FORMATION OF COVALENT CIRCLES OF LAMBDA DNA BY E. COLI EXTRACTS

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Recombination of DNA molecules by breakage and reunion[1-4] requires, at its final step, covalent joining of the DNA strands. Biochemical study of this process has been hindered by the rarity of recombinational events in most organisms.

Recently, a system has become accessible in which covalent linkage of DNA strands occurs with high frequency. In cells of *E. coli* infected with phage λ, or in λ-lysogenic cells superinfected with λ, a large fraction of the entering DNA is converted to a covalently circular form.[5, 6]

Lambda DNA is known to carry cohesive sites,[7] which have been identified[8] as terminal complementary single-stranded nucleotide sequences with free 5′ ends. Cohesion of the sites can produce circular molecules,[9] which in this paper will be called "hydrogen-bonded circles." It seemed likely that this form of λ DNA would serve as an intermediate in the formation of covalent circles.

We have thus looked for, and found, an activity in extracts of *E. coli* which converts λ hydrogen-bonded circles to a covalently circular form.

The requirement of hydrogen-bonded circles for the reaction is strict; linear λ DNA is not made into covalent circles. With crude cell extracts, the only other requirement is for a divalent metal ion.

Materials and Methods.—Bacterial and phage strains: Extracts were usually made from frozen cells of *E. coli* B (late log phase, Kornberg medium; General Biochemicals, Inc.).

Other *E. coli* strains used for the preparation of extracts were (*a*) *E. coli* C600 and C600 (λcI-857), both obtained from R. L. Baldwin; λcI-857 is a temperature-inducible mutant;[10] (*b*) two recombination-deficient mutants, AB2463 of Howard-Flanders and Theriot,[11] and JC-1569 of Clark *et al.*[12] Both these strains were obtained from M. Yarmolinsky. For growth of phage, the thymine-requiring strain CR34 (λcI-857)/λ (obtained from R. L. Baldwin) was used.

Chemicals: H^3-thymidine (6.7 c/mmole) was purchased from New England Nuclear Corp. C^{14}-thymine (40 mc/mmole) was purchased from Nuclear-Chicago, Inc.

E. coli B transfer RNA (sRNA) was supplied by General Biochemicals, Inc.

Actinomycin D (Merck, Sharp & Dohme) was a gift of the Cancer Chemotherapy National Service Center, National Institutes of Health.

Preparation of phage and DNA: H^3-thymidine-labeled λcI-857 was made by temperature induction of *E. coli* CR34 (λcI-857)/λ.

The bacteria were grown to $OD_{650} = 0.7$ at 34°C in M63 medium[13] supplemented with L-threonine and L-leucine (each at 40 μg/ml), vitamin B_1 (0.1 μg/ml), Casamino acids (0.5 mg/ml), and thymidine (10 μg/ml). After centrifugation, the bacteria were resuspended at fourfold higher concentration in the same medium with added H^3-thymidine (usually at 300 mc/mmole). They were shaken at 45°C for 12 min, then at 37° C until lysis (this technique is due to R. Doherty). Titers of $2–3 \times 10^{10}$/ml were obtained. The phage was purified by low- and high-speed centrifugation, followed by banding in CsCl.[14] It was then dialyzed and stored in 0.01 *M* Tris chloride, 0.01 *M* $MgSO_4$, pH 8.0. C^{14}-thymine-labeled phage was made in the same way, with C^{14}-thymine at a specific activity of 15 mc/mmole.

DNA was extracted by shaking the phage gently with redistilled phenol saturated with 0.01 *M* Tris chloride, 0.001 *M* EDTA, pH 8.0 (TE buffer); the aqueous phase was dialyzed three times for 12 hr at 4°C against 500 vol of TE buffer. Fresh DNA preparations contained 15–25% of material sedimenting more slowly than unbroken strands of λ DNA, when examined by alkaline sucrose gradient sedimentation.

Preparation of hydrogen-bonded circles: Tritium-labeled λ DNA was diluted to 5 μg/ml in 2 *M* NaCl, 0.01 *M* EDTA, pH 8.0, and incubated for 2 hr at 48°C. These conditions appear suited to give maximal formation of hydrogen-bonded circles.[15] The DNA was concentrated by precipitation with 2 vol of cold absolute ethanol. The precipitate was collected by centrifugation at 23,000 *g* for 20 min, drained, and resuspended in $^1/_{20}$ of the (incubation) volume of TE buffer (recovery about 70%). Finally the DNA was dialyzed against TE buffer to remove residual ethanol.

Between 60 and 80% of the molecules in these preparations were circular, as judged by sucrose gradient sedimentation at neutral pH.

Sucrose gradient centrifugation: Linear sucrose gradients of 5–20% were used, in three different media: (1) 0.01 *M* Tris chloride, 0.001 *M* EDTA, pH 8.0 (called "0.01 *M* salt" below); (2) 0.01 *M* Tris chloride, 0.001 *M* EDTA, 1 *M* NaCl, pH 8.0 (called "1 *M* salt" below); (3) 0.001 *M* EDTA, 1 *M* NaCl; each sucrose solution adjusted to pH 12.3 with NaOH, using a Radiometer G222B (low sodium error) glass electrode.

These solvents are similar to those used by Bode and Kaiser.[6] For some purposes, actinomycin D (3×10^{-6} *M*) was added to the pH 8 gradients. A marker of C^{14}-λ DNA was routinely added to the sample.

Centrifugation was usually carried out in the SW39 rotor of the Spinco model L centrifuge, at 38,000 rpm and roughly 5°C. For larger-scale preparations, the SW25 rotor was used.

For measurement of radioactivity, aliquots of sucrose gradient fractions were spotted on 2.4-cm disks of Whatman 3 MM paper. The disks were processed batchwise in a beaker, as suggested by Bollum,[16] first with cold 0.5 *M* $HClO_4$, then with cold 0.5 *M* HCl, then with two washes of 95% ethanol. They were then transferred to scintillation vials, dried under a heat lamp, and counted with a toluene-base scintillator (Liquifluor; Pilot Chemicals, Inc.).

Preparation of extracts: Extracts of *E. coli* B were prepared by blending the cells with glass beads.[17] The extraction buffer was composed of 0.02 *M* Tris chloride, pH 8.0, 0.002 *M* $MgSO_4$, 0.0002 *M* dithiothreitol. The crude extract (about 10 mg/ml protein) was separated from cell debris and residual glass beads by centrifugation for 20 min at 23,000 *g*. It could be stored at 0°C for some days, or at −20°C for some months, without appreciable loss of activity.

Smaller-scale preparations of extracts, as used for testing other strains, were made by sonication. The cells were grown in tryptone broth to $OD_{650} = 1.0$, centrifuged, and resuspended in the above extraction buffer at a density of 2×10^{10} cells/ml. The cells were then sonicated in 2-ml aliquots in an ice bath (Branson Sonifier, $^1/_2$-in. tip, setting 4, 1 min). Extracts made by the two methods had comparable activity.

To prepare an extract of induced C600 (λcI-857), the cells were grown at 34°C to $OD_{650} = 0.7$, transferred to a 45°C shaker for 12 min, shaken at 37°C for another 30 min, centrifuged, and sonicated as above.

Reaction conditions: Standard conditions for the synthesis of covalent circles were the following: a hydrogen-bonded circle preparation at a concentration of 50 μg/ml DNA; 0.01 *M* Tris chloride, pH 8.0; 0.001 *M* EDTA; 0.004 *M* $MgSO_4$; 0.01 *M* NH_4Cl; 1 mg/ml cell extract and 10 μg/ml sRNA (*E. coli*). sRNA was added to ensure inhibition of endonuclease I.[18]

The reaction mixture was incubated for 5 min at 20°C, then chilled, and EDTA was added (final concentration 0.008 *M*).

Treatment of the sample from this point depended on whether it was to be used for neutral or alkaline sedimentation. In the former case, the sample was shaken with phenol (saturated with TE buffer), the phenol phase separated by centrifugation, and the sample layered on a sucrose gradient without removal of dissolved phenol. In the latter case, 1 *M* NaOH was added to a concentration of 0.05 *M*, and the sample was layered on an alkaline gradient.

Purification of covalent circles: Phenol-treated reaction mixtures were sedimented through sucrose gradients (pH 8, 0.01 *M* salt medium—see above) containing 3 μM actinomycin. The shoulder of material (shaded area of Fig. 2*A*) sedimenting about twice as fast as marker λ DNA was isolated and dialyzed against TE buffer containing calf thymus DNA (5 μg/ml) to aid in removal of the actinomycin. Between 30 and 50% of the radioactivity in this fraction resided in covalent circles, as judged by alkaline sedimentation (Fig. 2*B*).

In some cases, this fraction was concentrated by ethanol precipitation (see above), and further purified by a second sucrose gradient sedimentation. Eighty to ninety per cent of this material was covalently circular.

Electron microscopy: Samples were prepared by a modification[19] of the technique of Kleinschmidt and Zahn,[20] and photographed in a RCA EMU-3G electron microscope.

Results.—Properties of the reaction: Covalent circles are most readily distinguished from other forms of λ DNA by sedimentation at an alkaline pH sufficiently high to denature the DNA. Bode and Kaiser[6] found that at about pH 12.2, covalent circles sediment 3.8 times as fast as linear λ DNA. In this region of a sucrose gradient centrifugation pattern, the background of contaminating counts can be so low that 0.1 per cent of covalent circles in a DNA sample is readily seen. For this reason reaction products were first sedimented at alkaline pH.

If a preparation of hydrogen-bonded circles of λ DNA is incubated with a crude extract of *E. coli* B under the conditions given in *Methods*, between 4 and 10 per cent of the DNA is converted to covalent circles. Figure 1 shows the results of such an experiment. The small peak at the left has moved four times as far as the main (denatured linear λ DNA) peak, and is most plausibly identified as the covalently circular form of λ DNA. Figure 1 also shows that if *linear* λ DNA is incubated with extract, no covalent circles (<0.1%) are formed.

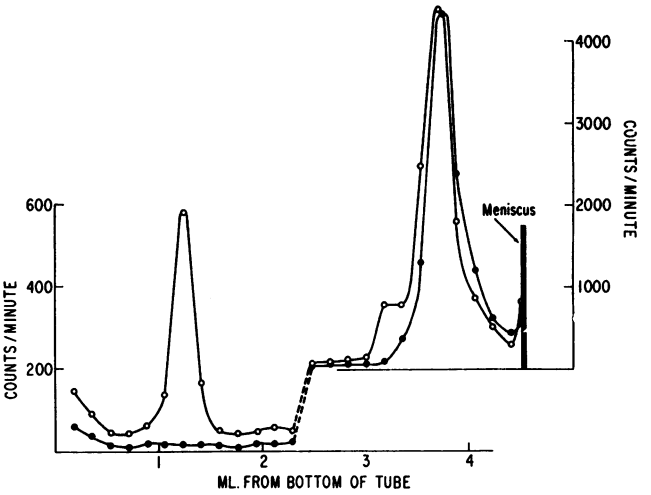

FIG. 1.—Conversion of λ DNA to covalent circles. Samples reacted as described in *Methods* were sedimented in alkaline sucrose gradients for 75 min at 38,000 rpm. *Open circles:* hydrogen-bonded circles of λ DNA reacted with *E. coli* B extract. *Filled circles:* linear λ DNA reacted similarly.

The reaction requires Mg^{++} (Table 1); other divalent cations have not been tried. There is an absolute requirement for preformed hydrogen-bonded circles, as seen above (Table 1 and Fig. 1). Lowering the amount of extract fivefold results in twofold less formation of covalent circles, suggesting that under the standard conditions a nearly saturating amount of extract is being used. The extract is inactivated by heating to 60°C.

TABLE 1

CONDITIONS FOR FORMATION OF COVALENT CIRCLES

	Input DNA converted to covalent circles (%)
Complete system	6
Complete system (extract conc. reduced to $^1/_5$ normal)	3
Linear λ DNA in place of hydrogen-bonded circles	<0.1
Omit Mg^{++}	<0.2
Extract heated to 60° for 5 min	<0.1

The complete system contained all reagents at the concentrations given in *Methods* and DNA with a total activity of 15,000 cpm. After incubation with extract the samples were sedimented in alkaline sucrose gradients for 75 min at 38,000 rpm.

TABLE 2

SEDIMENTATION OF COVALENT CIRCLES OF λ DNA

State of DNA	Centrifugation medium	Ratio of Distance Sedimented*: Covalent circles (this paper)	Ratio of Distance Sedimented*: Intracellular covalent circles (Bode and Kaiser[6])
Denatured	Alkaline	3.7	3.8
Native	0.01 *M* salt	1.7	1.9
Native	1 *M* salt	1.2	1.5
Native	0.01 *M* salt + actinomycin (3 μM)	2.1†	
Native	1 *M* salt + actinomycin (3 μM)	1.9†	
Neutralized from alkali	0.01 *M* salt	1.15‡	
Neutralized from alkali	1 *M* salt	1.15‡	1.6‡

For composition of media, see *Methods*.
* The distance sedimented is compared with that of a linear λ DNA marker in the same tube.
† In this case the distance sedimented is given relative to λ DNA in a parallel tube not containing actinomycin.
‡ Relative to a native λ DNA marker.

It was of interest to see whether the linkage activity would be grossly altered by changes connected with the physiology of λ phage. Studies on covalent circle formation were therefore carried out comparing extracts of *E. coli* B with those of *E. coli* C600, C600 (λcI-857), and the latter strain after induction of the phage by thermal shock (see *Methods*). The percentage of covalent circles made was in all cases the same, within a factor of 2 (over-all range 2–4%). While under these conditions the measurement more closely reflects the maximum extent of the reaction than a linear rate, it appears that no gross change in the level of linkage activity takes place.

Extracts of two recombination-deficient mutants, AB2463 and JC-1569, were similarly tested and also produced 2–4 per cent of covalent circles.

Prior to the experiments described above, the linkage activity had been detected with a more complicated assay which measures covalent linkage of two distinguishable DNA's. This assay, and further properties of the activity, will be described in a separate publication.

Properties of covalent circles: Covalent circles can be purified about fivefold by sedimentation in neutral sucrose gradients containing actinomycin, which speeds up their sedimentation (see Table 2 and below). They can be purified 10- to 20-fold by sedimentation in alkaline gradients, but their subsequent sedimentation at neutral pH is altered by the alkali treatment (Table 2). Purification at neutral pH (see *Methods*) was therefore routinely used to prepare material for the experiments below. Such a preparative centrifugation is shown in Figure 2*A*, and the alkaline sedimentation of the fractionated material in Figure 2*B*.

The sedimentation rate of these circles in alkali is identical to that of species I of Bode and Kaiser;[6] both move about 3.8 times as far as a denatured linear λ DNA marker (Table 2). Near neutral pH, however, these circles differ from species I; they sediment more slowly, both in 0.01 *M* and 1 *M* salt (Table 2). In 1 *M* NaCl our covalent circles sediment only 1.2 times as fast as λ DNA, a rate trivially different from that of hydrogen-bonded circles (1.14 times as fast as λ DNA). (It was thus necessary in this case to make sure that the covalent circles were still intact, as single-strand breakage could also have caused the reduced sedimentation rate. The material sedimenting 1.2 times as fast as λ DNA was therefore reisolated and sedimented in alkali. The majority sedimented at the rate characteristic of covalent circles.)

These results are most readily interpreted in terms of a twisted structure for both species I and our covalent circles. The enhanced sedimentation rate of the covalently circular form of polyoma DNA (above the factor of 1.14 expected from circularization alone) has been attributed[21] to such twists, and Bode (private communication) has drawn the same conclusion from the sedimentation behavior of species I. He has also observed highly twisted molecules in electron micrographs of species I. Bloomfield[22] has presented calculations showing how the sedimentation rate of twisted circular molecules increases with the number of twists. The sedimentation data can thus be rationalized if our covalent circles are less twisted than species I in both 0.01 *M* and 1 *M* salt, and if both are less twisted in 1 *M* than in 0.01 *M* salt. Our covalent circles would, in fact, appear to be essentially untwisted in 1 *M* NaCl.

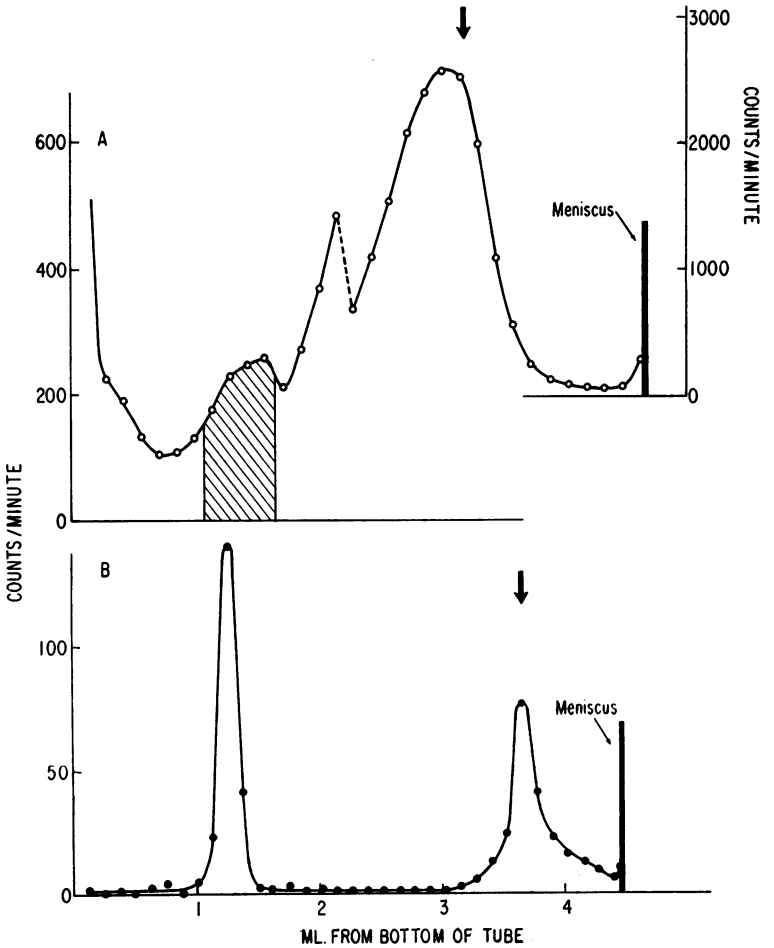

FIG. 2.—Purification of covalent circles. (*A*) A H^3-λ DNA sample reacted with an *E. coli* B extract as described in *Methods* was shaken with phenol and layered on a pH 8, 0.01 *M* salt, sucrose gradient containing 3 μM actinomycin. Sedimentation was for 135 min at 38,000 rpm. (*B*) The fractions designated by hatching in (*A*) were pooled, dialyzed, and an aliquot was sedimented in an alkaline sucrose gradient for 75 min at 38,000 rpm. The arrows mark the position of linear C^{14}-λ DNA markers.

It is noteworthy that the sedimentation rate of the covalent circles can be sharply increased by the addition of actinomycin. Relative sedimentation rates in sucrose gradients containing 3 μM actinomycin are shown in Table 2. (Under these conditions, the sedimentation rates of linear λ DNA and of hydrogen-bonded circles are increased by about 10%.) The marked increase in sedimentation rate, especially in 1 *M* NaCl, is most easily explained by an increase in the number of twists caused by binding of actinomycin. Qualitatively similar effects have also been seen with added acridine orange.

To test the possibility that the join in the covalent circles might be made by a protein or peptide "linker," an attempt was made to open covalent circles by treatment with pronase. A covalent circle sample was incubated with 10 μg/ml pronase (Calbiochem) for 30 min at 30°C in TE buffer. The proportion of covalent circles was decreased by less than 10 per cent. This experiment is not conclusive (the linker could be resistant to pronase), but taken together with the absence of breakdown by phenol treatment and by alkali, it suggests that a polypeptide join is rather unlikely.

Samples of λ covalent circles made and purified as described in *Methods* were examined in the electron microscope. The samples were first heated (60°C for 5 min in TE buffer) to destroy residual hydrogen-bonded circles. (Control preparations of hydrogen-bonded circles similarly heated showed no circular molecules.) A large proportion of molecules without ends was seen; many of the molecules were highly twisted. Two carefully selected examples are shown in Figure 3.

Discussion.—The evidence that covalently circular DNA molecules are being formed in this system is of several kinds:

(1) The molecules sediment very rapidly under alkaline denaturing conditions. This is characteristic of the double-stranded covalently circular form of DNA in other systems[23, 24] as well as species I of λ DNA.[6]

(2) The sedimentation rate of the molecules at pH 8 is strongly dependent on salt concentration in a range (0.01–1 *M*) where that of linear native DNA (or hydrogen-bonded circles) is not. Similar behavior has been found for intracellular covalent circles of λ DNA.[6]

(3) Molecules without ends are seen in electron micrographs of DNA samples previously treated so as to open hydrogen-bonded circles.

Taken together, these results strongly support a covalently circular structure. "Covalency" is here used in the sense which has become conventional in this field, to mean a structure which is resistant to conditions which denature the DNA. The nature of the links at the ends of the DNA is as yet unknown, as is the reaction mechanism by which they are made. Such questions will be most easily answered in studies with purified enzyme. A purification is now in progress.

The covalent circles made in this system apparently carry a lesser number of twists than those made intracellularly. We surmise that this is due not to any difference in the linkage reactions, but rather to the different media in which closure takes place. It was suggested above that the marked variation of sedimentation rate of covalent circles, near neutral pH, with salt concentration and added actinomycin could be explained by changes in the number of twists. Similarly, covalent circles formed in different media may be expected to carry different degrees of twist when sedimented under identical conditions. It will be interesting to see whether

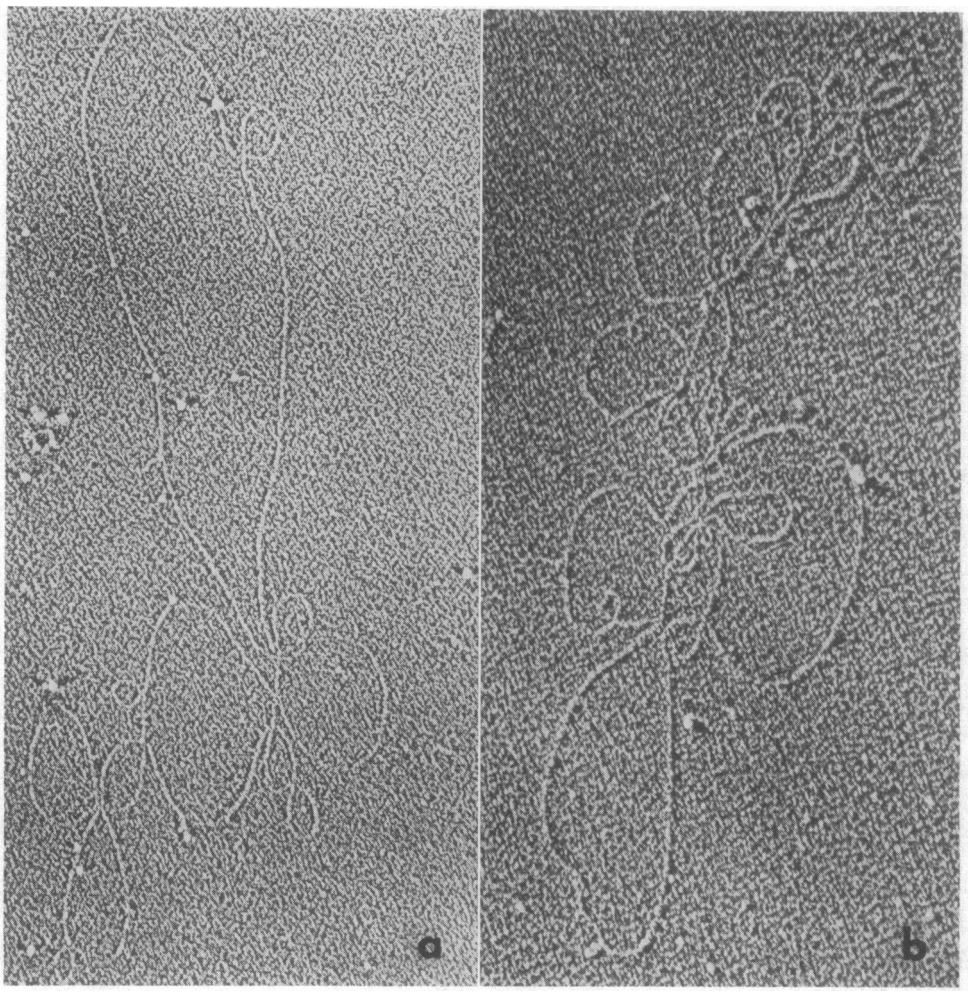

FIG. 3.—Electron micrographs of λ covalent circles made with the cell-free system. Magnification: (*a*) ×30,000; (*b*) ×43,000.

the number of twists can be systematically altered by variation of the medium in which the linkage reaction takes place.

We may speculate briefly on the biological role of this activity. It exists in *E. coli* strains nonlysogenic for λ, in lysogens, and induced lysogens. It thus appears to have no special connection to the λ system, and may more plausibly be considered a general repair activity designed for sealing breaks within DNA molecules. Experiments intended to test this possibility are under way.

The path which the λ linkage reaction follows—base-pairing of single-stranded regions to form hydrogen-bonded circles, with ensuing covalent closure—has elements in common with a mechanism of recombination proposed by Meselson[3] and Howard-Flanders.[25] While it is not clear that recombinational processes in other systems necessarily follow a similar route, it is suggestive that Anraku and Tomizawa[4] have found recombination of T4 DNA molecules to proceed by formation first of denaturation-labile links, then of covalent bonds.

Two recombination-deficient mutants have roughly normal levels of the linkage activity. Taken together with the finding[12] that no other known enzyme of DNA metabolism is deficient in these mutants, this strongly suggests that other enzymes involved in recombination remain to be identified.

Summary.—Extracts of *E. coli* convert λ DNA to covalent circles. The reaction requires magnesium ion and prior formation of hydrogen-bonded circles of the λ DNA. Extracts of λ-sensitive, λ-lysogenic, and induced λ-lysogenic strains have been found active, as have extracts of two recombination-deficient mutants. The covalent circles appear, by sedimentation, to be less twisted than those made intracellularly. Their sedimentation rate is sharply increased by addition of actinomycin.

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