Articles

Biochemical Topology: Applications to DNA Recombination and Replication

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Processes of DNA rearrangement such as recombination or replication frequently have as products different subsets of the limitless number of distinguishable catenanes or knots. The use of gel electrophoresis and electron microscopy for analysis of these topological isomers has made it possible to deduce physical and geometric features of DNA structure and reaction mechanisms that are otherwise experimentally inaccessible. Quantitative as well as qualitative characterization is possible for any pathway in which the fate of a circular DNA can be followed. The history, theory, and techniques are reviewed and illustrative examples from recent studies are presented.

THE FUNCTIONAL AND ESTHETIC APPRECIATION OF LINKED rings (chains, catenanes) and knots dates back to early civilizations. These forms have appeared in sculptures, suits of mail, braided coiffures, and formal gardens. Only within the last century, however, did topologists begin the first systematic study of knots and catenanes, developing equations for classification and preparing catalogues of the numerous topoisomers, forms differing only in a topological property.

In the last 25 years, linked rings have also found a place in both organic and biological chemistry. Interlocked organic rings were first synthesized in 1960 by Edel Wasserman, who termed them catenanes, from the Latin *catena* for chain (*1*). He pointed out the rich potential for creating different structures held together by topological bonds, bonds that, unlike any others, are maintained independent of direct interaction. Topological linkage and isomerization, however, are only rarely observed among simple organic molecules (*2*).

As manifestations of the rich topology of DNA, knots and catenanes have truly flourished, taking their place alongside the better-known topoisomers that differ only in linking number (*3*). [The linking number of closed circular duplexes is the sum of the writhing (Wr) of the DNA axis in space and the twisting of either strand about this axis.] Knots, catenanes, and linking number topoisomers (*4*) are the basis of a field of study that we term biochemical topology in analogy to the chemical topology of Wasserman, Walba, and others (*2*). Although enriched by the contributions of mathematicians and chemists, biochemical topology as pioneered by both Vinograd and Wang (*5*) is directed at the interests of biochemists and molecular biologists.

We describe the topological approach to biochemistry and give a selected overview of its recent contributions to the study of DNA replication and site-specific recombination. The exploitation of the unusual properties of catenanes and knots that made possible these contributions are the focus of this article; supercoiling and topoisomerases have recently been reviewed (*6*).

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The Topological Method

The strategy of the topological method is to deduce enzymatic mechanism or substrate DNA structure from a change in DNA topology associated with a reaction. For example, the sign inversion mechanism for topoisomerases, which postulates an enzyme-mediated passage of DNA segments through each other, was established by the demonstration of the predicted stepwise change in linking number (*7, 8*) and of the formation and resolution of catenanes and knots by topoisomerases (*8–11*). These experiments also distinguished the two classes of topoisomerases: type 2 enzymes, which in the course of reaction make double-strand breaks, and type 1 enzymes, which make single-strand breaks. We show below how important structural features of DNA have also been elucidated by a topological approach.

The full scope of the method is revealed when it is applied to structures or processes that are not topological per se but are made so by experimental design. The first important evidence for both the left-handed winding of DNA around histones in nucleosomes and the unpairing of promoter DNA on productive *Escherichia coli* RNA polymerase binding came from the fact that each reduces the linking number measured when circular DNA is covalently closed in the presence of bound protein (*12*).

The method can also be used indirectly to test, for example, whether one DNA site acts preferentially or exclusively in *cis* to a second site. Activity is usually compared for the two sequences located on the same DNA molecules (in *cis*) or on different ones (in *trans*). In such a test, however, the local concentration of one site relative to the other is generally much higher when the two are in *cis*. Through-space proximity can instead be held nearly constant by catenating the two DNA's for the *trans* configuration, allowing a direct assay of the role of connectedness in primary sequence. This strategy has been used in the study of intermolecular site-specific recombination (*13*) and could be productively applied to questions of enhancer activity and site selection by regulatory factors.

The topological method has three advantages: invariance, quantitation, and power. Invariance is a remarkable aspect of a topological property and its defining feature: no matter how much a DNA molecule is twisted, denatured, complexed, or distorted in any way short of breaking the backbone, its topology is unchanged during isolation and analysis. A break in one strand can change linking number of the double helix, but knot and catenane structures are unaltered even by extensive single-stranded gaps. Molecules for which these invariant properties are lost due to backbone scission

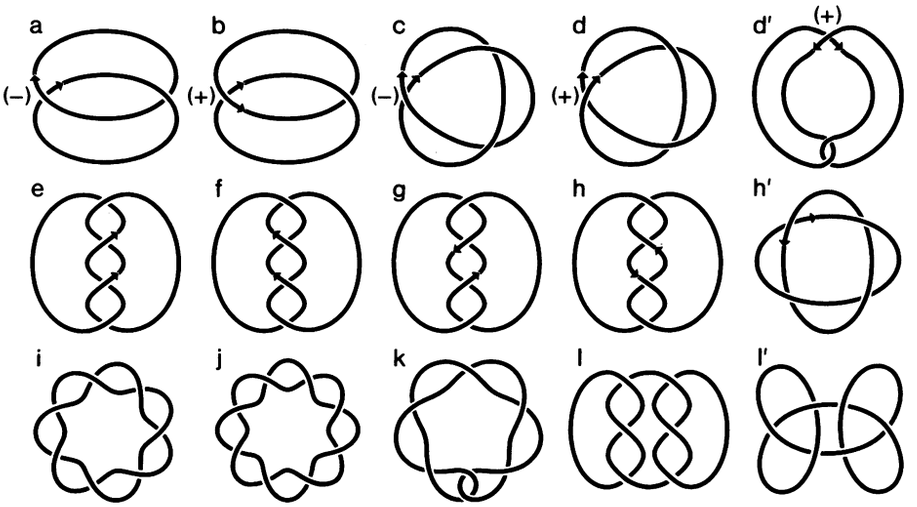

Fig. 1. Catenane and knot catalogue with representative examples of linked DNA forms. All forms shown are chiral (different from their mirror images). The node sign, displayed for a single node, or crossing, in each form in the top row is determined as follows. Arrows are drawn to indicate an orientation of the DNA primary sequence. If the overlying arrow can be aligned with the underlying one by a clockwise rotation of less than 180°, the node for duplex DNA has a value of −1. If a counterclockwise rotation produces alignment, the node has a value of +1. Top row: (a and b) The two forms of singly linked catenanes. (c and d) The two forms of the simplest knot, the trefoil. A trefoil is a torus knot, that is, it can be drawn on the surface of a doughnut-shaped object. Other members of the torus family of knots and catenanes are shown in (e to j). (d′) A redrawing of the knot (d), illustrating why a trefoil is also a member of the twist knot family. Twist knots are formed by strand passage between two looped ends of a molecule twisted on itself. Another twist knot is shown in (k). Middle row: (e to h) The four types of multiply interwound torus catenanes are represented as doubly linked forms. Drawings of this type make it possible to distinguish right-handed parallel (e), left-handed parallel (f), right-handed antiparellel (g), and left-handed antiparallel (h) double helical interwindings. (h′) Redrawing of catenane in drawing (h). Bottom row: (i) Right-handed torus knot with seven (+) nodes. (j) Right-handed torus catenane with eight nodes. (k) Right-handed twist knot with seven (−) nodes. (l and l′) Compound six-noded knot composed of two (−) trefoils.

rarely confuse the analysis, as they can easily be distinguished from intact molecules.

The quantitative analysis of DNA topology was important in establishing the pitch of the double helix (*14*), the mechanism of topoisomerases (*7, 8, 10*), and the number of supercoils in the synaptic complex of the Tn 3 resolvase (*15*). Such analysis is simpler than it might appear; biologically relevant results commonly fit a simple algebraic equation in which many terms can have only integral values (*3*). Moreover, most topological relationships can be appreciated by manipulating ribbon models for DNA rather than equations (*16*).

The power of the method lies in its ability to distinguish between models by revealing which of an often astronomic number of possible linked forms arise as products. For example, according to a model presented below, recombination mediated by the bacteriophage lambda Int (integrase) system should yield a particular class of knotted products. When 48 molecules were examined by electron microscopy, all were of the predicted class; included were two knots with 19 crossings, for which the correctly predicted structure represents only one of 10^8 possible forms (*17*)!

Topology provides a permanent record of DNA structure at the time of a breakage and reunion event, no matter how evanescent the structure. Nevertheless, we caution that topology should be combined with standard approaches whenever possible. Topological properties are invariant precisely because they are independent of deformation and therefore cannot specify size or shape. For example, the binding of either RNA polymerase or histones reduced the linking number measured when DNA was covalently closed as a circle, but local denaturation (by polymerase) or left-handed wrapping (by histones) reflected in the altered linking number was only identified on the basis of biophysical and biochemical data (*18*).

Linkage Analysis

It is convenient to portray molecules and define topological properties in plane projection (Fig. 1). When DNA is so flattened, segments cross to form what are termed nodes (*7, 16*). The two types of nodes, positive and negative, are defined by the convention described in the legend to Fig. 1.

Nodes can be used to characterize all forms of linkage. To analyze an electron micrograph of a nicked knot or catenane, the molecule is first redrawn with the minimum number of nodes. This process is illustrated in Fig. 2 for a five-noded structure, which upon redrawing is seen to have been a trefoil (three-noded) knot with two incidental crossovers. The path of the unfolded molecule is traced and at each crossing an arrow is attached to indicate the direction followed (*19*). The signs of the nodes are then determined by reference to the convention.

Any isomer of a knot or catenane is defined by the number, sign, and arrangement of the nodes that join the rings (catenane) or prohibit the molecule from unfolding into a circle (knot). An illustrated catalogue of knots and catenanes is available (*20*) and a

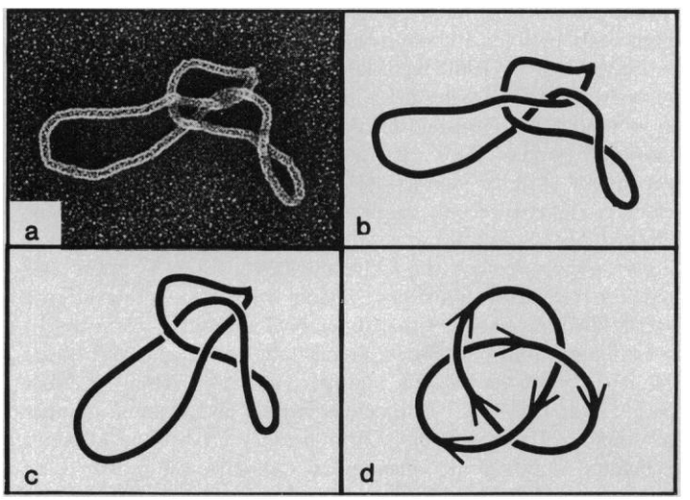

Fig. 2. Analysis of a DNA knot by electron microscopy. (a) Electron micrograph of DNA purified from the first rung of a knot ladder generated with T4 topoisomerase (*8, 26*). The purified DNA was denatured with glyoxal, then coated with *recA* protein as previously described (*26*). (b) Tracing of knot. (c and d) Redrawings, with successive removal of incidental crossovers (flops) to reveal trefoil in standard form (d) with three (−) nodes. For all DNA knots and catenanes commonly encountered, underpasses and overpasses alternate in the unfolded form; this rule provides a criterion for judging when a curve has been drawn with the minimum number of nodes.

method for classification of most experimentally encountered isomers has been rewritten for a biochemical audience (*21*). With these, it is possible to determine whether structures that look the same (or different) really are and how many related forms exist.

Knots and catenanes can be grouped into families and subfamilies that share common features (*20*). Members of the torus family, important for DNA replication and recombination, can be drawn without intersection on the surface of a torus, a doughnut-shaped object. Examples are shown in Fig. 1, a to j. These are the simplest linked forms because all the nodes are equivalent in sign and placement.

Like many others, torus molecules have, in addition to sign, a handedness ascribed by the familiar "hand rule" of physics. The critical point is that the two properties are related but independent; we show below that the supercoiling of native DNA, like its double helical twist, is right-handed, even though supercoiling is negative and twist positive. The interrelations of hand, sign, and orientation are shown by the four torus catenanes in Fig. 1, e to h. Although the first and third forms are right-handed and the second and fourth are left-handed, the molecules in each pair have opposite signs because of the opposite orientation of the rings. The fact that different subfamilies arise from distinct biological processes has made determination of catenane structure particularly informative.

Experimental Techniques

The two principal methods for determining the structure of knots and catenanes are agarose gel electrophoresis and electron microscopy; the molecules are usually first nicked to remove supercoils that confound analysis. Although only electron microscopy gives the complete stereostructure of individual molecules, electrophoresis provides a more rapid and quantitative overview of a population. Thus, it is best to use both methods.

Gel electrophoresis can resolve mixtures of covalently closed circles or nicked knots or catenanes into discrete ladders (*8, 22–24*). In the examples shown in lanes a and b of Fig. 3, each rung contains topoisomers having one more node than those in the rung immediately above. This correlation of mobility with complexity (node number) in general holds true for all forms of topoisomers and reflects the compaction brought about by interlinking DNA.

Figure 3 also illustrates the use of reference samples to calibrate topoisomer gels. Comparison of the bacteriophage lambda (λ) Int recombination products in lanes c and d with the marker knot ladder (lane b) shows that the former have a steps-of-two spacing; the Int knots have an odd number of nodes (lane c) and the catenanes an even number (lane d). Torus forms have exactly this node pattern; electron microscopy has proved that the products of Int-mediated recombination are exclusively of this family (*17*).

Higher resolution gels can even separate stereoisomers of common node number according to node geometry, such as the variable arrangement of different-sized rings in trimeric heterocatenanes (*25*). Two-dimensional gels in which the two dimensions are run under different conditions (*24, 26*) or in which between dimensions the DNA is digested in situ with a restriction enzyme (*13*) can resolve and identify complex mixtures of linked forms. In particular, the in situ digestion procedure distinguishes knots from catenanes and allows the constituent rings of a catenane to be analyzed separately.

Traditional techniques for electron microscopy are useful for studying knot or catenane samples (*24, 25, 27*), but provide insufficient visual information to reliably score the signs of nodes. The complete topological characterization of knots and catenanes required the introduction of the *recA* protein coating method (*28*). The cooperative binding of *recA* protein to single- or double-stranded DNA yields a thick complex that, when shadowed, allows reliable identification of DNA overlay and underlay at crossing points (Fig. 2). The phage T4 analog of RecA, the *uvsX* protein, can also be used for this purpose (*29*).

The *recA* protein method has now been improved to permit characterization of less than 1 nanogram of knotted DNA (*26*). A technique has also been developed for marking the orientation of RecA-coated catenanes, so that it is possible to completely determine the topology of any DNA catenane (*15*). Orientation is derived from an asymmetric partial denaturation map; *recA* protein is complexed to both duplex and denatured regions so that orientation and node overlay can be scored simultaneously. With this last development, all possible types of knots and catenanes can be identified and analyzed by electron microscopy. The formal process of node analysis has achieved physical reality.

Applications to the Termination of DNA Replication

DNA catenanes were first described by Wang and Schwartz, who in 1967 cyclized linear DNA's in the presence of excess preexisting circles (*30*) and thereby reproduced with DNA Wasserman's synthesis (*1*) of the first organic catenanes. The discovery of naturally

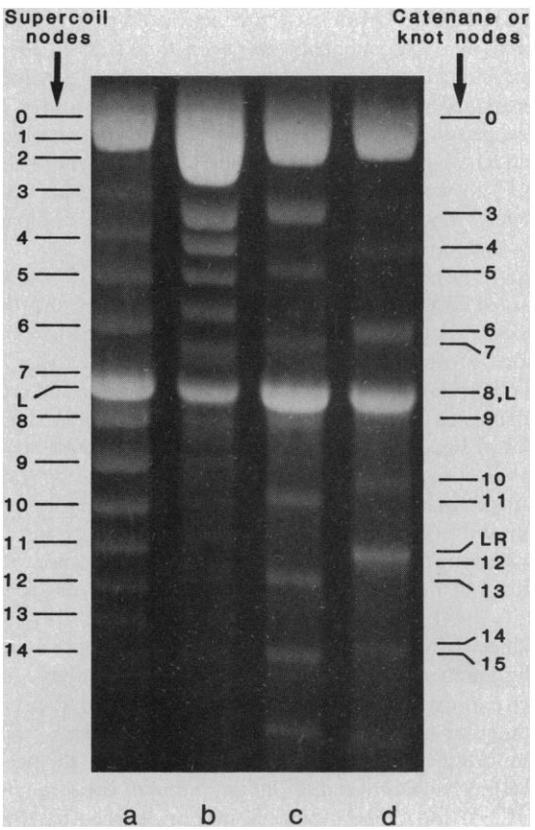

Fig. 3. Analysis of DNA knots and catenanes by gel electrophoresis. Samples of a 3.5-kb plasmid with two *att* sites 0.5 kb apart in direct (lanes a, b, and d) or inverse (lane c) order were subjected to electrophoresis in a 0.8 percent agarose gel (*23*). (Lane a) Marker ladder of relaxed linking number topoisomers. (Lane b) Marker ladder of knots generated by T4 topoisomerase. (Lane c) Knotted products of Int-mediated recombination. (Lane d) Catenated products of Int reaction. L, linearized substrate; LR, linearized recombinant ring. Linear species are by-products of DNase I nicking. [Courtesy of J. Bliska]

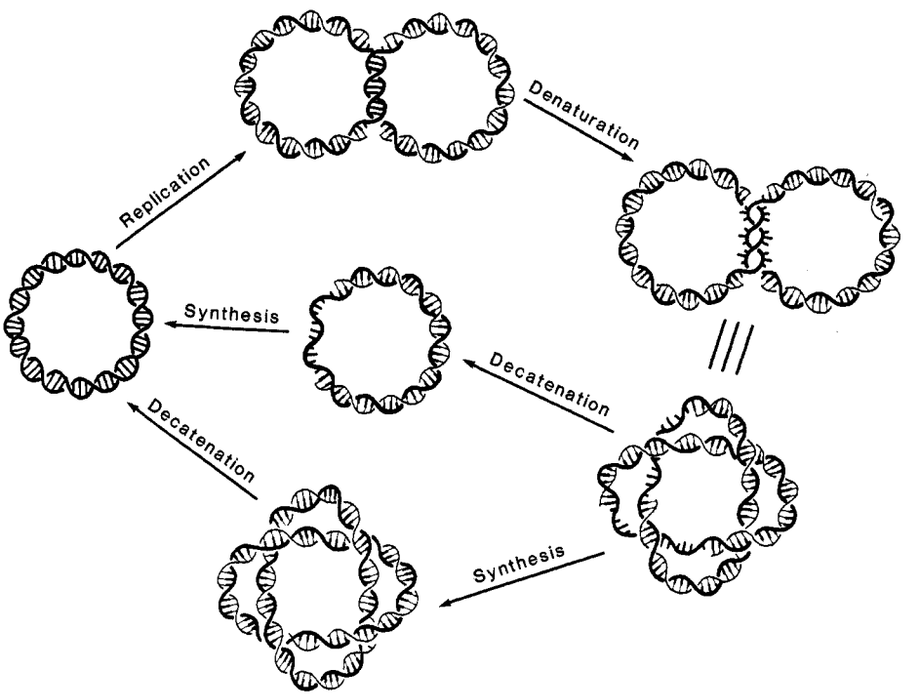

Fig. 4. Conversion of parental helical twist into daughter DNA interlinks at replication terminus. In the first stage of replication a swivel acts between approaching forks to reduce linking number concomitant with parental strand separation. Eventually steric and topological problems impede the swivel. Denaturation of the remaining parental duplex then converts the late Cairns form DNA to a catenane held together by a right-handed helix of interlinks; random motion of the DNA would distribute these nodes evenly over the molecule, as shown. If decatenation of the rings precedes the completion of synthesis, free gapped monomers are the final intermediates in replication (upper path). If the order of these two processes is reversed, supercoiled catenanes are broken down in the final step (lower path).

occurring DNA catenanes by Vinograd and co-workers (*31*) soon brought these linked forms to the attention of the biological community. By 1972 catenanes had been found essentially everywhere that circular molecules were known, including mammalian mitochondria, SV40-infected monkey cells, trypanosomes, human leukocytes, plasmid-containing bacteria, and the ring chromosomes of salamanders (*31, 32*). Most often rare (a few percent of the total DNA) and always apparently singly interlinked, these catenanes were generally thought to be by-products of DNA recombination or replication (*31–33*).

An important conceptual advance in the middle 1970's was the hypothesis of Skalka (*34*; cited in *35*) and of Gefter and Botstein (cited in *35*) that catenanes were essential intermediates in the production of progeny DNA circles. They proposed that, at the terminus of DNA replication, residual helical twist would cause the daughter rings to be held together by a single interlock. Releasing the DNA's by breaking either parental strand at the terminus before the completion of synthesis would compromise circularity and hence biological activity; catenation, they argued, was therefore unavoidable. They did not, however, explain why parental strand linkage remains when the final base pairs are denatured and copied. We now discuss this issue in topological terms and then return to experimental results.

A fundamental constraint of semiconservative replication is that the linking number of the parental DNA duplex must be reduced to exactly zero. Since linking number is a topological invariant, neither the denaturation of parental strands nor the synthesis of daughter strands can bring about its reduction. Cairns foresaw the need for a "swivel," a mechanism to reduce linking number concomitant with DNA synthesis (*36*). The rotational motion implied by the name swivel is not required; it is sufficient that one parental strand be passed through a transient break in the other to reduce their linkage. The catalysis of such processes is the hallmark of topoisomerases (*6*).

It is easy to imagine how a topoisomerase swivel could compensate for the loss of negative supercoils and ultimate introduction of positive supercoils caused by the denaturation of parental base pairs. By reducing linking number while the replication fork progresses, the topoisomerase would maintain the normal superhelical density in the unreplicated region. Although this would remove a torsional barrier to semiconservative replication, there would remain the additional problem of removing the last double helical twists at the replication terminus. The swivel is totally unsuited to perform this task.

If the topoisomerase acts in front of the replication fork to remove the last few twists, there is first of all a steric problem (*24*). As long as the terminus is bound up by a topoisomerase it cannot be replicated, but once a DNA polymerase assembly occupies this region it can no longer be readily relaxed. Every 10.5 base pairs required for topoisomerase binding could shelter a full helical twist.

Whether the swivel acts in front of or behind (*37*) the replication fork, there are several topological problems. A topoisomerase, which acts locally, will have difficulty sensing the global property (*38*) of parental strand linkage. Excess negative supercoils will block segregation as surely as will residual positive helical twist, yet compensatory supercoils would have to be introduced before the completion of synthesis to permit the exact dissolution of parental strand linkage. In addition, a topoisomerase cannot remove supercoils stabilized as wraps around binding proteins, including, among others, the topoisomerase itself. The fact that topoisomerases produce a distribution of topoisomers rather than a single species (*14, 22*) presents still another sticking point. Even if linking number were reduced on average to zero, most molecules in the population would have a nonzero linking number and the daughter rings would remain intertwined. Last, prefork denaturation by helicases and binding proteins will destroy the substrate for the swivel, converting parental twist at the terminus into interlocks between nascent rings.

Sundin and Varshavsky were the first to suggest a pathway for the termination of replication that took into account both steric and topological constraints (Fig. 4) (*24*). They proposed that the unreplicated terminus is first denatured, transforming each pair of single-stranded nodes comprising a parental helical twist into the topologically equivalent nodes of a catenane interlock. In a second stage, DNA synthesis and linkage reduction are completed independently, converting the catenated rings into progeny duplex rings.

Decatenation by a topoisomerase could finish the job of linkage reduction begun by a topoisomerase swivel. Decatenation, first

documented in vitro in 1980 with *E. coli* DNA gyrase (*9*), is a common activity of topoisomerases (*6*). If DNA synthesis is completed prior to decatenation, the staged segregation model requires a type 2 topoisomerase; if a gap or nick remains at the time of decatenation, a type 1 enzyme would suffice (*39*).

It is the mere fact that synthesis and decatenation are uncoupled in this scheme that permits the completion of linkage reduction for an entire population of molecules. Catenane nodes can be distributed over the rings, avoiding steric contraints. More important, so long as there is a thermodynamic or mechanical driving force for decatenation, the presence of a decatenase will lead to the eventual release of all progeny rings as free molecules.

In 1980, Sundin and Varshavsky devised a high-resolution gel system for identification of catenated forms of SV40 DNA (*24*) and observed species that, unlike any previously found, were highly intertwined (*24*). The SV40 catenanes isolated under standard conditions frequently contained single-strand interruptions in one or both rings; those isolated after hypertonic shock, however, were covalently closed and supercoiled (*24*). Since synthesis necessarily follows denaturation, these supercoiled catenanes, if replicative intermediates, must nonetheless have initially contained a single-stranded gap whose size would depend on the extent of prefork denaturation. The fact that catenanes accumulated during hypertonic shock contained, on average, ten interlocks suggests that approximately 100 bp of DNA remained to be synthesized when the DNA at the replication terminus was denatured (*24, 40*).

Whether repair-type DNA synthesis occurs before or after decatenation is not relevant to the dissolution of parental strand linkage, since the gap is in the daughter strand (Fig. 4). Although it is easiest to consider synthesis and decatenation as sequential, the two could proceed concomitantly if the decatenation is effected by the passage of replicated regions through each other (*37*). If decatenation is either concomitant with synthesis or very rapid relative to that process, replication will proceed without accumulation of catenated intermediates. The recent observation that, under certain conditions, catenanes are not detected during SV40 replication (*41*) does not, therefore, necessitate adoption of an alternative scheme for linkage reduction.

In 1984, Sternglanz and co-workers clearly defined for the first time the in vivo role of topoisomerases in replication. They found that, for the yeast *Saccharomyces cerevisiae*, the type 2 topoisomerase but not the type 1 enzyme was essential for cell viability (*42*). When a mutant with a thermolabile type 2 topoisomerase was grown at a nonpermissive temperature, only a single round of replication occurred; plasmid DNA was virtually quantitatively trapped as a multiply intertwined supercoiled catenane (*42*). Because DNA synthesis was not immediately arrested upon shift-up of the temperature, the type 2 enzyme must be essential for chromosome segregation but not for propagation of the replication fork.

The linear yeast chromosomes were also affected in type 2 topoisomerase mutants. At the nonpermissive temperature, the nucleus frequently became lodged in the neck between mother cell and daughter bud (*42, 43*), supporting the notion (*11, 24, 38*) that the problem of segregating parental strands concerns linear as well as circular chromosomes. Intertwining of daughter duplexes could potentially occur at the many sites where replication forks meet in a eukaryotic chromosome; there are severe torsional barriers to removing such duplex interlocks by simple rotation of long chromosome fibers (*38*). In addition, although linear DNA's cannot be catenated in a formal sense, they appear to be subdivided into topologically independent domains that can be supercoiled and in other ways act like closed DNA circles (*44*).

Studies by Uemura and Yanagida with the fission yeast *S. pombe* dramatically showed the failure of type 2 topoisomerase mutants to segregate DNA (*45*). At the nonpermissive temperature, nuclei were cleaved by septum formation, and strands of ruptured DNA spilled out of the cells. In *S. cerevisiae,* Botstein and co-workers showed that the failure to successfully segregate nuclei in type 2 topoisomerase mutants very likely was the lethal event in the cell cycle. Cell death occurred at the time of mitosis, but could be prevented by selective chemical disruption of the mitotic spindle (*43*). In the normal segregation process the mechanical force of the contracting spindle fibers might be used to make otherwise random strand passages by a topoisomerase unidirectional.

Results with prokaryotes parallel those described above. Catenanes arise as the in vitro products of Col E1, λdv, and pBR322 replication (*46*) and in vivo products of G4 growth (*27*). If the action of *E. coli* gyrase, a type 2 topoisomerase, is blocked in vivo, isolated nucleoids appear as dumbbell-shaped doublets, apparently reflecting incomplete segregation (*47*). Studies on the fate of catenanes generated by synchronous recombination in vivo also point to DNA gyrase as the principal decatenase in *E. coli* and indicate that mechanical forces are not needed to segregate catenated plasmids (*48*). Decatenation is rapid and spontaneous despite the high intracellular concentration of both DNA and DNA-aggregating agents (for example, polycations); such conditions in vitro shift the equilibrium toward catenation (*49*).

The similarity of experimental results with prokaryotes and higher and lower eukaryotes suggests that segregation via multiply intertwined catenated intermediates is a general mechanism for the completion of replication. The model, however, is not yet proved. In particular, in the absence of type 2 topoisomerase activity catenanes arising from any mechanism are expected to accumulate because of the lack of a functional decatenase. The origin of the in vivo catenanes can nevertheless be tested.

The staged segregation model predicts that these catenanes arise directly from denaturation of the parental double helix and should therefore be right-handed, of positive sign, and torus in form (see Figs. 1 and 4). In contrast, catenanes arising from a single round of recombination or, indeed, any mechanism that converts supercoil interwindings into catenane nodes (see below) will be right-handed but negative. Multiple rounds of recombination produce various nontorus forms (*26, 28, 50*), and catenation of free rings by a topoisomerase yields only single interlinks between any two rings (*13*). Thus, the prediction is specific and testable.

Applications to Site-Specific Recombination

Recombination is conveniently subdivided into the alignment of the DNA sites on the recombinase and the subsequent reciprocal exchange of DNA. Topology has been important not only in defining these two steps but also in determining the geometry of the substrate and the intermediate, the synaptic complex. Each of these four elements (alignment, exchange, substrate supercoils, and synaptic configuration) is reflected in the topology of the products.

We focus on the two best studied systems for site-specific recombination, Int and resolvase. The proteins of the λ Int system, which we collectively refer to as Int, integrate circular phage DNA into the host chromosome as well as excise the DNA after prophage induction (*51*). The recombination events occur via reciprocal exchanges between specific sites, called *att*, one on the phage DNA and another on the host chromosome. The resolvases of the Tn3 family of transposons (*52*) have a much more circumscribed role. In transposition between circular molecules, strand exchange and replication lead to formation of a cointegrate in which donor and recipient replicons are joined by transposon bridges (*52*). Resolvase then rescues the replicons from this intermediate by mediating

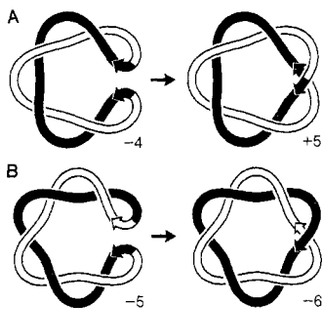

Fig. 5. Metamorphosis of supercoils into knots and catenanes by recombination. Crossover between sites (arrows) results in inversion of the sequence between inverted sites (A); directly repeated sites are resolved into two circular products (B). The sites delimit two domains, black and white; all substrate supercoils shown are interdomainal. The interdomainal substrate nodes are converted by inversion into knot nodes (A) and by resolution into catenane nodes (B). The right-handed, intertwining of the substrate is preserved in both products, but the (−) sign of the substrate nodes is kept only in the catenanes; the sequence inversion that leads to knotting also changes product nodes to (+). The recombinase is shown changing a single substrate interdomainal node in the process of strand exchange.

recombination between copies of a specific *res* sequence present once in each transposon. The result is that there are now two copies of Tn3, one in the recipient and one in the donor.

To evaluate recombination mechanistically requires the concepts of topological domain and site orientation. The regions of DNA whose interrelationship is altered by recombination define two domains; for exchange within a circular substrate they are the regions between the recombining sites. Sites can be oriented as inverted repeats (head-to-head) (Fig. 5A) or as direct repeats (head-to-tail) (Fig. 5B). If two sites are in a head-to-head orientation, the intervening domain is inverted by recombination. If they are head-to-tail, the domain between them is excised and both domains reclose as circles.

If the two domains are intertwined, either by substrate supercoiling or by the exchange process, the intertwining will be fixed by recombination; the single ring from inversion will be knotted (Fig. 5A), and the two rings resulting from excision will be catenated (Fig. 5B). In topological terms, knots and catenanes arise during recombination from interdomainal nodes, the intersections of the two domains (*16*). Intradomainal nodes also contribute to product topology but in the form of supercoils.

Resolvase

The first important result obtained on the topology of resolvase recombination was that the product of one round of recombination is a singly-linked catenane, regardless of substrate supercoil density (*13, 25, 53*). Since any variation in the intertwining of the two domains during synapsis or strand exchange would alter catenane complexity, both processes must be rigidly controlled.

It has been shown that the resolvase synaptic complex has exactly three negative supercoils between the *res* sites (see below); we consider here how the mechanism might remove all other interdomainal nodes prior to exchange. Site apposition via random collision (Fig. 5) is clearly ruled out; interdomainal nodes would be entrapped in proportion to substrate supercoil density, yielding catenated products of variable complexity. The most likely alternative is a scanning or tracking mechanism in which one site moves, or is moved, along the DNA until it meets the other site, triggering recombination.

The simplest scanning mechanism that fits the resolvase data is called looping and is illustrated in Fig. 6 (*13, 25*). One subunit of a functionally dimeric enzyme binds to a *res* site; another binds nonspecifically nearby, forming a DNA loop. As DNA slides past the second subunit supercoils are segregated into separate domains. By a one-dimensional random walk process, the second *res* site is eventually reached and exchange occurs. Continuous sliding along the primary sequence could explain why under optimal conditions resolvase markedly prefers nearest neighbor sites in a multisite plasmid and why both in vivo and in vitro it acts nearly exclusively on directly repeated sites in a single molecule (*13, 25, 52, 53*). Nonetheless, the looping model makes a strong topological prediction that is wrong.

The idea that resolvase slides continuously from one *res* site to another was tested by catenating two ϕX174 DNA reporter rings to a resolvase substrate (Fig. 7) (*25, 54*). The reporter rings should be excluded from the initially small DNA loop and remain so as it enlarges. The model therefore predicts that recombination should segregate both reporter rings to one of the product circles. In fact, the rings segregate independently, as do reporter rings generated by recombination (*25*).

Thus the continuous sliding aspect of the looping model is incorrect. In considering alternative mechanisms we nonetheless preserve scanning. One possibility is that resolvase occasionally bypasses a short region of DNA, for instance, by direct transfer to a nearby segment. The clear difficulty in such a gated looping model is that there is no obvious way to allow reporter ring inclusion between transfer sites while maintaining the restrictions on substrate and product structure that prompted adoption of a scanning model (*25*).

An attractive idea is that the structure of supercoiled DNA itself constrains the action of resolvase. Supercoiled DNA could circle continuously like a conveyor belt, the interwound segments slither-

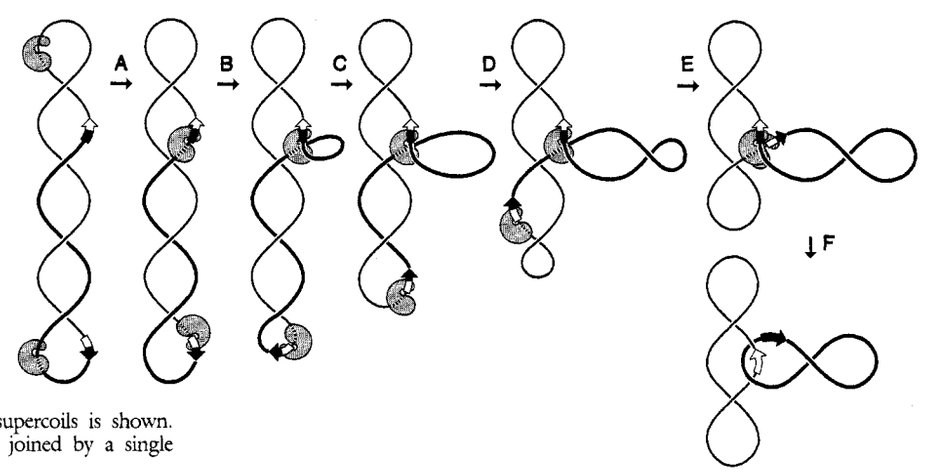

Fig. 6. The looping model for resolvase. The supercoiled substrate is shown with half-filled arrows representing the *res* sites. The domains that will be separated by recombination are differentiated as thick and thin lines. Two asymmetric resolvases (cashew-shaped) bind to DNA through nonspecific interactions, then translocate along the DNA (A) until they recognize a *res* site and bind tightly. Another part of resolvase remains nonspecifically bound and, by continuing the walk along the DNA (B), forms a loop of DNA between the points of specific and nonspecific interaction. For clarity, only one resolvase is shown forming a loop. As further translocation expands the loop (C and D), interdomainal supercoils are segregated within domains. At synapsis (E), all supercoils are intradomainal except those fixed by the protein. For clarity only one of the three constrained interdomainal supercoils is shown. Breakage and crossed reunion results in products joined by a single interlock (F). [From (*25*); courtesy of *Cell*]

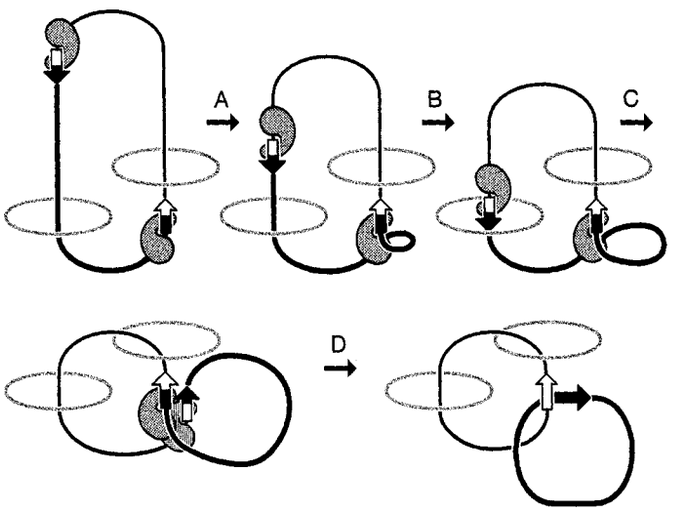

Fig. 7. Reporter ring test of looping model. An abbreviated looping model (supercoils omitted for clarity) is shown for a resolvase substrate to which two ϕX174 RF molecules (grey circles) have been catenated. According to the model the ϕX174 reporter rings should be excluded from the DNA loop formed at a *res* site (A) and remain so during loop expansion (B and C). Thus after recombination the reporter rings should always be catenated to the same progeny circle (D). [From (*25*); courtesy of *Cell*]

ing past each other until the *res* sites become aligned for recombination (*54*). If other ways of bringing *res* sites together are energetically unfavorable or tend to be unproductive in a negatively supercoiled molecule, the active synaptic complex would be identical to that obtained with looping and would always yield singly linked catenanes. Site orientation and connectedness preferences, but not nearest neighbor preference, are also directly explained by this model. If the slithering model is correct, supercoiled DNA is in effect introspective, continuously scanning itself for information; this process could also effect the interaction of distant sequences in other systems.

Topological studies have given precise information about the elaborate resolvase synaptic complex and the process of strand exchange. Synapsed *res* sites are aligned in parallel with three intervening negative supercoils held in place by resolvase (Fig. 8a). During strand exchange still a fourth interdomainal node is introduced, but since it is positive in sign its net effect is to cancel one of the synaptic negative supercoils upon release of resolvase. Because of the conservation of interdomainal nodes, the remaining two synaptic nodes make up the single catenane interlock of the product (Fig. 8b).

How this information has been derived illustrates well the topological method. We first define the issues quantitatively; the derivations are presented elsewhere (*16*). The algebraic sum of the nodes in a DNA molecule includes contributions from twist, writhe, catenation, and knotting. The change in this sum in a reaction is a fundamental topological parameter that is designated Me to emphasize that it is dictated by the mechanism of the reaction. Reactions of type 1 and type 2 topoisomerases, for example, have an Me of absolute value 1 and 2, respectively.

Me for recombination has intradomainal and interdomainal components. Only the latter, ^{ter}Me, is critical to catenane and knot structure generated by recombination. The conservation equation for recombination between directly repeated sites is:

$$Ca = {}^{ter}Wr + {}^{ter}Me$$

The algebraic sum of catenane nodes (Ca) is equal to the interdomainal writhe at synapsis (^{ter}Wr) plus the interdomainal nodes put in by the recombinase (^{ter}Me).

For resolvase, we measured directly that Ca is −2 and then deduced that ^{ter}Me is +1. Therefore ^{ter}Wr must be −3. Furthermore, according to the constraints on site orientation in the synaptic complex listed in Table 1, the sites must be aligned in parallel.

Ca was measured by partially denaturing the resolvase products and then coating with *recA* protein to allow visualization of the single-stranded landmarks as well as node overlays (*15*). The value of ^{ter}Me came from consideration of the structure of rare resolvase products, including a 4-noded knot (Fig. 8c) and a 5-noded catenane (Fig. 8d) (*28, 50*). Their structures were totally unexpected. Knots cannot be formed by any simple exchange between directly repeated sites and had, indeed, never been found in these circumstances. The 5-noded, figure-8 catenane contained an entrapped supercoil whose sign was positive even though the substrate was negatively supercoiled. Either resolvase had a very complex strand exchange mechanism involving a variable ^{ter}Me, or it could occasionally iterate a simple mechanism; the latter proved correct (*26*).

The singly linked catenane, the 4-noded knot, and the figure-8 catenane were hypothesized to be the products of successive rounds of processive recombination (Fig. 8) (*15, 16*). For this to be so, the

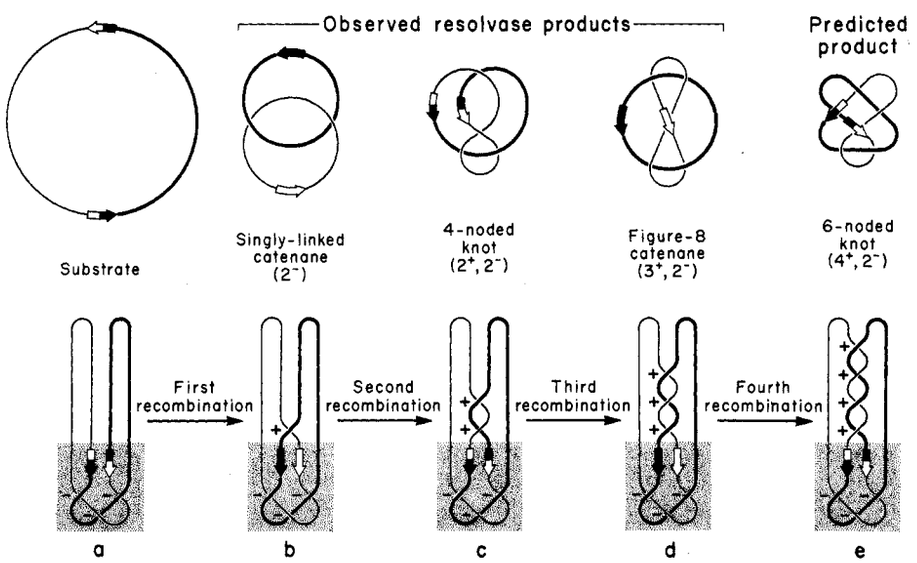

Fig. 8. Scheme for recombination by resolvase. The *res* sites (thick arrows) divide the substrate into two domains (thick and thin lines). In the upper row, the DNA's are represented in standard topological form with supercoils removed; below are intact forms of the same DNA's bound to resolvase (stippled rectangle) in a synaptic complex with three constrained (−) interdomainal supercoils. Successive rounds of recombination introduce a single (+) interdomainal node. Node composition is indicated in parentheses. [From (*26*)]

Table 1. Basic relationships of recombination variables.

Recombination type	Parallel sites*		Antiparallel sites*	
	^{ter}Wr	^{ter}Me	^{ter}Wr	^{ter}Me
Resolution of direct repeats	Odd	Odd	Even	Even
Inversion of inverse repeats	Even	Odd	Odd	Even

*Rules derived from model building for the values (odd or even) of the interdomainal nodes in the substrate (^{ter}Wr) and those introduced during the reciprocal exchange event (^{ter}Me) (*16*).

strand passage mechanism must introduce a single (+) node; that is, ^{ter}Me is +1 and ^{ter}Wr must be −3. Knots and catenanes alternate as products because an even number of rounds restores the original connectivity of the DNA. A 4-noded (2+, 2−) knot arises from a 2-noded (2−) catenane because the single (+) node that formed the catenane is not released in the processive mechanism and is combined with the second (+) node that forms the knot.

To test this scheme the structure was predicted for an as-of-then unseen product of a fourth round of iterative recombination, a particular 6-noded knot (Fig. 8e). By combining two-dimensional gel electrophoresis with the improved *recA* protein coating method it was shown that this product was indeed formed to the exclusion of all seven other possible 6-noded knots (*26*).

In sum, the resolvase synaptic complex has elaborate architecture but the exchange mechanism is simple. The three negative supercoils organized by resolvase in the synaptic complex probably extend over the more than 200 bp of DNA (*52, 55*) that define the *res* sites. Biochemical data suggest that perhaps a dozen resolvase molecules are in the complex (*13, 53*). Indeed there is a "synaptosome" for resolvase detectable by electron microscopy (*56, 57*) as also observed with Int (*58*). Echols and colleagues have suggested that the specific assembly of large nucleoproteins such as these ensures the fidelity of recombination (*58*); they may also promote the reaction by inducing DNA distortion that reduces the energetic barrier to the transition state.

The resolvase exchange mechanism could not be simpler. The value of ^{ter}Me must be odd for parallel sites (Table 1), and 1 is the smallest odd number. The occasional iteration, although of doubtful physiological relevance, yielded a periodicity in product structure that allowed the exchange mechanism to be deduced. The potential significance of the (+) sign for ^{ter}Me will be evident in considering the very different topology of the Int reaction.

Int

Recombination by Int is much less disciplined than that by resolvase. Directly and inversely repeated sites as well as sites in different molecules recombine to form products with a wide range of catenane and knot complexity (*51*). These differences between the two recombinases arise from the mechanism of synapsis. For Int, synapsis results predominantly from a random collision of *att* sites (*59*), although site apposition by a scanning mechanism such as slithering can also occur (*48, 54, 60*). Variable numbers of substrate supercoils are trapped between the sites by random collision and, as interdomainal nodes, are converted into catenane or knot linkage (Figs. 3 and 5).

The random collision mechanism, with its stochastic process of supercoil entrapment, was established by two lines of evidence. First, the average complexity of the products is directly proportional to substrate supercoil density (*48, 61*). Second, the some 100 knots and catenanes subjected to electron microscopy (*17*) are all of the predicted family (torus), handedness (right), and, where analyzed, sign, which is (+) for knots.

Int itself makes a constant contribution to product complexity by entrapment of a supercoil in the synaptic complex. This was implied by the formation of Int-*att* site complexes that shorten the substrate DNA (*58*) and reveal a 10-bp periodicity of enhanced sensitivity to deoxyribonuclease (DNase) (*62*). Very similar results are seen with the complexes of DNA around a histone protein core in nucleosomes.

The fact that, under nonphysiological conditions, Int recombines a nicked substrate (*63*) provides a topological means of identifying supercoiling of the entrapped DNA. Reaction with nicked DNA yields unlinked species and linked ones (a trefoil from inverted *att* sites or a singly linked catenane from directly repeated sites) in about equal proportions (*17, 61*). Since nicked substrates can, on average, have no free supercoiling, and since the enzyme introduces only one node during exchange (*17, 29*), product nodes arise from Int binding.

The trefoil Int products were all positive (*29*). Because of the sign change accompanying inversion (Fig. 5), this result is most simply explained if a negative interdomainal supercoil is bound in the synaptic complex (*29*). According to the rules in Table 1, in order for this protein-stabilized ^{ter}Wr to be odd, the synapsed *att* sites must be aligned in parallel. Henceforth, we assume that these features of synapsis and exchange for nicked substrates hold true for the standard supercoiled substrate; this extrapolation must be confirmed, however, because the topological change introduced by Int can differ for relaxed and supercoiled substrates (*61*).

It appears then that the Int synaptic complex contains one interdomainal negative supercoil, whereas the resolvase complex has three. This difference is easily rationalized. The available data (*51, 57, 58*) suggest that each of the identical *res* sites is wrapped into a supercoil, whereas only one of the two distinct *att* sites, that from the phage, is supercoiled. To meet the requirements for parallel site alignment (Table 1) a third supercoil must be introduced by *res* site synapsis.

The torus structure of the knots and catenanes produced by Int from a supercoiled substrate requires that ^{ter}Me is equal to 1 if the sites are aligned in parallel (*17, 64*). The experiments with nicked Int substrates (*17, 61*) suggested that ^{ter}Me for inversion is equal to +1. ^{ter}Me for resolution is therefore expected to be −1, because the same local action by a recombinase has opposite effects on linkage for inverted and directly repeated sites (*16*). Analogously, the different values for ^{ter}Me for resolution by Int (−1) and resolvase (+1) may reflect a common mechanistic component. Both proteins add across a phosphodiester bond in each strand; the polarities of the intermediate thus formed, however, are opposite. Resolvase is covalently bound to a 5′ and Int to a 3′ phosphoryl residue (*51, 52*), with each poised to reclose the broken bond upon completion of exchange. If during exchange the two recombinases move the covalently bound strand with the same local motion relative to the synaptic complex, the global effect would be that ^{ter}Me for resolvase and Int will, as found, be equal in magnitude but opposite in sign.

Griffith and Nash have pointed out that for a four-stranded synaptic DNA intermediate covalent attachment of a pair of recombinases 5′ or 3′ to the four-stranded region will also lead to opposite signs for ^{ter}Me (*29*). This argument incorporates the suggestions of both Wilson and McGavin that perfect alignment of identical sequences can be maintained by hydrogen bonding *between* duplexes (*65*). Although pairing across the major groove as proposed by McGavin is stereochemically more favorable than the minor groove pairing of Wilson, recent topological data are more consistent with the latter configuration for a four-stranded DNA intermediate (*29*). In the Nash models, four-stranded DNA is fixed in the central

Table 2. In vitro formation of structurally characterized knots and catenanes

Protein agent	DNA	Substrate products	Examples	References
E. coli topoisomerase I	Nicked	All knot types	Fig. 1, c, d, i, k, l	(*23, 27*)
DNA gyrase	Supercoiled	Singly linked catenanes	Fig. 1, a, b	(*13*)
T4 topoisomerase	Supercoiled	Twist* knots with all or all but two (+) nodes	Fig. 1, c, k	(*8, 66*)
		Compound twist knots	Fig. 1, l	(*66*)
Int	Supercoiled or nicked	(+) Right-handed torus knots and right-handed torus catenanes	Fig. 1, d, g, i, j	(*17, 29*)
Resolvase	Supercoiled	(−) Singly linked catenanes	Fig. 1, a	(*15*)
		Nontorus knots and catenanes (minor products)	Fig. 8, c, d, e	(*28, 50, 26*)

*The structure of this family is explained in the legend to Fig. 1.

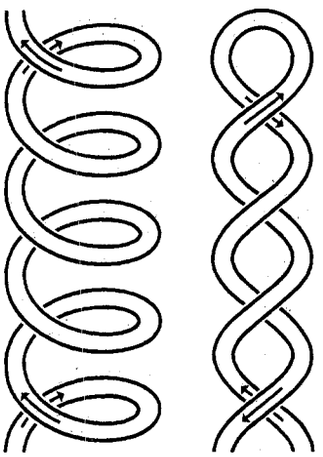

Fig. 9. The two forms of supercoiling. The supercoils in (A) and (B) are both (−), as can be seen from the node-defining arrows, but are left- and right-handed, respectively.

region between the position of the reversible nicks; the rotation of DNA strands around the borders of the four-stranded region would be equal in magnitude but opposite in direction for Int and resolvase.

According to a favored model of Nash (*29*), the overall linkage change (Me) for Int will be +2 for inversion. The first demonstration that recombination results in a fixed change in linking number showed just this increase in Int mediated inversion (*61*). Unfortunately, the value of Me for inversion cannot be deduced from this data alone because the overall linkage change was not also measured for knotted products (*16*). Determination of Me and ^{ter}Me for several recombinases could provide important support for the Nash four-stranded DNA intermediates; however, proof would require direct biochemical evidence.

Because the complexity of Int products is responsive to substrate supercoiling, Int is useful for analyzing the structure of DNA (*59*). DNA can be interwound as plectonemic supercoils or wound around an external axis as solenoidal coils (Fig. 9). The Int substrate is clearly plectonemic (*17*). Solenoidal negative supercoils form a left-handed helix; those that are plectonemic are right-handed (Fig. 9). Since the products of Int recombination are exclusively right-handed, and handedness is conserved by recombination (Fig. 5), the substrate supercoils must have been plectonemic.

Quantitatively, product complexity for a 3.5-kilobase substrate is nearly that predicted if synapsis is completely random and the linking number deficit of the substrate reflects only unbranched plectonemic supercoils (*48*). Thus, other forms of DNA that would lower supercoiling, such as left-handed helices, cruciforms, or denatured regions (*6*), are not present at appreciable levels, since they would reduce the number of interdomainal nodes that contribute to catenation.

The ability of Int to convert plectonemic supercoil nodes into invariant knot and catenane nodes also makes it a valuable probe for the structure of DNA in *E. coli* cells, provided that gyrase is blocked. In such experiments only about 40 percent of the linking deficit is in the form of plectonemic supercoils (*48*). The rest is driven into alternative forms, presumably by DNA binding proteins.

Perspectives

As ATP (adenosine triphosphate) is the energy coin of the cell, the node is the topological unit of exchange. Nodes from twist and writhe are in equilibrium and through the action of topoisomerases or recombinases can be changed totally or metamorphosed into knot and catenane nodes. Following the fate of these nodes is the essence of biochemical topology. Although recombination is far more complex than strand passage, results so far indicate that simple topological constants characterize the mechanisms of recombinases as well as topoisomerases.

Thus far the topology of the products of various systems has generally been different and always informative. This has been true for the products not only of DNA replication and site-specific recombination, but also of knotting by *E. coli* topoisomerase I (*23*) and by T4 topoisomerase (*66*) and of the combined action of *recA* protein and a topoisomerase (*67*). Thus each enzyme has a topological signature that reveals basic features of its mechanism and allows its handiwork in the cell to be ascertained by inspection.

The field continually builds upon itself. Table 2 lists the available methods for preparing defined knots and catenanes. As the structures of linked forms are determined they provide standards, controls and substrates for other studies. As the topology of processes is worked out, they become available as probes for DNA structure, DNA-protein interactions, and reaction mechanisms.

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Two-Dimensional Nuclear Magnetic Resonance Spectroscopy

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Great spectral simplification can be obtained by spreading the conventional one-dimensional nuclear magnetic resonance (NMR) spectrum in two independent frequency dimensions. This so-called two-dimensional NMR spectroscopy removes spectral overlap, facilitates spectral assignment, and provides a wealth of additional information. For example, conformational information related to interproton distances is available from resonance intensities in certain types of two-dimensional experiments. Another method generates ^{1}H NMR spectra of a preselected fragment of the molecule, suppressing resonances from other regions and greatly simplifying spectral appearance. Two-dimensional NMR spectroscopy can also be applied to the study of ^{13}C and ^{15}N, not only providing valuable connectivity information but also improving sensitivity of ^{13}C and ^{15}N detection by up to two orders of magnitude.

SINCE ITS DISCOVERY 40 YEARS AGO, NUCLEAR MAGNETIC resonance (NMR) spectroscopy has evolved continuously, becoming a powerful technique for studying molecular structures and interactions. In this article we describe a major development, two-dimensional Fourier transform pulse NMR (2-D FT NMR), which has extended the range of applications of NMR spectroscopy, particularly with respect to large, complex molecules such as DNA and proteins.

Jeener (*1*) first introduced the concept of 2-D FT NMR in 1971. This original experiment was analyzed in detail by Aue *et al.* (*2*) in a paper that provided the basis for the development of a tremendous number of new pulse sequences. One important application of the 2-D FT approach in NMR, suggested by Ernst and co-workers (*3*), is in magnetic resonance imaging (a new tool in diagnostic medicine), where it now has largely replaced the earlier projection-reconstruc-

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