to allow for 3Å salt bridges.

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And here's a movie of the same structure. It has a nice, orderly appearance, which is exactly what you'd expect for a structure which displays the fastidious behavior known for Form IV.

I would propose this as the structure for Form IV even if I believed that native circular DNA was a helix, which I don't believe. But it would appear to me that the time has come to stop pretending that Form IV is a "tangled mess", and to face the fact that it HAS to have SOME structure of some sort. If not this, then what? What other structure is possible? I can't think of any.

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This completes our review of the alkaline titration curve for Form I native circular DNA. We have shown that these data can be very satisfactorily accounted for by the TN theory, which states that the strands of circular DNA have no topological linkage. But, even though the TN theory makes sense, and is consistent with what we know about circular DNA, that doesn't make it true. What evidence is there that this structure exists in the real world?

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EXPERIMENTAL EVIDENCE

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Over the *decades* I have been repeatedly criticized for failing to take note of a veritable "mountain of evidence" proving the Watson-Crick structure.

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("Mountain of Evidence" movie)

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Oddly enough, however, when I demanded to be shown this “mountain”, the critics either become mute, or made reference to only a tiny group of manuscripts; four to be exact. So the mountain….

Slide 257 (“Mountain-to-molehill” movie)

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…appears to be a molehill.

We shall look at these papers shortly. But first, I must tell you which types of papers do NOT provide direct evidence for the Watson-Crick structure. There are innumerable papers which deal with DNA topology, and people naively presume that these somehow “prove” that DNA is a helix. The only reason they assume this is because they’re either too disinterested, or just too darned lazy to think the problem through. We shall not have that luxury here.

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These papers are exemplified by the work of JC Wang at Harvard, who scrupulously investigated the effects of various agents and conditions on DNA superhelicity. This means counting changes in numbers of superhelical twists, and relating the changes to the oft-referenced topology formula $L_k = T + W$.

This equation is no more than simple grade-school arithmetic. L_k is the so-called “linking number”, the number of times one strand crosses the other when the chromosome is forced to lie flat, in a plane. In the picture on the left, we’ve forced the chromosome to lie flat by employing imaginary thumbtacks. As long as the chromosome remains intact, L_k never changes. It’s a topological parameter which is permanently locked in at the moment of the chromosome’s creation, and can only be altered by breaking open a strand, changing the winding, and then re-sealing it.

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When we remove the thumbtacks, however, the winding changes, and we get the picture on the right. According to traditional Watson-Crick theory, what's happening here is that the chromosome has become supertwisted, to compensate for the alleged underwinding in the secondary structure. In the end, we have a new winding number, “T”, which stands for “twist”. Regardless of how underwound, or, for that matter, even *overwound*, the native chromosome may be, under physiological conditions “T” will generally be assumed to always be the standard Watson-Crick number, or exactly 10 base-pairs per full helical twist, with a pitch of 34 Å. This is the structure DNA prefers, and if it’s not present in the native chromosome, the chromosome will superhelically twist to bring it about. These supertwists are enumerated by the letter "W", which stands for "writhe".

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The relationship between Lk, T and W is precisely the same relationship we obsessed about with our rope models earlier. It's a very simple arithmetic relationship, as per the equation

$$L_k = T + W$$

In order to make this simple arithmetic formula work, the sense or direction of superhelical winding must be defined such that RH'd supertwists are "-" and LH'd supertwists are "+".

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Let's consider the Topology Equation for a typical small virus or plasmid chromosome, such as ϕx174 RF, which has some 5000 base pairs. If a segment of DNA of this length is linear, it will have the standard W-C helicity, *i.e.*, 10 base pairs per helical turn, or a total of 500 secondary helical twists. If, however, as traditionalists insist, the circular ϕx174 RF chromosome is created in the "underwound state", then L_k will be LESS than 500. How much less? Well, the number of supertwists can be estimated from experiments, including direct electron microscopic observations, and it's found to be about 25. Their direction is also known: Since intercalating agents DECREASE the absolute value of the number of supertwists, it can readily be deduced that the supertwists are RH'd, or negative in value. As I hopefully showed you with the rope models, the deficiency in the number of secondary helical twists must be exactly the same as the number of supertwists, so that the mathematics is totally simple:

$$L_k = T + W$$
$$= 500 - 25, \text{ so}$$
$$L_k = 475$$

We thus learn, from the Topological Equation, that the native chromosome is underwound by 25 W-C twists, or 5% underwound.

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All this assumes that the chromosome has the Watson-Crick structure. What if it actually *is* in the TN, Topologically-Nonlinked conformation, as I and a goodly number of others believe? What becomes of the Topology Equation?

Numerous studies, by researchers such as Wang, measure changes in superhelicity in the presence of various physical and chemical agents. These sorts of studies form a very substantial part of the so-called "Mountain of Evidence" in support of the Watson-Crick theory. What if DNA turns out to *not have* the Watson-Crick structure? Do these studies have to be discarded?

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The answer is, no data has to be discarded, and very little has to be done to the topology equation. If DNA has no net helical twists, being topologically 50% RH'd and 50% LH'd, then L_k actually has a value of *zero*. For a chromosome the size of ϕX174, with 5000 base pairs, since we previously presumed L_k to have a value of 475, we need only subtract 475 from both sides of the equation to correct the error, giving us the admittedly somewhat silly-looking new topological equation shown in the slide:

```
Equation:        Lk =   T  +  W
Old values:     475 = 500 - 25
Subtract 475:     0 =  25 - 25
```

Note that after subtracting 475, we wind up with the somewhat-silly-looking equation "0=25-25". Obviously, in this case, the equation no longer serves much purpose, but it's still true. What it's now saying, is that since the flattened chromosome on the left is exactly 50% RH'd and 50% LH'd, with Lk therefore having a net value of zero, the parameter T, in the drawing on the right, now represents any excess of either RH'd or LH'd twists above 50%, and must be exactly and precisely equal to the number of supertwists, W. In more complex language, we may say that every RH'd, or negative supertwist adds, in one-to-one fashion, a single RH'd twist to the secondary winding. Conversely, every LH'd, or positive supertwist *removes* a RH'd twist from the secondary winding.

All this is merely a long, drawn-out way of pointing out that the data of Wang, and numerous others like him, do not need – if I may be permitted a medical analogy – emergency surgery if DNA turns out to not have the W-C structure; they merely need a slight chiropractic adjustment wrt interpretation.

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What about papers which DIRECTLY investigate the Lk of the native chromosome? As I've already said, there are really only four such papers which have ever been quoted — far more of a "molehill" than a mountain.

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The first pair of papers, by Sebring *et al* and Jaenisch *et al,* reach the conclusion that DNA is a helix, through observations of the electron micrographic appearance of replicative intermediates of small circular chromosomes. This drawing, which is artistically dreadful, but topologically accurate, illustrates their observations. The parental loop of un-replicated DNA is observed to be superhelical, and the pair of daughter loops are relaxed, or "open circles". This, we are told, proves that DNA is a helix.

Huh? Where's the logic here? The fact that the daughter loops are open circles is consistent with the fact that they have not yet been sealed shut, but what does the superhelicity of the parental structure prove?

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The logic is obvious bizarre, and we've already gone over it previously. It goes something like this:

"DNA MUST have the Watson-Crick structure, because no other structure is possible".

"Circular DNA with the W-C structure is invariably superhelical"

"Therefore anything which is superhelical must have the W-C structure".

Am I wrong? I don't think so. They're saying that since DNA MUST have the Watson-Crick structure, it therefore has the Watson-Crick structure!

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This is just “Planet of the Apes” again! I’ve gone to a lot of trouble to show you that a Topologically-Nonlinked chromosome would undoubtedly have a goodly number of RH’d supertwists. Not only does the TN theory *explain* the superhelicity of native DNA, but it explains it without making silly assumptions, such as the unwarranted assumption that DNA, for some mysterious reason, is "underwound".

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Since the conclusion reached from these electron micrographs of replicative intermediates is that DNA has the W-C helical structure simply because it's superhelical, which is logically absurd, you can perhaps now see that this sort of research proves absolutely nothing. It's not a mountain. It's a molehill.

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Here's a reminder of what we discussed in great detail earlier. (PAUSE) You'll need to keep this in mind as we move to the next paper.

It’s also worth belaboring the point that in the living systems in which DNA is found in nature, there are invariably basic proteins present, which certainly play an indispensible role in maintaining the normal architecture of DNA *in vivo*. It’s only when these proteins are stripped away *in vitro* that the differences in the relative stability of RH’d and LH’d DNA cause superhelical twisting.

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In terms of pervasive influence, the most thought-suppressing, creativity-destroying paper on this subject was by Crick himself.

The circular logic employed in the above-referenced studies of replicative intermediates was used again by Crick in this paper. However, Crick’s stature in this field was so huge, that once he said it, it became the Law, and almost no one dared to challenge his conclusions afterwards.

Let us examine Crick’s false logic, to see where he went wrong.

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In considering the title question of his article, “Is DNA *Really* A Double Helix?”, he came to the conclusion that the best evidence was from topoisomerase experiments. Topoisomerase both nicks and re-seals circular DNA. Whenever it nicks the DNA, the DNA becomes relaxed—all tightly-wound superhelical structures will immediately unwind, and the chromosomes will assume the all-right-handed W-C structure. BUT…they will not necessarily adhere precisely and exactly to the W-C winding number of a single, 10-base-pair helical turn per 34Å of length. During the period that they are in the nicked state, random thermal motion in the medium will cause changes in the winding, so that when the molecules are re-sealed, there will generally be some small but significant number of supertwists, according to the topology equation we just discussed.

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Note that since the superhelical winding is the result of thermal buffeting of the chromosome, the direction can be either RH’d OR LH’d, *i.e.,* the random thermal motion

in the medium might, at one point in time, bring about a small degree of unwinding of the W-C structure, and a moment later it might bring about a small degree of OVER-winding. The first will cause the appearance of RH'd, or negative supertwists, after the molecule is re-sealed, and the second will cause the appearance of LH'd supertwists.

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NOW, if DNA is treated with topoisomerase to equilibrium, a family of so-called "topoisomers" will be seen on gel electrophoresis of the steady-state product. This drawing qualitatively depicts this steady state accurately, with the DNA structures represented by the various bands drawn adjacent to them. The direction of migration in the electric field, as indicated by the arrows, is up.

The different bands represent different "topoisomers", which differ by one twist in their superhelicity.

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The slowest band, now highlighted at the bottom of the slide, is totally relaxed DNA, which has been nicked by topoisomerase, and has coincidentally been sealed with exactly and precisely the standard W-C pitch of 10 base-pairs per 34 Å of length. It has no "overwinding" or "underwinding" issues, and it therefore has no supertwists.

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Just above it, migrating just a little bit faster than it, are the minimally supertwisted forms, *i.e.,* topoisomers containing only one or two supertwists. Note that the direction of supertwisting, that is, whether the supertwists are "+" or "-", does not matter in this experiment. A topoisomer with +2 superhelical twists will have about the same density as one with –2 superhelical twists.

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Note also that the bands are close together at the bottom, because the difference in density between topoisomers having low degrees of supertwisting is not great.

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The bands do not become well-separated until a larger number of superhelical twists is present, as depicted by a single exemplary topoisomer in the middle of the drawing.

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At the very top of the drawing is the native chromosome, untreated by topoisomerase, added as a marker.

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The most highly-supertwisted enzyme-generated topoisomers band just beneath it. As at the bottom of the gel, the bands at the top are not very well-separated, because the difference in density between topoisomers have 24 or 25 supertwists is not great.

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Now, how does Crick conclude, from this data, that native DNA is a helix? His words, verbatim from his manuscript, are, quote, "no other interpretation of the bands is even remotely plausible". What does this mean?

It doesn't mean what he'd like you to think it means. In order to understand this, we must go back a few paragraphs in his manuscript. He points out, correctly of course, that the strands of plasmids do not separate when alkali-denatured, which, he says, proves that they are topologically-linked. <u>Note that this is supposed to be the CONCLUSION of his manuscript, but we see here that it's the underlying assumption, raising the question of what the purpose of this manuscript is in the first place</u>.

I hope that if I've shown you anything at all, it's at least that alkali denaturation data do NOT necessarily prove that the strands of circular DNA are linked. The alkali data which Crick specifically quotes, to persuade you that DNA is helical, are from the very same papers we have used to show that it might *not* be helical after all. Obviously more research is needed. But no useful new research appears in the Crick paper.
Yet essentially everyone who read it thought what he wanted you to think.

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And this is what that is:

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1. The DNA of a typical 5000 base-pair plasmid is a right-handed helix, since the strands do not separate at high pH.

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2. This DNA has 25 superhelical twists, and therefore must have been sealed shut in the 5% underwound state.

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3. The agarose gel of the products of topoisomerase treatment reveals something like 25 bands, corresponding to 25 topoisomers, each of which evidently contains 1 less superhelical twist than the one in front of it in the gel.

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4. Since this is a perfectly good explanation for the bands, we may conclude that DNA is a helix.

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Do I have to tell you that this is nonsense? #1 is *presumptuous* — whether or not DNA is helical is what we're supposedly investigating, and here he tells us it's already proven!

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#2 is presumptuous, since it also presumes that DNA is helical, and fails to consider the possibility that there are other explanations for the 25 superhelical twists.

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3, and #3 only, is true, but it proves nothing about the native structure, since it explains only the differences between the products of enzyme degradation of the native structure.

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#4, disguised as a so-called "conclusion", is in fact not a conclusion at all!

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It's merely the unjustified starting presumption in #1, regurgitated *as if* it was a conclusion.

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Here's a slide which is sure to offend many, but not anyone possessed with even rudimentary wisdom. Yes, you've learned how to throw genes into huge vats of E. coli, and, lo-and-behold, MONEY comes out of the other side. But YOU HAVE NO IDEA WHAT'S ACTUALLY GOING ON IN THOSE VATS, DO YOU?

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In case I'm not getting through to you, let us take only a moment to predict the outcome of the topoisomer experiment according to the TN theory. We have to do this, because Crick says "no other interpretation of the bands is even remotely plausible". Well, we're going to give another interpretation.

We start with the native chromosome, which is highly supertwisted, as I have explained and re-explained. If you still don't get it, then I've failed as an educator, but the native TN chromosome is supertwisted all the same, whether or not you understand it, and whether or not I've failed in my attempts to explain it.

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As soon as it's nicked by topoisomerase…

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…it totally relaxes. From this point forth, it makes no difference whatsoever what the state of helicity, or lack thereof, of the starting product was, because it's no longer present in the reaction mixture.

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As the topoisomerase reaction reaches equilbrium, the other topoisomers are generated.

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If you can't understand that this Crick paper is a sham, then you're missing something essential to the pursuit of real knowledge, and you really need to get back to the lucrative but mindless world of applied biotechnology for profit. Don't worry…in the money-obsessed world we live in today, no one will fault you for loving wealth more than truth.

Slide 298

This brings us to our final paper on this subject, namely Stettler *et al,* from the laboratory of Charles Weissmann. Weissmann was a co-founder of Biogen, a company with billions of dollars in annual sales. He was one of the first to become very adept at throwing genetically-valuable enzyme genes into vats of E. coli and other trained unicellular organisms, and extracting from those vats lucrative gene-engineered products for international sale and distribution. As I have already opined, however, these commercialists haven't a clue as to what actually goes on in the nuclei of these trained animals, at least not from the structural point of view.

Nevertheless, we cannot ignore the Weissmann paper, even though essentially no one ever cites it, because it was the final nail in the coffin of common sense among those molecular biologists who should have cared about the structure of DNA. I often wonder whether anyone but me ever bothered to really read it. Certainly the editors of JMB didn't read it, or if they did, they must have been half-asleep.

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There is history behind this paper. According to my older and wiser colleagues, Francis Crick was visibly upset by the commotion started by the publication of the Rodley and Sasisekharan "side-by-side" structures. He apparently felt personally threatened, and put out a public call for a response. The suggestion he made, at that time, namely the late 1970's, was that someone—*anyone*—should prepare complementary single-stranded circular DNA and re-anneal it. Let's introduce some terminology immediately: The separated strands of duplex circular chromosomes are often referred to as the "+" and "–" strands respectively. Since Crick was sure DNA had the W-C structure, and since re-annealing of separated "+" and "–" circles couldn't possibly give rise to a topologically-LINKED structure such as the Watson-Crick structure, Crick was confident that the re-annealed product would promptly be shown to be abnormal in its physical properties, and the side-by-side structure could be laid to rest.

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Two scientists took up the "Crick challenge". One was Robert W. Chambers, who, at that time, was the acting chairman of the Biochemistry Dept at the New York University School of Medicine. Chambers assumed the chairmanship upon the retirement of Severo Ochoa, the man who had won the 1959 Nobel Prize for his discovery of polynucleotide phosphorylase, with which he was able to crack the genetic code.

Chambers was a highly-respected scientist, best-known at that particular time in his long career for elucidating one of the first 3D structures for a transfer RNA. His colleagues referred to him as "Bullet Bob", because he was sharp as a whip, and he never made a mistake.

I knew Chambers quite well, since that was the department in which I earned my doctorate degree in biochemistry. If not for this, his greatest discovery would never have been known, because he chose not to publish it, and I alone have struggled to keep it from sinking into the depths of obscurity.

Slide 301

The other man to take up the "Crick challenge" was Chambers' colleague of many years, Charles Weissmann. Weissmann had only recently left NYU, where he had been a professor of biochemisty, to found Biogen. In doing so, he had placed himself in the vanguard of those who have renounced the ancient wisdom which teaches that striving for knowledge and striving for money are incompatable. "Now", we are told, "they are suddenly compatible". Ever since then, it has become progressively more difficult for anyone who is not wealthy, through public stock offerings, to get anyone to listen to him, or to take him seriously. This is an extremely dangerous trend, and one for which the world will, in the end, pay a high price, since money destroys all truth.

Slide 302

The Weissmann experiment, which was all-but-directly-commissioned by Crick, was, in effect, uncontrolled, and the conclusions in the discussion section were not supported by the data which was presented. I cannot review the entire paper here, but I can cover the main points.

The overall experimental design was to kill all interest in Rodley's side-by-side structure, and any other topologically non-linked structures, by *intentionally creating such a structure*, and proving that it did NOT have the physical properties of normal DNA. This is illustrated in the slide, the top of which shows my rope model of two separated single-stranded circular half chromosomes. Weissmann proposed that they should be re-annealed, giving rise to a base-paired duplex product, as shown in the top right panel. He then proposed to study the physical properties of the product, to prove that it was *not* normal DNA, but something entirely different.

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Note that the W-C "double helix" CANNOT arise in this experiment, because the separate single strands we start with cannot become plectonemically-entwined unless they are broken open, wrapped around one-another, and then re-sealed – something which is NOT going to be done in this experiment.

The duplex product here can *only* be topologically-NON-linked. Thus, if it has normal physical properties, then DNA *is* topologically-non-linked. Otherwise, TN DNA is excluded as a structure for normal DNA.

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Here's the experimental protocol. DNA from either the plasmid PβG or the virus PM2 was nicked with DNase I under conditions designed to introduce about one nick per chromosome. Two identical chromosome substrates are depicted schematically on the top of the slide, each containing one blue strand and one red strand.

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The two purple arrows show the effect of random cleavage by DNase I, an old-fashioned, pre-genetic engineering enzyme which indiscriminately cleaves essentially anywhere on either strand. As indicated by the arrows, in the chromosome on the left, the blue strand has been nicked, and on the right, the other, *i.e.,* the red strand has been nicked.

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Having now been nicked, the single-strands can be readily separated by alkali denaturation, yielding the products shown on the bottom of the slide. The nicked strand, upon denaturation, becomes linearized. Thus, the chromosome on the left yields a blue linear strand and a red circular strand…

Slide 307

…whereas on the right, we see the opposite, namely a blue circular strand and a red linear strand.

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The mixture of linear and circular DNA was then sedimented through an alkaline sucrose gradient, which resulted in the data shown on the right side of the slide. The most important peaks, whose identities were confirmed by electron microscopy, were a peak of single-stranded linear DNA, and, just ahead of it…

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…a peak of single-stranded circular DNA. Both these peaks were mixtures of DNA from both strands of the chromosome. That is, the slower peak contained a mixture of "+" and "–" linear strands, while the faster peak contained a mixture of "+" and "–" circular strands.

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The fractions enriched in "+" and "–" circular strands were pooled immediately, and re-annealed under the following conditions:

pH 8.5, 60°, 20 minutes.

Please keep these numbers in mind, because we'll be getting back to them shortly.

Next, the product was purified and analyzed by gel electrophoresis.

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Here's one of Weissmann's gels, showing Form I and Form II markers on the left, a single-stranded DNA marker on the right, and a peculiar *new* form of DNA in the middle, designated "Form V". "Form V" migrated faster than Form I, but slower than single-stranded DNA. What is it?

Weissmann's stated conclusion was that in creating the so-called "Form V" he had, in fact, created the true "side-by-side" structure, that is, a BASE-PAIRED duplex structure whose strands were NOT plectonemically-intertwined. From this slide, we can see that *whatever* it was that he created, it certainly was NOT normal DNA, since it had the wrong electrophoretic mobility. This was supposed to prove that the "traditional" Watson-Crick structure, appearing as "Form I" in the left lane of the gel, did not have the Rodley "Side-By-Side" structure.

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Here's the problem. Keep in mind the pH and temperature at which Weissmann did his re-annealing: pH 8.5 and 60°. I concede that Weissmann created a duplex structure, but was it really a properly base-paired structure? I think maybe not. Bearing

in mind that circular DNA is not the same as linear DNA, we may start by asking: Are these conditions under which circular DNA will re-anneal?

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To get a handle on this, let's look again at the reannealing data from the masters of re-annealing, Robert Warner and his associates.

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To make this task easier, let's remove all the data except the 60° curve. Note there's a pH optimum around pH 11, where there is essentially 100% reannealing in only 10 minutes. As pointed out previously, notice please that even small changes in pH markedly discouraged reannealing.

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Thus, at the lowest pH in these data, pH 10.6, reannealing was already getting rather sluggish, with only about 30% reannealing in the time allotted . What do these data predict at pH 8.5, the pH chosen, apparently quite arbitrarily and capriciously, by Weissmann for *his* reannealing? Well, you can't tell unless you extrapolate this data to 8.5.

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The trouble is, extrapolation of these data quickly take us right off the chart.

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The extrapolated data line crosses the X-axis at pH 10.4.

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Note that even at this relatively high pH, the predicted percent reannealing in this experiment is…ZERO percent! There would be absolutely NO re-naturation under those conditions. And Weissmann is 2 entire pH units BELOW this! That is, Weissmann's supposedly re-annealed DNA was created at a pH 2 entire units *below* the pH of zero renaturation!

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What can we conclude from this, other than that incubating DNA at pH 8.5, 60°, would result in *no* renaturation? And yet the experiment generated some sort of weird duplex product, which the authors called "Form V". What was it? To this day, it remains a complete mystery.

Slide 320

Now, those of you who are really, really astute and observant may be saying, at this point, that my logic is flawed. You may be asking yourself the following: "Since Form IV, although denatured wrt base-pairing, is a structure within which the two strands remain physically *associated*, what gives Dr. Biegeleisen the right to say that RE-naturation data for Form IV can be applied to re-naturation of Weissmann's single-stranded circles, where the two strands are completely separated?"

That's a good question. Bear in mind, however, that ***iff*** the strands of Form I are topologically-non-linked, then so are the strands of Form IV, regardless of the fact that they remain somehow stuck together physically. If so, then the results of a reannealing experiment may *not* depend so much upon the relative physical locations of the strands at the start of the experiment, but only upon establishment of the precise pH, temperature and ionic strength required for the optimization of the re-annealing reaction.

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Along these lines, there is one thing which can be said with certainty. Form I is a base-paired structure, whereas both separate single-stranded circular DNA and Form IV are NON-base-paired structures. There's got to be a transitional form between them. Denatured DNA *must* pass through this transitional state if the single-strands are to re-anneal. I'll say as much as can be said about the transitional state, then show a movie.

The transitional state I refer to is a juxtaposition of the two strands, base-to-base, with a wheel-like rotation taking place, so the proper Watson-Crick base-pairs can come into alignment. Without this, reannealing cannot occur.

The conditions established by Robert Warner for optimal renaturation are *not* optimal for the stability of either *de*-natured or *re*-natured DNA, but lie somewhere in the middle. Therefore, I have adopted the arbitrary terminology "Form 0" for the transitional form. This is a sort of pluripotent form which, with small changes in prevailing conditions, can lead to any of the other forms.

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In "Form 0", weak base pairs repeatedly form and then rupture. At any given moment in time, such as the moment illustrated here, the odds are that few bases will fortuitously lie adjacent to other bases with which they can form proper hydrogen bonds.

Under those narrow sets of conditions which are optimal for renaturation, however, it is both necessary and sufficient to presume that the single-stranded circles are rendered capable of rotating wrt one-another…

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("Form 0", the movie)

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…until the complementary base-pairs are properly re-aligned, and hydrogen bonding is re-established.

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Since Weissmann's conditions for re-annealing were far, far from anything which could reasonably be expected to result in significant restoration of a base-paired structure, what, then, is this Form V stuff?

I can't answer this question any better than anyone else, but I certainly can take an educated guess. My guess is that it's merely Form IV. We know that both Forms I and IV can exist over the entire physiological pH scale, but Form I requires precise alignment of the bases, whereas Form IV is totally indiscriminate, requiring only proximity of the two strands.

If you're familiar with Form IV science, you may protest, saying that Form IV is extremely dense, and ought to migrate much faster than the band marked “V” in this gel. I once thought that too, but then I did some research which taught me otherwise.

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For most of my life I’ve had no access to anything even remotely resembling a biochemistry laboratory, but for one brief period of time, a biology teacher named Timothy Purnell, a true gentleman and a true friend of science, made his equipment available to me, and I seized the opportunity to do some research. For several months I turned my dining room into a lab, which did *not* ingratiate me to my wife, but which did generate some interesting data.

I attempted, during these months, to separate the strands of circular DNA by heating at various pH’s and ionic strengths. The long-and-the-short of it is that you CANNOT heat-denature circular DNA under any circumstances, except at pH’s so high that it would denature whether or not it was heated.

The gel on the left shows a typical experiment from this period. The DNA, from the plasmid pUB, was a gift from Richard Roberts.

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Unfortunately it was not topologically-pure, but contained a mixture of Forms I and II, which is visible in every lane except at the very highest pH’s. The Form I band is the one in front, since it is superhelical and moves a bit faster in the gel.

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At the highest pH’s, starting at pH 12.5, we see what may appear to be a *widening* of the Form I band, with concomitant fading of the Form II band. In fact, the Form I band is not really widening, but is merging with a new band of alkali-denatured DNA, which fully-reveals itself in the final lane, at pH 13.4.

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The single remaining band at pH 13.4 is very faint, reflecting the poor binding of ethidium bromide for single-stranded DNA. Note that *all* the single-stranded DNA, both single-stranded circular as well as linear and Form IV, are contained in this single band. We scrupulously studied these gels at various times, from beginning-to-end, to rule out the possibility that the Form IV moved so quickly that it might be missed, but nothing was missed. The Form IV is definitely in this faint band, mixed with the single-stranded linear and circular forms.

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The gel on the right is essentially the same experiment, differing only in that the ethidium bromide was incorporated directly into the agarose, whereas on the left, the gels were stained after the electrophoresis was run. With the ethidium bromide incorporated directly into the agarose, the single-stranded band is now split into two bands, revealing its heterogeneous composition. Presumably, one of these bands contains the Form IV, the other a mixture of single-stranded linear and circular forms.

Note that Form IV, on these gels, moves only marginally faster than Form I, in sharp contrast to the behavior we saw previously in CsCl density gradients, where the Form IV dropped like a rock. Why?

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The answer comes from the laboratory of Pouwels and his group in the Netherlands. These researchers published a series of very important studies on the properties of circular DNA in the 1960's. Shown here is their data concerning the effects of ionic strength on the sedimentation coefficients of the various forms.

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Look at the Form IV data. In the physiological range of salt concentration, at the left of the graph, it has the well-known high-density conformation, sedimenting nearly twice as fast as Form I. But as the ionic strength is *decreased*, the *s* value steadily diminishes, until, at a salt concentration of 0.001 M…

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…the *s* value of Form IV actually drops *below* that of Form I!

This is a really fascinating result, with obviously-important implications for our concepts of the structure of Form IV, but for now, it sufficient to note that at the ionic strength of a typical agarose gel,

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…indicated by this double-headed vertical arrow, the density of Form IV is actually quite low. Clearly, this accounts for its surprisingly low electrophoretic mobility in agarose gel electrophoresis.

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Returning to Weissmann's results, we see now that this supposedly-new form, which he calls "Form V", may be nothing more than Form IV, created by indiscriminately re-annealing DNA under conditions which were *extremely* sub-optimal for the regeneration of Form I. That is, these were conditions which allowed for an atypical, non-base-paired duplex structure to form, but did not allow for the wheel-like rotation necessary for the re-establishment of proper base-pairing.

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How about a control experiment? The control experiment is the backbone of the scientific method. Weissmann claimed that his "Form V" was a base-paired structure, which is a critically-important claim. Therefore, for the control experiment, Weissmann used ϕx174 DNA from the virion, which contains only ONE of the two strands found in the RF, or replicative form. With only one strand, no Form V should appear, if Form V is, as he claimed, a base-paired structure.

And this was the design of the "control" experiment: He found a new set of conditions under which DNA from the plasmid PβG turned into the so-called "Form V", and simultaneously incubated the *single*-strand of ϕx174 under the same conditions. He reported that the ϕx174 remained single-stranded. Therefore, he concluded, the

appearance of Form V requires the presence of complementary DNA, so that there can be specific base-pairing.

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What's wrong with this? A lot! First of all, *why* did he use a different species of DNA for the original experiment than for the control? He could have generated his so-called "Form V" with ϕx174 RF, which contains both strands of the virus, and then used the virion DNA for his control. But instead he used DNA from two different species. Very strange.

But it gets worse. Much worse!

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Look at the conditions of incubation of the control experiment: It's not just the DNA which is different; *everything's* different! The solvent, suddenly, is organic: 50% formamide; the pH has been altered; it's now only 8, and the temperature of incubation has been precipitously dropped to 20°. Huh? What's going on here?

This is called a "control" experiment, yet it employs a different DNA, in a different solvent, at a different pH, at a different temperature, for a different period of time. What madness caused JMB to publish this? Did anyone even bother to read it?

Although the text certainly did not say so, the fact of the matter is that the data, in the form in which it's presented, suggests that ϕx174 virion DNA *did indeed* become "Form V" under the conditions of the original experiment, and that new conditions were therefore sought out, where the experimental DNA turned into Form V, and the control DNA did not. I'm *sure* that's *not* the explanation for this data, but if that isn't what happened, then it would be nice if the authors would *tell us* that that's not what happened. But the paper is silent on this, so we're all left hanging, and must draw our own personal conclusions about it.

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But there's more still. To prove that Form V was a base-paired structure, it was studied by thermal denaturation, which should have revealed a cooperative transition at the melting point.

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The Form II melting curve is normal, revealing the expected cooperative transition at the melting temperature.

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But the data for the so-called Form V reveal essentially *no cooperative transition at all* upon denaturation, suggesting that Form V, whatever it is, is not a base-paired structure. Yet the conclusion of the authors of this paper was that Form V *is* a base-paired structure. What's going on here? And why? Who's idea was this?

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EXPERIMENTS WHICH PROVE THAT DNA IS NOT HELICAL IN CELLS

Slide 343

This brings us to the final chapter in this increasingly annoying story, namely the experiments which prove, or strongly suggest, that DNA is NOT helical in living systems. We'll start with Bob Chambers, *i.e.,* Bullet Bob. In a word, Chambers inadvertently did the Weissmann experiment correctly, and got the opposite result. That is, he proved, quite accidentally, that separate "+" and "-" circles can indeed renature to Form I.

His vehicle was ϕx174. This was a far more sensible choice of experimental DNA than Weissmann's choice, since, as I pointed out above, the ϕx174 DNA could have been used for both the experiment *and* the control.

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As I mentioned earlier, Chambers and Weissmann had been colleagues at NYU for years. Chambers knew that Weissmann intended to take up the Crick Challenge, and he entered the fray. It was thus a race. Weissmann had more money and more help, and he finished first—the race was over. But Chambers had gotten quite far at the point when he learned, through the biochemistry grapevine, that Weissmann's work was accepted for publication in JMB. Chambers had already completed the most difficult part of the work, which was to prepare the mixture of purified single-stranded circles, *i.e.* the "+" and "–" strands.

So what could he do? He took his painfully-acquired single-stranded circles, the product of months of work, and retired them to the refrigerator.

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About 3 months later he thought of another, entirely different use for them, but before using them, he decided to subject them to a careful analysis to make sure they were still what they had been when they went into that refrigerator.

This analysis began with an analytical velocity gradient centrifugation to determine the sedimentation coefficients of the material present. To his *great* surprise, he found that a substantial portion of the DNA had spontaneously re-annealed back into Form I.

Could it have been Form V? Remember, Chambers and Weissmann knew one another personally for years, and each was well-aware of what the other was doing. Remember too that it was Weissmann's published report on Form V which caused Chambers to quit the race. It would therefore be a mistake to presume that old "Bullet Bob" couldn't distinguish between Form I and Form V. I know, from my personal discussions with him, that he knew *all about* Weissmann's Form V, and he was very sure that his spontaneously, re-annealed form was, in fact, Form I.

Now the question was, "How did Form I get in there?"

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After establishing, to his satisfaction, that Form I had indeed appeared in the midst of his "+" and "–" circles, Chambers reported his result to me, because he knew that I believed in the non-helicity of DNA. Clearly, I thought—and said—this proved that DNA was, in fact, *not* helical, which I'd been saying for years.

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The obvious explanation for the appearance of Form I was the scenario portrayed on the top line of this slide. But, to my astonishment, Chambers *still didn't agree.* In fact, although this was, in my opinion, an observation of surpassing importance, Chambers never even published it! Why not? Because he believed that there was *another* explanation for the results, as per the *bottom* row of pictures on the slide. Chambers told me that, according to his formulation, there was a 3-step process:

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(1) For the first step, one of the strands broke open spontaneously. This, of course, is not only possible, but likely over time.

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(2) The broken strand wrapped itself around an intact complementary strand. This too is entirely possible, and might even be expected over time, even in the refrigerator. So far, Chambers was proposing nothing unusual. But then…

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(3) … he proposed that the nicked strand, once wrapped around its complement, spontaneously *sealed itself.* This, he explained, was a reaction mediated by an *enzyme.* What enzyme? WATER! What? "How can this be?", I asked? Chambers took me to a blackboard and drew a detailed reaction mechanism, with water as the "enzyme".

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His hypothetical reaction mechanism looked impressive on the blackboard, but off the blackboard, out here in the real world, it was thermodynamically absurd. The fact that DNA spontaneously accumulates nicks on storage is painfully well-known to every nucleic acid scientist. How can a population of chromosomes, which spontaneously accumulates nicks, simultaneously accumulate a re-sealing of those same nicks? It's impossible!

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Every graduate student and postdoc has, as some point, awoken in the morning to find his painstakingly-prepared Form I had degraded into Form II, as depicted on the top of this slide, but what graduate student, in the history of science, has ever awoken to find his sloppily-prepared Form II had magically re-sealed itself into pristine Form I? IT'S NEVER HAPPENED!

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Now here's the "kicker": By a stretch of the imagination, it could be proposed that nicked and re-sealed DNA were energetically equivalent, so that water *really could* catalyze the re-sealing of nicked strands. It's true, after all, that topoisomerase requires no energy source. Even this notwithstanding, however, what would cause the DNA in the Chambers experiment to become 5-7% *underwound* before sealing, to produce a single topoisomer containing the native supertwist? *It's absolutely impossible,* and

without the exactly-correct amount of underwinding, there's no way the product could ever have been identified by either ultracentrifugation *or* electrophoresis.

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At some level Chambers understands the importance of what he has observed. In accordance with this, he has, in the past, given me permission to describe his work in my publications, even though he still cannot accept the most obvious conclusion. I last spoke to him about a year ago. He's long retired, and he no longer has his laboratory notes from this period, but he stands by his observations, to this day.

Since the work is un-published, you are free to doubt or disbelieve. But the Wu study, to be described next, is published, and difficult to controvert. About the best you can do is just ignore it. That, in my opinion, would be tantamount to standing on scientific thin ice.

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(TITLE slide for Tai Te Wu)

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Tai Te Wu, whom I believe has elucidated the most probable structure for DNA in living systems, went to medical school in Hong Kong, then got his PhD from Harvard. He did DNA research at Cornell Medical College for some years, but made himself so unpopular because of his unorthodox opinions about DNA structure that he felt ostracized. He now practices immunology at Northwestern, and seldom makes public statements about DNA.

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I'm going to read through this now, even though it's hard to comprehend without pictures. I'll show you some of the pictures shortly, but most of them are provided in part II of this series, entitled "The Probable Structure of the Protamine-DNA Complex", which fully-develops the structure we shall now briefly describe.

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The article is called "Secondary Structures of DNA", and it's from PNAS.

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In this paper, Tai Te Wu discussed the pitch:diameter ratio for the Watson-Crick double helix at 66% and 92% humidity, as revealed by x-ray crystallography. He proposed that the diffraction pattern of DNA at high humidity was less suggestive of a single Watson-Crick duplex, than it was of a *pair* of identical duplexes, each one stretched out to twice the normal length, with their base-pairs mutually intercalated.

In other words, as I shall illustrate in a moment, he was proposing that at high humidity, DNA crystallized as a *four stranded* structure, consisting of two intertwined Watson-Crick-type duplexes.

It is evident from the publication of the paper in PNAS in the first place, and secondly from the lack of any serious criticism subsequent to its publication, that the idea was considered to be an entirely reasonable interpretation of the x-ray data, although not

necessarily the interpretation of choice for DNA in the real world, where genetic considerations seemed to greatly favor a 2-stranded model.

There is no doubt, however, that even at that early time, Wu was in fact thinking of DNA in the real world. In his own words:

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"At 92 percent, the surrounding of the DNA fiber resembles that inside a cell, while at 66 percent, the state of the fiber becomes completely artificial. The differences…should then provide the necessary clue for us to resolve the intricate secondary structure of DNA *in vivo.*"

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After a thorough analysis of the x-ray diffraction patterns of DNA at the two humidities, Wu concluded that "if the structure of the DNA fiber at 66 percent consists of a double helix, than at 92 per cent it must consist of two double helices". In the Wu structure, the distance between base pairs in either double helix was twice as large (*i.e.,* about 6.8 angstroms) as in the Watson-Crick structure, but in the end, the stacking of bases at the more familiar 3.4 angstrom spacing was preserved, by mutual intercalation of the base pairs of the two adjacent duplexes.

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Finally, and most surprisingly, he predicted that at 100% humidity, *i.e.,* the condition prevailing in living cells, the two mutually-intercalated duplexes would lose all vestiges of helical twist and exist as a pair of what he referred to as "straight ladders", having their base-pairs mutually-intercalated.

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Wu's paper, in 1969, attracted little attention, and caused no great uproar. But in the years that followed, he began to understand that superhelical DNA, as illustrated in the slide, provided the basis for the 4-stranded structure he had proposed. He came to the conclusion that viral and plasmid DNA did indeed consist of two mutually-intercalated double helices, each one stretched-out so that the base-pair spacing was twice normal, but with mutual intercalation of base-pairs restoring the familiar 3.4 Å spacing between adjacent base pairs in the 4-stranded structure.

Since this subject is covered in detail in the second presentation in this series, I will show the pictures but without much comment.

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This is Wu's own highly schematic diagram of his proposed structure for circular chromosomes. I found it confusing when I first looked at it, so I'll try to walk you through it quickly. The single-stranded, circular, antiparallel sugar-phosphate backbones for the entire chromosome are represented by the elongated circles on the sides. I should note that although Wu presumes a superhelical twist will be found in purified, protein-free circular DNA in the laboratory, he makes no attempt to portray that superhelical twist here, depicting only what he refers to as the "straight ladder" aspect of the 4-stranded structure.

The rectangular shapes attached to the backbones are not individual bases, but base-*pairs,* the small number shown obviously representing a much larger number than can be drawn in a picture like this. In order to clarify the drawing, the base-pairs in the back have been arbitrarily shaded, and the base-pairs in the front have been left white. This hopefully makes the mutual intercalation of base-pairs easier to see.

The longitudinal backbone distance between adjacent base pairs is 6.8 Å, which, after the intercalation, gives the more familiar 3.4 Å spacing expected for DNA.

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Thanks to the generosity of Mercury Computer Systems, I have been able to create a true and detailed virtual model of this structure. This is what it looks like. It's merely untwisted, stretched-out Watson-Crick DNA. Without the helical twist, the base-spacing becomes the same as the residue spacing in the sugar-phosphate backbone, which is always in the neighborhood of 7 Å.

Again, let me remind you that this structure will be described in much more detail in part II of this series.

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Here's a movie of the structure.

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This is the second duplex, which will intercalate into the first.

Slide 368

And here's the final structure.

Slide 369

When animated, it's really quite lovely to look at. It's simple, logical and even aesthetically pleasing. Hopefully, your eye perceives the direction of spin as being to the right, because if your eye is playing that perspective trick on you, and telling you that the structure is spinning to the left, then it will NOT seem simple & logical, but complex & confusing.

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Unlike the other proponents of non-Watson-Crick DNA structures, Wu did not stop with a theoretical paper. He went on to design an ingenious experiment to prove that his structure was real, and widespread in nature. Here is the experimental design, in his own words:

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"Since the complementary DNA strands of most plasmid molecules are of about equal molecular weight and their charges are the same, it will be very difficult to separate them on agarose gel electrophoresis."

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"However, one strand of the plasmid DNA is the sense strand and one the antisense strand. While RNA transcription is occurring, D-loops are formed, with mRNA paired with one strand of DNA."

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"Since under agarose gel electrophoresis conditions, RNA:DNA bonds are tighter than DNA:DNA bonds (J. Casey and N. Davidson, *Nucl. Acids Res.* **4**, 1539-1552, 1977), these RNA:DNA bonds will be maintained and promote the separation, on the gel, of the weaker DNA:DNA structure."

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The principle, which is really quite ingenious, is illustrated in this drawing, depicting 2 "D-loops". The DNA is black, and the freshly-synthesized m-RNA in the D-loops is red. The DNA, for the purposes of this illustration, is somewhat arbitrarily represented as having a gently undulating, Rodley side-by-side structure, with no full helical twists.

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The D-loops are, of course, greatly exaggerated in size here, but the principle is well-represented by the drawing, which shows that transcription only takes place on one of the two strands, namely the "sense" strand.

Because the RNA in the D-loops is linear, having none of the topological constraints imposed by circularity, it will effortlessly wrap itself around its DNA template, whereby the resulting RNA:DNA hybrid duplex will assume the all-right-handed Watson-Crick structure, since there's nothing to prevent it from doing so. Since these hybrid duplexes have the most stable known conformation, namely the W-C RH'd double-helix, it is not surprising that they do *not* dissociate during electrophoresis.

Note, however, that the main body of the chromosome remains in the TN conformation, which, topologically-speaking at least, is 50% LH'd. In the absence of the nuclear proteins necessary to support this structure, it has a relative instability compared to the more-stable RH'd W-C structure in the D-loops.

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In the presence of substantial amounts of bound m-RNA, the sense-strand no longer has the same structure as the anti-sense strand, the latter having no bound RNA. Therefore, as electrophoresis progresses, the two strands, which now have different densities and structures, will slowly separate, as hopefully suggested by the large arrow.

In order to insure a clear-cut result, Wu grew his cells for 3 days, to stationary phase, so there would be no replicative intermediates to confuse the results. He also set the electrophoresis current to the low setting of 10 mA, so that the electrophoresis could be continued for 48 hours.

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This is Fig. 7 from Wu's paper. It looks like a single large agarose slab, but if you look at the time notations at the top, you'll see that this is actually a composite of 4

gels. In order to get through this before we all die of old age, let's use the magic of computer graphics to separate the gels, so we can see exactly what was happening at each time.

Slide 378

These are the gels, separated for easy comprehension, and excluding the 48 hour gel, since all relevant changes had taken place by 36 hours of time.

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In each experiment there are 3 lanes. The right-hand lane, in each case, contains the Form I experimental DNA. This is the plasmid DNA grown to stationary phase, so that there are no replicative intermediates, but – presumably—lots of m-RNA-containing D-loop structures.

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The middle lanes contain the control experiment. This is the same DNA, but intentionally cleaved with the restriction endonuclease Pst I, obtained from New England Biolabs. These chromosomes are thus linearized, and no longer have any of the peculiar topological properties of circular DNA. The purpose of this control is to answer to any critics who may wish to suggest that Wu's circular DNA was accidentally nicked. The control DNA is intentionally nicked, and any behavior of DNA in these experiments which is the result of strand cleavage should therefore be seen in both the experimental *and* control lanes.

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The left-hand lanes contain 6.0, 3.6 and 2.4 kb molecular markers.

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Well, here are the results. At 12 hours, the intact Form I plasmid band is beginning to show signs of splitting into two bands. At 24 hours, the split is definite, and at 36 hours, the bands have separated entirely, and begun to diffuse. This diffusion is characteristic of single stranded circular DNA molecules such as ϕx174 single-stranded virion DNA.

The staunch Watson-Crick "helicist" will surely try to attribute the separation of these strands, widely believed to be hopelessly topologically linked, to accidental strand breakage. Unfortunately for them, that explanation will NOT WORK here.

Slide 383

Look at the middle lanes, containing the intentionally-linearized control chromosomes. They remain as single, sharp bands, even after 36 hours.

Slide 384

The really astute observer, cognizant that D-loops are surely also present in the linearized control chromosomes, may at this point ask, "If D-loops change the relative electrophoretic mobility of the two strands of the chromosome, why don't the linearized chromosomes also undergo strand separation in these gels?" This is an excellent

question; one which Wu does not go to any great lengths to answer. I would propose that the correct explanation is the same one I gave a moment ago, namely that the TN structure is, topologically speaking, 50% left handed, and is therefore inherently unstable relative to the linearized control chromosome. The latter, once nicked, is free to assume the all-right-handed W-C structure. This is the the most stable known structure for duplex DNA, and evidently does not undergo strand dissociation in these gels.

Slide 385

At grave risk of being accused of demagoguery, I have prepared a semi-animated version of the Wu experiment, which dramatizes the splitting of the circular DNA sample into two bands.

Slide 386

I think that this little bit of cinematic melodrama is justified, because mere print publication of this remarkable discovery, in the year 1999, had no impact on science whatsoever.

Slide 387

Therefore the discovery needs to be publicized.

Slide 388

Some skeptics might be prepared to accept the splitting of the DNA into two bands, but might still demand to know, with more certainty, what those two bands actually consisted of. Wu addressed that question in a very direct and compelling way. His experimental plasmid chromosome, from the plasmid pHTB4, contained the sequence shown in the slide.

5′ ttcgcccagcttcgctcagct … aatatgcactgtacattcca 3′
3′ aagcgggtcgaagcgagtcga … ttatacgtgacatgtaaggt 5′

Two probes - - and I won't bother to read out the sequences, but they are identical to the chromosomal sequences matching their colors - - were synthesized by the Northwestern University Biotechnology faculty. On Southern blotting, the former probe hybridized to the faster moving band, whereas the latter hybridized to the slower moving band.

Slide 389

Wu did not stop there. Next he repeated the entire experiment with a second plasmid, called pUC19, with the same result, that is, two bands on electrophoresis. He then cut out the bands, purified their DNA, and used them as templates in DNA sequencing. This time, instead of asking his colleagues at Northwestern to synthesize primers for him, he used commercially-available primers specific for the pUC19 plasmid. The result was that each primer initiated DNA synthesis efficiently on DNA from only one of the two bands.

For those of you not familiar with DNA sequencing, the object of doing it in this particular case is not to determine the actual DNA sequence, but merely to determine whether or not the primer recognizes and binds to the DNA. If so, sequencing will

proceed. If not, there will be no sequencing. If the DNA in the bands is mixed, then the result will reflect that.

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Here are the details of the DNA sequencing experiments:

Plasmid pUC19 was purchased from New England BioLabs.

Wu also purchased two primers for pUC19 DNA sequencing. I won't read the sequences, but here they are:

#1233 AGCGGATAACAATTTCACACAGGA
#1224 CGCCAGGGTTTTCCCAGTCACGAC

These were also purchased from New England BioLabs.

DNA molecules from the slower and faster bands, respectively, were then sequenced using these two primers separately with Sequenase, purchased from United States Biochemicals.

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Here are the results. For those of you who are not familiar with the details of DNA sequencing methodology, I won't waste time describing the technique. The important point here is not the sequence itself, but the recognition of the template DNA by the primer. Please note that we're *not* looking at the dots in this figure, but rather the horizontal bands which appear in the various lanes, giving them an appearance reminiscent of metaphase chromosomes in replicating eucaryotic cells. If the bands are dark, then DNA sequencing occurred, demonstrating that the primer recognized the DNA. If the bands are light or non-existent, however, then DNA synthesis was reduced or absent, demonstrating that the primer did *not* find its complementary sequence. This is how Wu described the result, from figure 8 of his paper:

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For each primer, the four lanes are A, C, G and T. For the slower band, primer #1233 (shown in the left four lanes in Fig. 8(a)) gave much more intense signal…

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…than primer #1224 (shown in the right four lanes). Again, remember, we're looking at the darkness of the horizontal bands, not the dots adjacent to each lane.

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On the other hand, for the faster band, the reverse was true as shown in Fig. 8(b).

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(NO AUDIO – HIGHLIGHT MOVES TO THE RIGHT).

Slide 396

Thus, the slower band consisted mostly of one strand…

Slide 397

…while the faster band consisted mostly of the complementary strand. Some cross-contamination could not be avoided since the two bands were very close on the agarose gel.

Slide 398 (SUMMARY)

Well, this concludes Part I of our slide presentation, on the history and science of topologically-non-linked DNA. The purpose of this portion of the presentation is *not* to persuade you to adopt my ideas about DNA structure, but rather to get you to think. What *is* the structure of DNA? Have we thought the problem through, or are we just blindly assuming that the W-C structure, first discovered in synthetic laboratory crystals, *must* be the structure of DNA everywhere else it's found?

I've showed you that the so-called "mountain of evidence" in support of the W-C structure is, in fact, a mole-hill, and that, moreover, it's a mole-hill of false logic.

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The logic, as we have seen, goes something like this:

"DNA MUST have the Watson-Crick helical structure, because no other structure is possible."

"Circular DNA must also have the W-C structure. Since it's invariably superhelical, it must also be underwound."

"Therefore, any DNA which is found to be superhelical must have the underwound W-C structure".

Sounds childish, doesn't it? And yet this is precisely the logic which has prevailed in this field. What do you think? Do you *really* believe that all superhelical chromosomes *must* have the underwound Watson-Crick structure?

Slide 400

What about DNA replication? Do you really believe that all, or even part of the chromosome, is constantly spinning at 20,000 rpm? Or not?

Never mind what *I* think. What do *you* think? Does DNA replication proceed like this?…

Slide 401

…or this? What strikes YOU as more plausible? And *why* should small circular chromosomes always be superhelical?

Slide 402

Is it because they are mysteriously "underwound", so that they need to be nailed to the surface to stop them from twisting? Isn't that an awkward presumption?

Slide 403

Or could it simply be that the superhelical twist arises logically and naturally as a result of the nature of the structure, being topologically 50% left-handed, and 50% right-handed, and having been taken out of its normal environment where nuclear proteins support its non-twisted structure? What do you think?

Slide 404

You may object to my speculations about the failure of the strands of circular DNA to separate at high pH, making reference, as they do, to a hypothetical Form IV structure whose phosphate groups are in the middle…

Slide 405

…but at least my structure was proposed by the world's greatest expert on the chemical bond.

Slide 406

The establishment dismisses all this, alleging that Form IV is just a "tangled mess", with *no structure at all.* It doesn't matter what I think, or what they think. WHAT DO *YOU* THINK?

Slide 407

Then I've shown you the evidence. Crick's paper starts by assuming that DNA is a double helix, then concludes, at the end, that DNA is…..*a double helix.* Do you accept this as a logical argument?

Slide 408

Weissmann's paper describes an attempt to reanneal DNA at what appear to be arbitrarily and capriciously-chosen conditions of pH and temperature, and claims to have created TN DNA, reporting that it is not normal. The experiment is essentially uncontrolled, since the purported control experiment uses a different DNA, in a different solvent, at a different pH, temperature, and time of incubation. What's missing in this picture? Do you believe that this experiment really proves anything at all?

Slide 409

Bob Chambers refused to publish his discovery. Single-stranded complementary circular DNA spontaneously re-annealed back to a form having the properties of native Form I in his refrigerator over a 3-month period. I say the top mechanism is the only way to explain it. Chambers says it's the bottom mechanism, even though it's thermodynamically absurd. What do YOU think? Is Chambers' mechanism correct, or not? Did he merely make a mistake, and should the data just be ignored? You must decide for yourself.

Slide 410

Finally, I showed you the work of Tai Te Wu, who demonstrated, in an experiment which was rigorously and doubly-controlled, that the Form I band can be split into two bands by electrophoresis under the right circumstances, and that the two bands correspond respectively to the two fully-intact, circular single strands that together comprised the original chromosome. Do you believe it? Or not? Are you able to just ignore it, regardless of whether true or false? I can't decide for you. You must decide for yourself.

Slide 411

If you wish to see a detailed virtual model of the probable structure of DNA in at least one biological setting, with almost-certain implications for DNA structure in many other settings, please see Part II of this series, entitled "The Probable Structure of the Protamine-DNA Complex".

Thank you for watching and listening, and, above all, for being willing to think. When there is no promise of immediate financial reward, or of academic positions and honors, it can be very difficult.

Slide 412

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R→L transition; left-handed DNA

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The End.