The mechanism by which the type 2 topoisomerase from bacteriophage T4 mediates knotting of negatively supercoiled DNA was deduced from an analysis of product topology. The knotted products were nicked and then subjected to electrophoresis in order to separate species on the basis of the minimum number of crossings in the knotted form. Knots with defined numbers of crossings were purified and the configuration of these crossings determined in the electron microscope by the RecA coating method. The product knots were exclusively of the twist form, in which an interwound region is entrapped by a single interlock of two looped ends. The interwound region was of negative sign in >98% of the molecules examined, whereas the single interlock was equally likely to be positive or negative. These results are interpreted in terms of a model for knot formation in which random strand passage mediated by the topoisomerase links bent or branched portions of a superhelix that has a specific interwound geometry. Superhelix interwinding and DNA contacts stabilized by excess enzyme molecules explain the very high frequency of knotting.

Topoisomerases, by carrying out DNA strand passage reactions, fulfill a vital role in the replication and segregation of double helical DNA (1-4). In vitro, topoisomerases readily knot and unknot as well as catenate and decatenate DNA (5-11). In vivo, however, knotted and catenated DNAs accumulate to significant levels only in cells deficient for topoisomerase activity (2-4, 12). Although the active segregation of chromosomes might be postulated to drive decatenation in the cell, the same hypothesis could not explain the near absence of knots in wild-type cells, nor the rapid unlinking of catenated plasmids generated by recombination (13, 14). Rather, it seems likely that the intracellular state of the DNA is the predominant influence on the outcome of topoisomerase-mediated strand passage events, particularly for intramolecular processes. In order to investigate the influence of DNA structure on the action of topoisomerases, we have therefore undertaken a study of DNA knotting.

Topoisomerase-mediated knotting of duplex DNA in vitro has been well characterized for the Escherichia coli topoisomerase I (15, 16). The reaction, which requires a nicked or gapped substrate, is a simple strand passage reaction. Topoisomerase I breaks one strand of the duplex opposite a preexisting interruption in the other, allows a duplex segment to pass through the transient double-stranded break, and then rejoins the broken strand. The form of the knot is determined not by the enzyme, but by the random folds and twists of the DNA that become locked in place by strand passage. The resulting catalogue of products contains each entry of a mathematician’s knot table.

In light of the substantial insight into topoisomerase I action gained from studies of its knotting reactions (15-18), we set out to investigate the knotting of DNA by a type 2 enzyme from T4. Type 2 topoisomerases pass entire duplexes through each other and usually require ATP. In contrast to topoisomerase I, high concentrations of T4 topoisomerase can efficiently knot covalently closed DNA, provided that the substrate is supercoiled and no ATP is added to the reaction (6). If ATP is added, the T4 topoisomerase carries out multiple rounds of reaction, relaxing the substrate and untying any knots formed. Thus knotting by the T4 enzyme reflects a limited reaction with a supercoiled substrate, resulting, as we demonstrate here, in a unique family of products.

Our studies reveal a fundamental similarity between the type 1 and type 2 reactions; both enzymes knot DNA by carrying out random strand passage reactions. Both enzymes also augment greatly the frequency of knotting by what we interpret as a bridging of segments of the DNA chain. Striking dissimilarities in product distribution arise, nonetheless, due to conformational differences between relaxed and supercoiled DNA substrates. Moreover, a detailed analysis of the product array from the T4 topoisomerase reaction serves to solidify our picture of the structure of supercoiled DNA in solution as a branched interwound superhelix.

**EXPERIMENTAL PROCEDURES**

**DNA and Enzymes—** Plasmids pBR325, pBR51, and pA² have been described (19-21). T4 topoisomerase partially purified from an extract of phage-infected cells by DNA cellulose and hydroxyapatite chromatography was a gift of Ken Kreuzer (Duke University, Durham, NC). It was purified to homogeneity by chromatography on Sephacryl S-300 as previously described (22).

**Knotting Reactions—** Knotting was carried out in a mixture containing 50 mM Tris (pH 7.6), 60 mM KCl, 10 mM MgCl₂, 20 µg/ml DNA, and 40 µg/ml bovine serum albumin (22). The solution was prewarmed to 32 °C prior to addition of 1.75 µg of T4 topoisomerase/µg of DNA. After a 4-min incubation at 32 °C, the reaction was quenched by addition of EDTA to 10 mM. Following extraction of the mixture twice with phenol, the volume of the quenched reaction product was then reduced 2-fold by extraction with sec-butyl alcohol. The concentrated solution was then extracted twice with ether and the DNA precipitated with sodium acetate and ethanol. The precipitated DNA was resuspended to 0.1 reaction volume in TE buffer (10 mM Tris, pH 8.0, and 1 mM EDTA).

**Nicking Reactions—** After knotting, supercoils were removed from DNA by limited nicking (23) in a reaction containing 50 mM Tris (pH 7.6), 6 mM MgCl₂, 100 µg/ml each DNA and ethidium bromide, and 1.9 µg/ml DNase I. Electrophoretic analysis indicated that under these conditions, a 30-min reaction at 30 °C yielded approximately 90% nicked, 5% linear, and 5% supercoiled DNA. The reaction was quenched by addition of EDTA to 10 mM and NaCl to 150 mM. The
mixture was then extracted twice with phenol, the volume reduced 4-fold by extraction with sec-butyl alcohol, and the concentrated solution extracted twice with ether.

Gel Electrophoresis and Quantitation—Nicked knotted DNA samples (1 μg for analytical gels and 40 μg for preparative reactions) were subjected to agarose gel electrophoresis buffered with Tris-acetate containing 0.05% SDS1 or with Tris-borate (89 mM Tris, 89 mM borate, 2.5 mM EDTA, pH 8.3) (2, 21). Following staining with 0.5 μg/ml ethidium bromide, the banding pattern was recorded on Polaroid film and the negative scanned with a Molecular Dynamics densitometer.

Purification of Knotted DNA—Alternative methods (24) were used for the purification of knotted DNA with equivalent results. In one, samples were subjected to electrophoresis in a 0.7% agarose gel in Tris-borate for 38.5 h at 60 V. The gel was stained and a trough cut behind the trefoil species. The trough was filled with hydroxyapatite (Bio-Gel HTP) and electrophoresis carried out with reverse polarity for 2.25 h at 60 V. The hydroxyapatite was then packed into 0.5-ml columns, and each column was washed with a buffer of 10 mM potassium phosphate, pH 6.8, and 1 mM EDTA. The DNA was then eluted with 400 mM buffer and extracted sequentially with phenol, sec-butyl alcohol, and ether.

In the other procedure, samples were subjected to electrophoresis in a 0.75% agarose Tris-acetate gel for 43 h at 42 V. The gel was washed twice in water to remove SDS and then stained. Agarose slices containing knotted species were cut from the gel and dissolved in 10 mM phosphate buffer saturated with potassium iodide. The DNA samples were then loaded onto 0.5-ml columns of hydroxyapatite (Bio-Gel HTP) equilibrated with 10 mM buffer. The DNA was eluted as above, extracted with phenol, and dialyzed twice against TE at room temperature.

Electron Microscopy—Nicked, knotted DNAs were denatured by glyoxal treatment at 62 °C for 90 min (25). The single-stranded DNA was desalted and coated with RecA in the presence of 5 mM ATP and 5 mM magnesium acetate, as described previously (26). The DNA-protein complexes were then fixed with glutaraldehyde and tungsten shadowed for electron microscopy (25). Criteria for tracing knots and scoring nodes were as reported previously (15).

RESULTS

To explore the mechanism of the T4 topoisomerase knotting reaction, we first characterized the products by gel electrophoresis. A supercoiled 3800-bp plasmid, pBNW3.8d, was knotted with purified T4 topoisomerase and singly nicked with DNase I; nicking removes supercoiling but leaves knotting intact. The DNA was then subjected to gel electrophoresis and stained with ethidium bromide (Fig. 1A, lane 3). Under the conditions used, mobility in the gel was proportional to the minimum number of crossing, or nodes, in the knotted form.

Four features of the knotting reaction were evident from the electrophoretic analysis. 1) Topoisomerase-generated knots were generated with both odd and even numbers of crossings, forming a node ladder with steps-of-one spacing (Fig. 1A, lane 3). By comparison, catenanes resulting from the same substrate by recombination mediated by the λ integrase (Int) have even numbers of crossings exclusively (Fig. 1A, lanes 4 and 5). 2) The top rung of the T4 topoisomerase knot ladder was composed of trefoils, knots with 3 crossings. The trefoil (Fig. 2, a and b) is the simplest knot, since knot crossings are always integral and knots cannot be tied with fewer than 3 crossings. 3) The amount of a given product species declined with node number (Fig. 1A, lane 3). 4) Nicking of the T4 topoisomerase reaction required supercoiled substrate, since nicked or relaxed pBNW3.8d yielded less than 1% knotted product (data not shown).

Larger supercoiled substrates, pRR51 (5950 bp) and pA2 (6400 bp), yielded an even higher percentage of knotted products, 50–75% in a standard reaction. Fig. 1B shows the pA2 pattern. The distribution of knotted species for these larger plasmids was broad, ranging from 3-noded knotts to knotted products with 18 or more crossings. The spacing of the ladder rungs remained one node.

These electrophoretic analyses indicated that, like E. coli topoisomerase I, the T4 type 2 enzyme could tie knots with variable numbers of crossings of odd or even number. However, electrophoretic separation according to the minimum number of duplex crossings could not indicate whether the T4 enzyme produced all of the knots seen with topoisomerase I, since the number of crossings is an incomplete descriptor of all but the 4-noded knot. There is only a single knot with 4 nodes. There are, however, two enantiomeric knots with 3 nodes and eight knot isomers with 6 nodes. To determine which particular isomers arose in knotting reactions with the T4 topoisomerase, we undertook an electron microscopic analysis of the reaction products.

In order to visualize overlying and underlying DNA segments at knot crossings, we thickened the DNA by coating it with RecA. Since RecA reacts more readily with single- than double-stranded DNA, gel-purified knots were denatured to separate the knotted single strand of each duplex from the nicked strand. This does not affect the topology of the knot. The single-stranded DNA was then reacted with RecA, adsorbed to carbon-coated grids, shadowed, and examined by electron microscopy.

We began our analysis with the 3-noded knots or trefoils. Trefoils can be of only two forms, distinguished by whether the three, irreducible nodes are of positive or negative sign (Fig. 2, a and b). The sign convention is explained in the legend to Fig. 2. The trefoils formed by T4 topoisomerase were overwhelmingly of one enantiomeric form; 85 out of 87 trefoils examined were negative in sign; only two were positive. Examples are shown in Fig. 3.

The strongly biased array of product trefoils formed by the T4 topoisomerase is in sharp contrast to the racemic mixture of (+) and (−) trefoils from an E. coli topoisomerase I knotting reaction (15). Our gel analysis revealed, however, that the T4 enzyme, like topoisomerase I, generated the 4-noded knot, which contains 2 (+) as well as 2 (−) nodes. Furthermore, the T4 topoisomerase is known to relax both positive and negative

---

1 The abbreviations used are: SDS, sodium dodecyl sulfate; bp, base pair(s).
supercorks (6) and can thus carry out binding and catalysis at both (−) and (+) nodes. These facts, the topology of the 3- and 4-noded knots, and a catalytic mechanism that is blind with regard to node sign can all be accommodated by a model postulating random strand passage events between portions of a negatively interwound supercoiled DNA substrate, as illustrated in Fig. 4 and detailed below.

If conformational fluctuations of the supercoiled substrate or topoisomerase-stabilized DNA bridges bring one loop of DNA across a second loop, a (+) node and a (−) node are formed. If the topoisomerase breaks one duplex at either the (+) or (−) node and allows the crossing duplex to pass through the gap before resealing the broken DNA, a knot of the twist family is produced (Fig. 4). The (−) sign of the crossings in the twisted region of the knot reflects the (−) supercoiling of the plasmid substrate. In the process of strand passage supercoils have been converted from geometric forms that can be unfolded to topological forms that cannot.

Two distinct classes of twist knots will arise depending on which of the overlap nodes is the site of reaction (Fig. 4). If the topoisomerase carries out strand passage at the (+) node, converting it to a (−) node, the knot will contain an odd number of nodes, all negative in sign. The 3- and 5-noded examples are shown, respectively, in Fig. 2, a and d. If the topoisomerase instead acts at the (−) overlap node to convert it into a (+) node, the knotted product will have a twist region containing an even number of (−) nodes and a linking region with two (+) nodes, as in the 4- and 6-noded knots in Fig. 2, c and e.

FIG. 2. **Topology of DNA knots.** Projection drawings of knotted molecules are shown. Knots are classified as to number and configuration of the irreducible number of nodes, or crossings. Knots a and b have 3 nodes, knot c has 4 nodes, knots d and g have 5 nodes, and knots e, f, and h have 6 nodes. If two molecules have an equal number of nodes, but one cannot be stretched to superimpose on the other or its mirror image, the two knots are distinct and diastereomeric. If one knot can only fit the mirror image of the other, the two are enantiomers distinguished by node signs. Node sign, shown for a single node in the top row of knots, is determined separately for each crossing. Arrowheads denote an arbitrary orientation along the entire DNA axis. A node is negative in sign if the overlying strand can be aligned with the underlying one by a clockwise rotation of less than 180°. The node is positive if a counterclockwise rotation results in alignment.

FIG. 3. **Electron microscopy of trefoil knots.** Purified trefoil knots made by T4 topoisomerase were denatured, coated with RecA protein, adsorbed to grids, shadowed with tungsten, and photographed in the electron microscope. A, pRR51 knots. B, pRR51 (left) and pA5 (right) knots. Each micrograph is accompanied by a tracing showing the relative overlay of strands at each crossing and by a redrawing of the knot with only the minimum number of crossings. Each knot has 3 irreducible (−) nodes.
By this model, product trefoils are of (-) sign not because the enzyme must invert a (+) node to form a knot, but because inversion of a (-) node will form a knot with an even number of nodes in its fully unfolded form. Rare (+) trefoils could be formed by the chance entrapment of a single (+) node by the enzyme and strand passage at a (-) node (27).

This model leads to the strong prediction that the knots formed by T4 topoisomerase should be restricted not only in node sign, but also in knot form. Only twist knots are expected to arise by the mechanism shown in Fig. 4. For knots with an odd number of crossings, all crossings should be of the same sign (Fig. 5). Among 5-noded knots, the proposed mechanism predicts that the T4 topoisomerase will produce only one of the four possible forms. The expected knot is a twist form with 5 (-) nodes (Fig. 2d), whereas the twist knot with 5 (+) nodes and the torus knots with 5 (-) nodes (Fig. 2g) or 5 (+) nodes should not appear. When 5-noded knots generated by T4 topoisomerase were examined, all 39 molecules were twist knots with 5 (-) nodes (Fig. 5). None of the other possible 5-noded knots, all of which are formed by topoisomerase I, were observed.

Although the analysis of the 5-noded knots offered substantial support for the supercoiled DNA-directed knotting model described above, an anomaly in the electrophoretic gel profile prompted us to extend our analysis to the 6-noded knots. Close examination of the electrophoretic ladders of T4 topoisomerase knots revealed two knotted species in the region of the gel where 6-noded molecules migrate (Fig. 1B). A simple reaction of the type illustrated in Fig. 4 should give rise to only a single type of 6-noded knot, a twist knot with 2 (+) and 4 (-) nodes (Fig. 2e). We noted, however, that the total amount of 6-noded species was greater than that expected by extrapolation from the amount of knots with fewer crossings. The 3-noded, 4-noded, and 5-noded knots form a series of decreasing abundance (Table I). In contrast, the total amount of 6-noded knots from the two larger substrates, for which a second 6-noded species is more evident in gel profiles, is greater than the amount of 5-noded knots.

The relative overabundance of 6-noded species suggested that there might be a pathway for forming 6-noded knots that is inaccessible to knots with fewer crossings. The 3-noded, 4-noded, and 5-noded knots form a series of decreasing abundance (Table I). In contrast, the total amount of 6-noded knots from the two larger substrates, for which a second 6-noded species is more evident in gel profiles, is greater than the amount of 5-noded knots.

The relative overabundance of 6-noded species suggested that there might be a pathway for forming 6-noded knots that is inaccessible to knots with fewer crossings. The 3-noded, 4-noded, and 5-noded knots form a series of decreasing abundance (Table I). In contrast, the total amount of 6-noded knots from the two larger substrates, for which a second 6-noded species is more evident in gel profiles, is greater than the amount of 5-noded knots.

The relative overabundance of 6-noded species suggested that there might be a pathway for forming 6-noded knots that is inaccessible to knots with fewer crossings. The 3-noded, 4-noded, and 5-noded knots form a series of decreasing abundance (Table I). In contrast, the total amount of 6-noded knots from the two larger substrates, for which a second 6-noded species is more evident in gel profiles, is greater than the amount of 5-noded knots.

The relative overabundance of 6-noded species suggested that there might be a pathway for forming 6-noded knots that is inaccessible to knots with fewer crossings. The 3-noded, 4-noded, and 5-noded knots form a series of decreasing abundance (Table I). In contrast, the total amount of 6-noded knots from the two larger substrates, for which a second 6-noded species is more evident in gel profiles, is greater than the amount of 5-noded knots.

The relative overabundance of 6-noded species suggested that there might be a pathway for forming 6-noded knots that is inaccessible to knots with fewer crossings. The 3-noded, 4-noded, and 5-noded knots form a series of decreasing abundance (Table I). In contrast, the total amount of 6-noded knots from the two larger substrates, for which a second 6-noded species is more evident in gel profiles, is greater than the amount of 5-noded knots.

The relative overabundance of 6-noded species suggested that there might be a pathway for forming 6-noded knots that is inaccessible to knots with fewer crossings. The 3-noded, 4-noded, and 5-noded knots form a series of decreasing abundance (Table I). In contrast, the total amount of 6-noded knots from the two larger substrates, for which a second 6-noded species is more evident in gel profiles, is greater than the amount of 5-noded knots.

The relative overabundance of 6-noded species suggested that there might be a pathway for forming 6-noded knots that is inaccessible to knots with fewer crossings. The 3-noded, 4-noded, and 5-noded knots form a series of decreasing abundance (Table I). In contrast, the total amount of 6-noded knots from the two larger substrates, for which a second 6-noded species is more evident in gel profiles, is greater than the amount of 5-noded knots.

The relative overabundance of 6-noded species suggested that there might be a pathway for forming 6-noded knots that is inaccessible to knots with fewer crossings. The 3-noded, 4-noded, and 5-noded knots form a series of decreasing abundance (Table I). In contrast, the total amount of 6-noded knots from the two larger substrates, for which a second 6-noded species is more evident in gel profiles, is greater than the amount of 5-noded knots.

The relative overabundance of 6-noded species suggested that there might be a pathway for forming 6-noded knots that is inaccessible to knots with fewer crossings. The 3-noded, 4-noded, and 5-noded knots form a series of decreasing abundance (Table I). In contrast, the total amount of 6-noded knots from the two larger substrates, for which a second 6-noded species is more evident in gel profiles, is greater than the amount of 5-noded knots.

The relative overabundance of 6-noded species suggested that there might be a pathway for forming 6-noded knots that is inaccessible to knots with fewer crossings. The 3-noded, 4-noded, and 5-noded knots form a series of decreasing abundance (Table I). In contrast, the total amount of 6-noded knots from the two larger substrates, for which a second 6-noded species is more evident in gel profiles, is greater than the amount of 5-noded knots.

The relative overabundance of 6-noded species suggested that there might be a pathway for forming 6-noded knots that is inaccessible to knots with fewer crossings. The 3-noded, 4-noded, and 5-noded knots form a series of decreasing abundance (Table I). In contrast, the total amount of 6-noded knots from the two larger substrates, for which a second 6-noded species is more evident in gel profiles, is greater than the amount of 5-noded knots.
The left is a twist knot with the right is a granny, the compound knot composed of two trefoils, crographs each with in Fig. were observed, we conclude that these knotted products indeed result from random catalysis by T4 topoisomerase acting topoisomerase knotting reactions (6) appear quite similar. The most abundant species and the molecules form a steps-of-twist knots. The odd-noded simple twist knots contained only on an ordered DNA structure. Nonetheless, the knotted products of T4 topoisomerase reaction are clearly distinguishable from the products of processive recombination by Tn3 resolvase, which have only an even number of nodes and can be neither torus nor twist (26). Coincidentally, however, experiments in which the T4 topoisomerase knots served as topological standards demonstrated that the products of processive recombination by the Gin invertase constitute the same subset of knots as is produced by the T4 enzyme (29).

Paradoxically, the non-random nature of the T4 topoisomerase products provides substantial support for a random strand passage mechanism for the enzyme. Our proposed mechanism, in which substrate configuration plays a critical role in determining knot structure, is illustrated in Fig. 4 for a single catalytic cycle. Of 148 molecules examined, 146 can be viewed as twist knots with (−) nodes in the interwound region (Table II). This result can only be readily understood by assuming that the sign of the twist region reflects the supercoiling of the substrate. There is in fact evidence from electron microscopy, the structure of the Int products, and computer simulation of DNA structure that the linking deficit of DNA is expressed in the form of interwound (plectonemic) rather than solenoidal supercoils (28, 30–32). The current results confirm this important conclusion for DNA in solution.

Knotted plasmid DNAs have also been analyzed from a mutant E. coli strain in which a deletion of the topoisomerase I gene and a compensatory mutation in the gyrase B gene lead to the accumulation of knotted DNAs at a level 10 times that of wild-type strains (12, 33). Those knots analyzed by the RecA coating method appear, like the T4 enzyme products, to be (−) twist knots, but there is an unexplained preponderance of knots with an odd number of nodes. Increased knotting has also been detected in wild-type strains upon addition of high concentrations of the DNA gyrase inhibitor, norfloxacin;" the structure of these knots is not known. The production of (−) twist knots in the mutant strain suggests a mechanism of knot formation similar to that proposed here for the in vitro reaction with T4 topoisomerase and further indicates that DNA inside of E. coli cells is a (−) interwound superhelix.

**Knotting Frequency and Specificity**—As pointed out originally by Liu et al. (6), the efficiency of knotting (up to 75%) by T4 topoisomerase in vitro is far greater than expected on the basis of theoretical calculations. The probability of knotting can be estimated for molecules modeled as a series of connected but freely jointed (Kuhn) segments of sufficient length to account for the bending stiffness of the DNA. Each such Kuhn segment is twice the persistence length. Using the accepted value for the length of the Kuhn segment of free DNA (1000 Å) and for the effective diameter of DNA (36), we calculate that at equilibrium only about 1–2% of relaxed DNA would be knotted for plasmids in the size range (3800–6400 bp) we used.

These calculations fail to take into account the presence of excess topoisomerase molecules and the supercoiling of the substrate. It has been suggested that a restriction on the volume of the DNA chain imparted by both supercoiling and excess bound protein forces a more tortuous path for the

---

**Table II**

**Topology of knots tied by T4 topoisomerase**

The predicted knot topology is that derived from the scheme shown in Fig. 4.

<table>
<thead>
<tr>
<th>Number of nodes</th>
<th>Predicted knot node composition*</th>
<th>Number of found/total knots scored</th>
<th>Predicted knot form shown</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>3 (−) (twist)</td>
<td>85/87</td>
<td>Figs. 2a and 3</td>
</tr>
<tr>
<td>4</td>
<td>2 (−), 2 + (twist)</td>
<td>ND*</td>
<td>Fig. 2c</td>
</tr>
<tr>
<td>5</td>
<td>5 (−) (twist)</td>
<td>39/39</td>
<td>Figs. 2d and 5</td>
</tr>
<tr>
<td>6</td>
<td>4 (−), 2 + (twist) or</td>
<td>12/12</td>
<td>Figs. 2e and 6</td>
</tr>
<tr>
<td>3 (−), 3 (−) (granny)</td>
<td>10/10</td>
<td>Figs. 2f and 6</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>146/148</td>
<td></td>
</tr>
</tbody>
</table>

*Knot type is indicated in parentheses.

*Not determined (only one possible structure).

possible 6-noded knots (4), yet only the two predicted forms were observed, we conclude that these knotted products indeed result from random catalysis by T4 topoisomerase acting on an ordered DNA structure.

**DISCUSSION**

**Knotting Mechanism**—When analyzed by gel electrophoresis, the products of E. coli topoisomerase I (15) and T4 topoisomerase knotting reactions (6) appear quite similar. Each reaction yields knotted products in which the trefoil is the most abundant species and the molecules form a steps-of-one electrophoretic ladder. Nonetheless, the knotted products of T4 topoisomerase reaction are clearly distinguishable from the knots tied by topoisomerase I. Whereas topoisomerase I produces every knot theoretically possible (15), we have demonstrated here that the T4 topoisomerase reaction gives rise to a unique subset of the products seen with topoisomerase I. The results are summarized in Table II.

The 3-, 4-, 5-, and 6-noded knots produced by T4 topoisomerase are all either simple twist knots or composites of two twist knots. The odd-noded simple twist knots contained only (−) nodes; the even-noded simple knots also had 2 (+) nodes. Despite the topological sign difference the simple knots shared an overall geometry: in each there is a (−) interwound region of variable length held in place by a single interlock (see Fig. 2, a, c–e). Only two exceptional knots of (+) sign, both 3-noded, were found among 148 examined. Such molecules probably arise during those rare circumstances when the high concentration of bound topoisomerase captures a (+) node in the otherwise negatively supercoiled DNA substrate (27).

The knots formed by the T4 topoisomerase differ from those formed by the Int recombinase, which are exclusively (+) torus knots with an odd number of nodes (28), and those formed during progressive recombination by Tn3 resolvase, which have only an even number of nodes and can be neither torus nor twist (26). Coincidentally, however, experiments in which the T4 topoisomerase knots served as topological standards demonstrated that the products of processive recombination by the Gin invertase constitute the same subset of knots as is produced by the T4 enzyme (29).

**Fig. 6.** Electron microscopy of 6-noded knots. Electron micrographia of purified 6-noded knots of pA2*, together with tracings and redrawings with the minimum number of crossings. The molecule on the left is a twist knot with 4 (−) and 2 (+) nodes. The molecule on the right is a granny, the compound knot composed of two trefoils, each with 3 (−) nodes.
chains. This is equivalent to a reduction in the effective Kuhn length and, in turn, explain the observed high frequency of knotting (6). Indeed, Monte Carlo calculations show that the probability of knotting increases greatly with the number of Kuhn segments (34), reflecting the greater flexibility of a DNA composed of an increased number of segments. However, we calculate that the effective Kuhn length would have to be reduced to an implausibly low value of less than two turns of the double helix to explain our pBNW3.8d results. Furthermore, if supercoiling and excess protein were simply equivalent to increasing the effective number of Kuhn segments, knots of all topological types would be produced. Instead only a particular subclass of knot topoisomerases are observed.

Frank-Kamenetskii and Vologodskii (35) proposed an alternative mechanism for the efficient knotting by T4 topoisomerase. Knotting frequency varies inversely with effective DNA diameter (36), and they suggested that excess protein provides an attractive potential between DNA segments and thereby a smaller effective DNA diameter. An attractive potential does indeed increase greatly the calculated frequency of knotting (35). Their model, however, also does not account for the specificity of product knot structure.

In searching to explain both the efficiency and specificity of the T4 topoisomerase knotting reaction we first consider the consequences of high amounts of T4 topoisomerase. *E. coli* topoisomerase I is known to play a stoichiometric role in its knotting reaction; knotting requires a 20-fold greater level of topoisomerase I than is necessary for strand passage (15). High levels of topoisomerase are also needed for knotting by the T4, *Drosophila*, *Bonbyx mori*, and HEA type 2 enzymes (6, 9, 10); we routinely use a stoichiometry of 15-20 molecules of T4 topoisomerase (M = 260,000) per plasmid.

Since a topoisomerase must bind two separate DNAs in order to pass them through each other, it should act as a bidentate ligand and thus cross-link, or bridge, disparate points along the DNA axis. Indeed, the type 2 topoisomerase can knot nicked DNA roughly 7-fold. The knots produced by simulation were found to be reduced to an implausibly low value of less than two turns of the double helix to explain our pBNW3.8d results. Furthermore, if supercoiling and excess protein were simply equivalent to increasing the effective number of Kuhn segments, knots of all topological types would be produced. Instead only a particular subclass of knot topoisomerases are observed.

Thus, supercoiling alone cannot explain the high degree of knotting. Moreover, the requirements for a high amount of topoisomerase and the absence of ATP argue persuasively for some stoichiometric role of the protein. Just as persuasively, the (−) twist topology of the product knots clearly indicates that (−) supercoiling supplies the interwound part of the knot. Our conclusion then is that the high frequency of knotting requires a structural contribution from both plectonemic supercoiling and protein cross-linking.

Given that bending of the superhelix to bring nearby points on the DNA into contact should be energetically disfavored, one would expect knots with many nodes to predominate among the products, contrary to what is observed. Rather it seems likely that the branching of interwound DNA (30) provides a ready means for facilitating contact between disparate points without requiring extensive bending of the superhelix, as shown in Fig. 4. The equation derived in the appendix that relates substrate supercoil structure to the number of nodes in the knots produced by a type-2 topoisomerase confirms the reduction in knot complexity by branching. Moreover, the predicted (5.9) and measured (5.4) average number of knot nodes for pBNW3.8d are in very good agreement (see Appendix; Ref. 40).

In summary, the topology of the T4 topoisomerase knots is explained by the branched (−) plectonemic supercoiling of the substrate and random strand passages by the enzyme. The type 2 topoisomerase is greatly increased over that calculated from random passage in relaxed molecules by both supercoiling and topoisomerase-induced crosslinking of the substrate.

REFERENCES


3 S. Levene and N. R. Cozzarelli, unpublished results.