lowered, the spectrum of the tris phosphine undergoes the changes shown in Figure 3. In this case, as the rate of interconversion is slowed, the methylene protons remain chemically equivalent, but become magnetically nonequivalent due to different spatial relationships with each of the three PF_3 groups. A computer calculation of the low-temperature spectrum was found to agree quite well with the pattern observed at −93°. The proton spectrum of the bis phosphine also shows broadening at lower temperatures, and the limiting pattern is even more complex.

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Interlocked Deoxyribonucleic Acid Rings

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In an ordinary molecule, different parts of the molecule are held together by chemical bonds. The situation is different for two interlocked rings (a dimeric catenane), depicted diagrammatically in Figure 1. There is no chemical bond between the two component rings, yet the separation of the two rings requires the breakage of some chemical bonds, at least transiently, in one of the two rings. Therefore the two rings are said to be bonded by a topological bond or non-bond.[1] The possible existence of such a species was probably realized very early, after the discovery of cyclic compounds. The earliest known discussion of such a species was attributed to Willstätter in 1912,[1] although serious considerations on the formation of such a species were initiated only two decades ago when the formation of large polymeric rings was realized.

The first catenane was reported by Wasserman in 1960.[2] The formation of a cyclic acyloin from the linear molecule $EtO_2C(CH_2)_{32}CO_2Et$ by reduction with Na, in the presence of a high concentration of a 34-membered ring compound, $C_{32}H_{63}D_5$, was believed to give a catenane, depicted in Figure 2.

The formation of such a dimeric catenane is due to the threading of one ring by a linear chain which cyclizes subsequently. It is intuitively clear that the probability of interlocked ring formation is appreciable only when the sizes of both rings are sufficiently large. Steric consideration alone predicts that no interlocked rings would form if the number of carbon atoms in either ring is less than 20.[1]

Interlocked DNA Rings

It was discovered by Hershey and his coworkers in 1963 that the DNA molecule of the bacteriophage λ (molecular weight 30.5×10^6) possesses two "cohesive ends" which can join to give a ring with a contour length of ~170,000 Å.[3] It turned out that the cohesive ends are protruding single-stranded oligonucleotides with base sequences complementary (but not identical) to each other.[4] Therefore the left end may join with the right end by the formation of a short segment of double-stranded DNA. If the ends are on the same molecule, a ring results. If the ends are on different molecules, a dimer or higher aggregate results. Since the discovery by Hershey, *et al.*, many other DNA molecules have been found to possess cohesive ends, some with sequences very similar to or identical with those of λ, and some with quite different base sequences.[5]

The physicochemical aspects of DNA ring formation were studied by Wang and Davidson.[6] The DNA of λ is a fairly large molecule. In solution it resembles more or less a loose ball of thread with a root mean square radius of ~5000 Å. Since interpenetration of molecules can readily occur, the question whether a significant amount of interlocked rings are formed when the DNA molecules cyclize has been frequently raised.

The answer to the question is fairly straightforward. As long as one starts with a system of linear molecules, the fraction of interlocked rings which can

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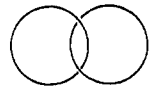

Figure 1.

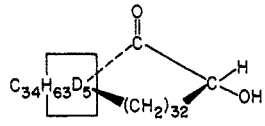

Figure 2.

be obtained by the cohesion of the ends is no more than a few per cent by mass, at any DNA concentration. This is because at high concentrations of DNA the major species resulting from the joining of ends are linear aggregates of very high molecular weight.[6] At low concentrations ring formation is favored, but intermolecular overlapping and interpenetration decrease.

Therefore the way to obtain a significant amount of interlocked rings is to allow the cyclization of linear molecules at *low* concentrations in the presence of a *high* concentration of preformed rings.[1,7] Wang and Schwartz first demonstrated the formation of interlocked rings by cyclizing 5-bromouracil (5-BrUra) labeled λ DNA in the presence of phage 186 DNA rings.[7] The system was chosen for the following reasons. Firstly, since 186 DNA and λ DNA have different kinds of cohesive ends, no joining of a λ cohesive end and an 186 cohesive end can occur.[7] Secondly, the substitution of bromine atoms in 5-BrUra labeled λ DNA for methyl groups in ordinary DNA decreases the specific volume of the DNA, since a Br atom is denser than a methyl group. This allows the separation of a 5-BrUra-labeled DNA from an unlabeled DNA by a technique widely used in molecular biology: density gradient centrifugation.[8]

If a concentrated salt solution (CsCl is frequently used because of the high density attainable) is spun at high speed (say 40,000 rpm) in an ultracentrifuge, the centrifugal force causes sedimentation of the solute molecules, and a concentration gradient is soon generated in the direction of the centrifugal field. The force opposing the generation of such a gradient is diffusion. Therefore, a sedimentation-diffusion equilibrium is eventually established. For a given solution at a given temperature and centrifugal field, the final equilibrium concentration gradient is completely predictable from the thermodynamic properties of the solution. Since the density of a solution is dependent on its concentration, a concentration gradient also means a density gradient. If a macromolecular species is present in such a density gradient of the proper range, it will form a band of a finite width dependent upon its molecular weight. In other words, the distribution of the macromolecular species will be centered around a position at which it is "buoyant."

For a 5-BrUra-labeled and an unlabeled DNA in a density gradient, the lower specific volume of the former means that it would assume a position of higher density in the gradient, while the latter would assume a position of lower density. If interlocked rings are formed between the labeled and unlabeled DNAs, species of intermediate "buoyant densities" would be observed.

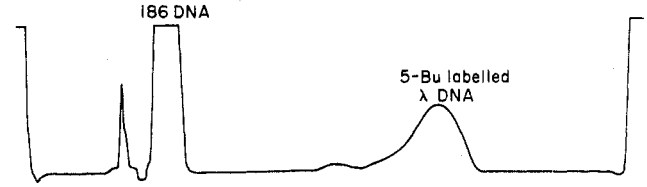

Figure 3. 5-BrUra-labeled λ DNA was cyclized in the presence of cyclic 186 DNA and the sample was analyzed by density gradient centrifugation. The 186 DNA peak is off scale due to the high initial concentration of this DNA. The sharp peak to the left of the 186 DNA band is the air–CsCl solution meniscus. The band between 186 DNA and 5-BrUra-labeled λ DNA is the dimeric catenane with interlocked λ DNA and 186 DNA rings. Its position can be predicted from the known molecular length of the two DNAs.[5] Taken from Figure 4a of ref 7 through the courtesy of *Biopolymers*.

Figure 3 depicts the result of such an analysis. A species of intermediate buoyant density is clearly seen. Since Wang and Schwartz have carefully demonstrated that no stable aggregate can form between linear 186 DNA and λ DNA, the intermediate species observed is undoubtedly due to the presence of interlocked rings.[7]

DNA Catenanes *in Vivo*

A DNA molecule, being the genetic material, differs from most of the other molecules in that it replicates *in vivo*. For a catenane, if the replication products of each component ring are rings, a very complex catenane would result after several generations of replication (see below). This led Wang and Schwartz to suspect that catenanes are probably nonviable.[7] This early conjecture appears to be unfounded.

The existence of DNA catenanes *in vivo* was first discovered by Vinograd, *et al.*[9-11] It was found that mitochondrial DNA from a number of sources contained an appreciable amount of interlocked rings. Catenanes have also been observed by others in plasmid DNA,[12] in intracellular animal and bacterial virus DNA,[13-16] and, most dramatically, in kinetoplast DNA from *Trypanosoma cruzi* and *Leishmania tarentolae* (Plate I).[17-19]

Formation of DNA Catenanes by Replication

There are two mechanisms for the formation of DNA catenanes *in vivo:* replication and genetic recombination.[20,21] Several models have been pro-

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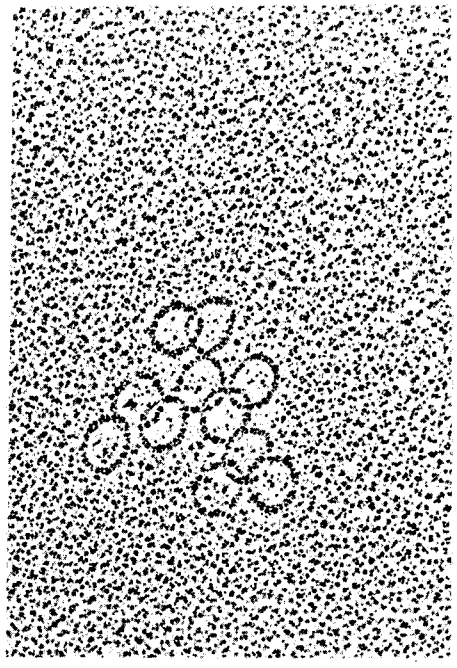

Plate I. An electron micrograph of interlocked kinetoplast DNA rings from Leishmania tarentolae. Taken from Plate IIf of ref 19 through the courtesy of the *Journal of Molecular Biology*. I thank Dr. L. Simpson for providing me with the micrograph.

posed for the replication of a circular DNA. It is easy to visualize that, if a linear replicative intermediate is involved, there is a certain probability of yielding interlocked rings when the linear intermediate recyclizes in the presence of other DNA rings. The "rolling circle" model for DNA replication, *e.g.*, involves a linear replicative intermediate.[20,22,23]

The problem of catenane formation by replication is more intriguing when replication schemes which do not involve linear intermediates are considered. Take the model first proposed by Cairns, for example (Figure 4).[24] Normally two segregated daughter molecules are expected. In other words, complete separation of the two parental strands results. If there are N helical turns in the parent molecule, the two strands must rotate around each other N times during one round of replication. This can only be achieved if there is a *swivel point* in the parent molecule, around which free rotation can occur. In Figure 4b, the swivel point is represented by an interruption in one of the parental strands. Unwinding of the strands is achieved by rotation around one of the single bonds opposite to this interruption.[25] The events immediately preceding the final segregation of the daughter rings are at present unknown. It is reasonable to expect that replication cannot pass through the interruption, otherwise one of the daughter molecules would be broken. If the interruption is sealed while one (or more) parental helical turns remains, then, following the disruption of base

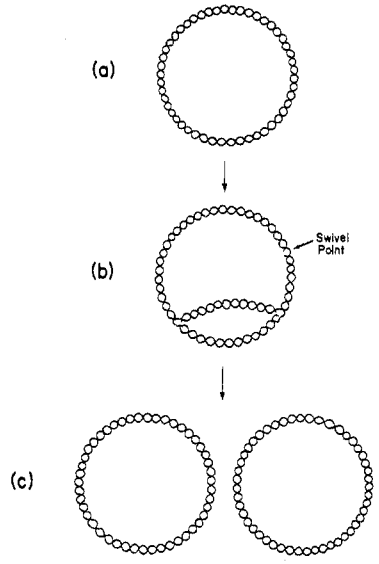

Figure 4. The Cairns' model for the replication of a DNA ring into two separate daughter rings. The events preceding the segregation of the daughter rings are unknown. An error can conceivably give two interlocked rings instead.[21]

pairs between the parental strands and the completion of the daughter strands, two interlocked rings would result.[15] How does the replication apparatus achieve the complete separation of the parental strands, normally *without* the formation of interlocked rings, is therefore an even more intriguing question. So far very few experiments have been directed toward the elucidation of this problem.

Once a dimeric catenane is formed, the replication of either ring in a normal fashion would yield a trimeric catenane. This reaction can be denoted as

$$\text{CtpC} \xrightarrow{\text{replication}} \text{C'tpCtpC'}$$

The symbol tp denotes a topological bond and the superscript "prime" denotes the daughter molecules from the first round of replication. Note that if a trimeric catenane is derived from a dimeric catenane by replication, the two rings on the sides are always identical twins (a dimeric catenane XtpY would give either Y'tpXtpY' or X'tpYtpX'). The replication of the trimer can either give A (C'' denotes a daughter molecule derived from C') or B depending upon whether a ring on the side or in the center is replicated.

$$\begin{matrix} \text{C''tp} \\ \text{C''tp} \end{matrix}\text{CtpC'} \qquad \text{C'}\begin{matrix} \text{tpC'tp} \\ \text{tpC'tp} \end{matrix}\text{C'}$$

A B

Formation of DNA Catenanes by Recombination

Interlocked DNA rings can also result from several modes of genetic recombination.[20] Catenation *via* a two-step reciprocal recombination mechanism[21] is illustrated in Figure 5. The second step shown, the internal recombination within the circular dimer C_2, can give either a catenane or two separate rings. A reciprocal recombination between two interlocked rings always gives a dimeric ring, C_2. Entropic considerations of ring formation predict that, if an equilibrium is established between C_2 and C_1tpC_1, the formation of the former is favored over the latter

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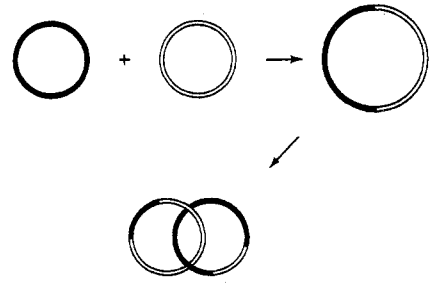

Figure 5. A diagrammatic drawing showing the formation of a catenane by a two-step reciprocal recombination.[21]

by approximately a factor of 30.[6] Therefore, if recombination events are frequent in a cellular system, interlocked rings are not expected to be stable. It has been observed recently that for the animal virus SV40 the newly synthesized DNA contains a higher fraction (a few tenths of 1%) of catenated dimers, which then decays gradually, probably to circular dimers or monomers.[16]

Catenanes can also form by a nonreciprocal recombination event between two separate rings. For example, if one ring is linearized by an enzyme which makes two staggered single-chain cuts to give a λ DNA type molecule with two cohesive ends, a catenane may result when the linear DNA molecule recyclizes.

A special case of nonreciprocating recombination involving a partially replicated molecule is of some interest (Figure 6). The breaking and rejoining of the DNA duplex anywhere in the "θ" shaped replication intermediate may give a catenane at the end of the replication, depending upon the topology of threading between the breaking and rejoining events.

The exact origin of interlocked rings in a particular cellular system is therefore coupled to the particular mechanisms of recombination and replication. The best documented case is for the formation of catenanes of φX174 duplex DNA, where both replication and nonreciprocal recombination appear to be involved in catenation. If the mechanisms of replication or recombination are altered by certain factors, the fraction of interlocked DNA rings may change as a result. This is probably why the fraction of catenanes in the mitochondria of leukocytes of some leukemia patients is higher than that in normal persons,[10] and that the fraction of catenanes in kinetoplast DNA decreases when a trypanocidal drug, ethidium bromide, is administered.[19]

Probability of Formation of Interlocked Rings

Estimates can be made on the probability of formation of interlocked rings *in vitro*. It is helpful to describe briefly a simple and useful model for a DNA chain—the random-coil model. The long DNA chain is represented by a coil of many segments, each of length b, and joined by universal joints such that any two adjacent segments can assume any orientation with respect to each other. It can be shown that the root-mean-square radius R [defined as $(\Sigma m_i r_i^2/\Sigma m_i)^{1/2}$, where m_i is the mass of the ith element and r_i is its distance to the center of mass] of a linear chain composed of n segments is $(nb^2/6)^{1/2}$ or $(bL/6)^{1/2}$, where $L = nb$ is the contour length of the chain. For a DNA ring, R equals $(bL/12)^{1/2}$. Hydrodynamic measurements indicated that b is of the order of 10^3 Å in aqueous solution, depending upon the ionic strength.[26] L can be readily obtained from the molecular weight, since two adjacent base pairs are separated by 3.4 Å in the normal B configuration.

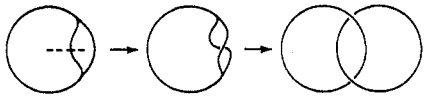

Figure 6. A diagrammatic drawing showing the breaking and rejoining of a segment of a partially replicated DNA ring. If two separate rings would normally result, the breaking and rejoining events would give two interlocked rings with a high probability.

Frisch and Wasserman considered the formation of interlocked rings by the threading of a ring with a linear molecule which subsequently cyclizes.[1] The probability of dimeric catenane formation is considered to be the probability of overlap of the segment distribution of the two molecules forming the catenane times a geometric factor $\bar{\beta}$. The factor $\bar{\beta}$ accounts for the fact that only a fraction of molecules with overlapping segment distribution gives interlocked rings. By trial with models, they estimated that $\bar{\beta}$ is approximately ½.

Wang and Shwartz considered the problem from a somewhat different point of view.[7] Consider the equilibrium

$$X + Y \rightleftharpoons XtpY$$

While each of the two separate rings X and Y can be anywhere in the volume v of the solution, in the catenane XtpY the center of mass of one ring must be confined within a distance d (and therefore a volume $\delta v = (4\pi/3)d^3$) of the center of mass of the other rings. When all concentrations are in molecules per unit volume. the equilibrium constant of the above reaction is simply δv. If d is taken as the sum of the root-mean-square radii R_X and R_Y, δv can be readily calculated.

For the formation of interlocked rings between 186 and λ DNA, these estimates give the correct order of magnitude, with the experimental result lower by approximately a factor of three.[7]

It should be pointed out that the unfavorable entropy for catenation comes primarily from the much higher degree of freedom of the *separate* rings. If the component rings are initially linked together by a certain reagent and breakage and rejoining of one ring then occurs, there is a high probability that a catenane would result. The breakage and rejoining of a partially replicated DNA discussed previously would give a catenane with a high probability for the same reason.

Properties of Interlocked Rings. Unless the two component rings are very small so that steric factors become important, the chemical properties of the component rings are *not* affected by the interlocking. Physical properties which involve the molecule as a whole (hydrodynamic properties, *e.g.*) are of course different from those of the component rings. The sedimentation properties of interlocked rings will be discussed below.

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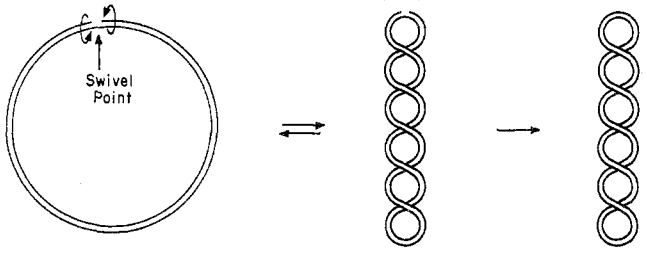

Figure 7. For a DNA double helix (represented by the two parallel lines) with a single-chain scission, relative rotation around a single bond opposite to the scission (swivel point) is allowed, with the DNA molecule twisting upon itself as a result. The change is reversible, but the twisted form is entropically unfavored. If the single-chain scission is repaired while the molecule is in a twisted form, the twists are topologically locked into the molecule.[31]

The sedimentation coefficient of a molecule in a centrifugal field is defined as its sedimentation velocity per unit acceleration. For simplicity, consider a dimeric catenane as two component rings joined at one fixed point. Furthermore, each ring is considered as a random coil. With these simplifications, the relative sedimentation coefficients of the catenane and its component rings can be computed, based on a theory first put forward by Kirkwood. If the two component rings are of the same size, the sedimentation coefficient of the catenane $s_{C_1tpC_1}$ is 1.15 times that of a simple ring of the same molecular weight, s_{C_2}.[27,28] The ratio is not much affected when the sizes of the component rings are different.[28]

Based on such a model, one would predict that a catenane should have a sedimentation coefficient somewhat higher than that of a simple ring of the same molecular weight. Recent measurements, with purified catenanes, showed that $s_{C_1tpC_1}$ is close to s_{C_2}. For a dimeric λb2b5 DNA catenane, with a molecular weight of 26×10^6 for each of the component rings, s_{CtpC}/s_{C_2} was measured to be 1.03.[29] Similar results were obtained for Hela mitochondrial DNA catenane, with a molecular weight of 10.6×10^6 for each component ring.[30]

For a double-stranded DNA ring, a unique topological constraint arises if both strands are uninterrupted.[31] To illustrate this point, consider a double-stranded DNA ring with only one single-chain scission. The position of the single-chain scission (nick) serves as a swivel since rotation around one of the single bonds on the strand opposite to the nick can take place freely. Suppose that one side of the nick is fixed in space and the end on the side is rotated around a single bond opposite to the nick. It is easy to see that the molecule would twist on itself (Figure 7). Since a twisted configuration is entropically unfavorable, freeing of one end would release the twists by rotation around the swivel in the other direction. If the nick is sealed while the molecule is in a twisted configuration, then there is no longer a swivel, and the twists are topologically locked into the molecule. A double-stranded circular DNA with both strands continuous is referred to as covalently closed and the symbol C* is used to denote such a molecule. If twists exist in such a molecule, it is referred to as twisted or superhelical. Such molecules have some interesting properties, but will not be discussed here. (For a review, see ref 31.)

It is easy to see that, for a C*, the two strands cannot be separated, even when all hydrogen bonds between the DNA base pairs are disrupted. If *one* single chain scission is introduced, disruption of base pairing (denaturation) would result in a single-stranded ring, C^s, and a single-standed linear molecule. L^s. These reactions are listed below.

$$C^* \xrightarrow{\text{denaturation}} C^* \text{ (denatured)}$$

$$C^* \xrightarrow{\text{one single-chain scission}} C \xrightarrow{\text{denaturation}} C^s + L^s$$

$$C^* \xrightarrow[\text{scission}]{\text{double-chain}} L \xrightarrow{\text{denaturation}} 2L^s$$

For a catenane, similar reactions can be carried out, *e.g.*,

$$C^* + L$$

$$\Big\uparrow \text{one double-chain scission}$$

$$C^*tpC^* \xrightarrow[\text{scission}]{\text{one single-chain}} C^*tpC \xrightarrow{\text{denaturation}} C^* \text{ (denatured)}tpC^s + L^s$$

$$\Big\downarrow \text{one single-chain scission in each ring}$$

$$CtpC \xrightarrow{\text{denaturation}} C^stpC^s + 2L^s$$

The sedimentation coefficients of many catenated DNA species, including C*tpC*, C*tpC, C*(denatured)tpC^s, CtpC, and C^{s}tpC^s, have been measured.[29] In general, for any catenane XtpY, its sedimentation coefficient, s_{XtpY}, is related to the sedimentation coefficients s_X and s_Y of the component rings by the empirical relationship

$$s_{XtpY} = s_Y[(M_X + M_Y)/M_Y][1 + (M_X/M_Y)^{1.78}(s_Y/s_X)^{1.78}]^{-0.56}$$

where M_i is the molecular weight of species i. For a dozen or so different species of interlocked λ DNA rings, the calculated s agrees with the experimental value to within 3%.[29] For interlocked mitochondria DNA rings, the agreement is also satisfactory.[30] Based on these results, the sedimentation coefficient of any simple catenane can be predicted with some confidence if the sedimentation coefficients of the component rings are known. The degradative reactions depicted above can therefore be used in identifying a catenane, and, since some of the degradative products have distinct sedimentation properties, in the quantitative assay of catenanes.

Summary

Interlocked DNA rings form a unique class of topologically bonded compounds. Their formation *in vitro* and their sedimentation properties have been studied in some detail. The coupling between their formation *in vivo* and the mechanisms of two genetic processes of fundamental importance, replication and recombination, make their origin an interesting question.

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