The various activities of the recBC enzyme of *Escherichia coli* are differentially sensitive to reaction conditions. Although spermidine up to 40 mM and putrescine up to 40 mM in the presence of Mg$^{2+}$ produce no significant alteration in the activities of the enzyme, low concentrations of Ca$^{2+}$ have a dramatic effect. In the presence of 1 mM Ca$^{2+}$, 1 mM Mg$^{2+}$, and 5 mM ATP, the DNA-dependent ATPase is unaffected, but the double-strand DNA exonuclease and the single-strand DNA exonuclease and endonuclease activities are completely inhibited. Duplex phage T7 DNA which has been exposed to enzyme under these conditions remains wholly duplex and full length, although about five nicks are made randomly in each duplex. When DNA binding protein from *E. coli* is included in the reaction with enzyme and DNA, the products are, at early stages of the reaction, duplex DNA molecules with one or two single-stranded tails at a terminus; after prolonged incubation, solely single-stranded fragments of 5,000 to 35,000 nucleotides are found. (Full length strands are roughly 40,000 nucleotides long.) These products can be rationalized by a model in which the enzyme binds initially to the terminus of a duplex DNA molecule and then tracks along the DNA, unwinding the strands of the helix as it moves. This denaturation would be transient and localized around the enzyme molecule so that the DNA would renature after the enzyme has passed through a particular region. However, the renaturation can be prevented by the presence of DNA binding protein during the reaction. During the passage of an enzyme molecule through a DNA molecule, a limited number of nicks are randomly introduced in a manner essentially unaffected by binding protein. This mode of action is distinct from that observed for the degradation of duplex DNA with Mg$^{2+}$ but not Ca$^{2+}$ present (MacKay, V., and Linn, S. (1974) *J. Biol. Chem.*, 249, 4286-4294).

Exonuclease V of *Escherichia coli* is the product of the *recB* and *recC* genes (1) and plays a central role in pathways of genetic recombination and recombinational repair (2). Purified enzyme exhibits several catalytic activities in vitro. It acts as an ATP-dependent exonuclease with duplex- or single-stranded DNA; it functions as an ATP-stimulated endonuclease with single-stranded DNA but not with duplex DNA; finally, it acts as a DNA-dependent ATPase, although concurrent DNA degradation is not necessary for ATPase activity (3-5).

Under optimal conditions for exonuclease activity with duplex DNA, enzyme molecules initiate degradation asynchronously and then act processively, degrading one DNA molecule completely before switching to another (5). Consequently, intermediates in the degradative reaction are present transiently and at relatively low concentrations. However, by using conditions that favor the formation of intermediates, MacKay and Linn (6) have determined the structure of these DNA molecules and proposed a mechanism by which they are formed. The model suggested that the enzyme unwinds the strands of the duplex from one terminus, cleaving fragments of several hundred nucleotides from one strand while remaining bound to the terminus of the degraded strand. After producing a single-stranded tail up to 5000 nucleotides long, the enzyme switches strands and degrades the tail from its terminus, producing a shortened, wholly duplex molecule. This cycle is repeated until the molecule is entirely degraded to small single-stranded fragments which are finally broken down to acid-soluble oligonucleotides in a reaction that is specifically inhibited by DNA binding protein (DBP) (6, 7).

This model had several interesting features with regard to current models for genetic recombination; moreover, it predicted that the enzyme uses energy derived from the hydrolysis of ATP to unwind the DNA helix. However, even under conditions favoring the formation of intermediates, the action of the enzyme remained principally degradative, ultimately producing acid-soluble oligonucleotides. The formation of acid-soluble oligonucleotides might not be desirable during recombination, however, so we have sought conditions under which the DNase activities of the enzyme are controlled while the ATPase-mediated unwinding reaction is retained.

The activity and mechanism of action of several DNases have been found to be dependent on the particular divalent ion present in the reaction (8-11). Moreover, a complex containing recBC enzyme and DNA polymerase I obtained from gently lysed spheroplasts has been reported to be present in a "crude ribosomal fraction" which was rich in membrane vesicles (12, 13), and mutations have been isolated in the gene for termination of transcription (rho) which also cause defective recombination and an altered Ca$^{2+}$-Mg$^{2+}$-dependent membrane ATPase (14, 15). The latter observations might be taken to imply some relationship between the recBC enzyme and the cell membrane and/or the Ca$^{2+}$-Mg$^{2+}$-dependent membrane ATPase so we have examined the effect of divalent cations, particularly Ca$^{2+}$, upon the recBC enzyme. As reported below, not only are various activities of the recBC enzyme...
enzyme differentially sensitive to the reaction conditions, but the mechanism of action of the enzyme also appears to change. In addition, evidence is reported that the unwinding of the DNA helix is indeed coupled to ATP hydrolysis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Commercial bacterial alkaline phosphatase (Worthington) was freed of nuclease on DEAE-cellulose (16). Glycerol gradient fraction of recBC enzyme was used throughout; its purification and assay for DNase and ATPase activities have been described. Units of recBC enzyme refer to double strand DNA exonuclease units (3). E. coli exonuclease I was the hydroxylapatite fraction (17). E. coli DNA (2.9 to 4.5 x 10^6 cpm/nmol), H-labeled DNA from phage fd (3.5 x 10^6 cpm/nmol), and H-labeled DNA from phage PM2 were prepared as described in (19-21). All DNA concentrations are expressed as nucleotide equivalents.

**Sucrose Gradient Centrifugation—**DNA sedimentation analyses were performed with a Spinco SW50.1 rotor at 4°C, run at 50,000 rpm. Neutral gradients were used to separate DNase and ATPase activities to the same extent with roughly 40% of each remaining at 50 mM. Putrescine at concentrations greater than 10 mM slightly inhibited the ATPase activity with roughly 70% of the activity present at 50 mM. Putrescine at concentrations greater than 10 mM slightly inhibited the ATPase activity with roughly 70% of the activity present at 50 mM. Inhibition of the exonuclease was not observed at less than 40 mM putrescine. At 1 to 20 mM putrescine stimulated the double strand DNA exonuclease up to 20% but had no effect upon the ATPase.

The polyamines spermidine and putrescine were also tested for modulation of the activities of the recBC enzyme in the presence of 10 mM MgCl2. Neither behaved like Ca2+. Spermidine had no effect when present at less than 3 mM; higher concentrations inhibited both the double strand DNA exonuclease and ATPase activities to the same extent with roughly 40% of each remaining at 50 mM. Putrescine at concentrations greater than 10 mM slightly inhibited the ATPase activity with roughly 70% of the activity present at 50 mM. Inhibition of the exonuclease was not observed at less than 40 mM putrescine. At 1 to 20 mM putrescine stimulated the double strand DNA exonuclease up to 20% but had no effect upon the ATPase.

**RESULTS**

**Modulation of recBC Enzyme Activities by Divalent Cations**—In the presence of Mg2+ alone, the duplex DNA exonuclease activity of recBC enzyme was maximal at 8 to 12 mM Mg2+, while the ATPase activity had a slightly broader optimal range (Table I). ATPase activity was accompanied by DNase activity at all Mg2+ concentrations. With Ca2+ alone, no DNase activity was observed at any Ca2+ concentration from 0.01 to 100 mM; however, some ATP hydrolysis was observed with Ca2+ between 1 and 10 mM (Table I). In contrast to the ATPase activity observed with Mg2+, ATPase activity in the presence of Ca2+ was not totally dependent on the presence of DNA.

Adding Ca2+ with Mg2+ present had a marked inhibitory effect on recBC DNase activity but little effect on the ATPase activity (Table I). With a Ca2+/Mg2+ ratio of 0.1, DNase activity was reduced by 83%, whereas the ATPase activity was 13% inhibited. At Ca2+/Mg2+ ratio greater than 1, no acid soluble DNA was detected, while ATPase hydrolysis remained near maximal.

### Table I

<table>
<thead>
<tr>
<th>CaCl2</th>
<th>MgCl2</th>
<th>DNase</th>
<th>ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>4</td>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>1</td>
<td>28</td>
<td>53</td>
<td>25</td>
</tr>
<tr>
<td>5</td>
<td>80</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>50</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

"Omit DNA.

The above results were obtained with 60 μM ATP under reaction conditions that favor the degradative activity of the enzyme (3). With intracellular concentrations of ATP (approximately 3 mM) (24) and only Mg2+ present as the divalent cation, the recBC duplex DNA exonuclease activity has been found to become considerably inhibited (25), whereas the DNA-dependent ATPase was stimulated by roughly 15-fold (3) and the initiation of degradation became more easily synchronized (6). With 5 mM ATP in the presence of Mg2+ and Ca2+, however, no acid-soluble material is produced, but recBC enzyme still hydrolyzes ATP (Table II). (At the high ATP concentration, no ATP hydrolysis is observable with only Ca2+ present.) In order to study this reaction, all subsequent recBC enzyme reactions contained 5 mM glycylglycine-NaOH (pH 7.0), 1 mM CaCl2, 1 mM MgCl2, 0.1 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol, DNA, and enzyme. Under these conditions, ATP is hydrolyzed, but no acid-soluble product has been detected with any DNA substrate tested.

To determine more precisely the amount of residual single strand DNase activity under these conditions, recBC enzyme was incubated with a preparation of fd phage DNA that contained approximately equal amounts of circular and linear DNA.
molecules and then the DNA was examined by sedimentation in alkaline sucrose. With Mg\(^{2+}\) alone, the DNA was extensively degraded, but with Ca\(^{2+}\) and Mg\(^{2+}\) present, the sedimentation of the enzyme-treated DNA was indistinguishable from that of untreated DNA (Fig. 1). Thus, the single strand DNA endonuclease and exonuclease activities of the recBC enzyme were both completely inhibited in the presence of Ca\(^{2+}\), Mg\(^{2+}\), and 5 mM ATP.

Possible double strand endonuclease activity upon circular DNA was investigated by treating closed, circular duplex DNA from phage PM2 with recBC enzyme and then sedimenting the DNA in alkaline sucrose. No endonuclease activity was detected whether or not DBP was present during the incubation (Table III). Under optimal conditions for duplex DNA exonuclease activity, closed, circular duplex DNA was also found to be resistant to recBC activity (3).

Residual double strand DNA exonuclease activity was determined by sedimenting phage T7 DNA in neutral sucrose before and after treatment with recBC enzyme. With Mg\(^{2+}\) alone, the DNA was extensively degraded (Fig. 2, cf. Ref. 6). With Ca\(^{2+}\) and Mg\(^{2+}\), the sedimentation of untreated DNA

<table>
<thead>
<tr>
<th>CaCl(_2)</th>
<th>MgCl(_2)</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>nmol acid-soluble</td>
<td>nmol ATP formed</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.16</td>
<td>3.9</td>
</tr>
<tr>
<td>20</td>
<td>&lt;0.01</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>1</td>
<td>0.26</td>
<td>4.8</td>
</tr>
<tr>
<td>10</td>
<td>&lt;0.01</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>1</td>
<td>0.01</td>
<td>4.3</td>
</tr>
<tr>
<td>5</td>
<td>&lt;0.01</td>
<td>4.6</td>
</tr>
<tr>
<td>1</td>
<td>&lt;0.01</td>
<td>4.6</td>
</tr>
<tr>
<td>3</td>
<td>&lt;0.01</td>
<td>4.7</td>
</tr>
</tbody>
</table>

**FIG. 1.** Sedimentation of phage fd DNA in alkaline sucrose after digestion with recBC enzyme. Reaction mixtures contained in 100 \(\mu\)l: 5 mM glycylglycine-NaOH (pH 7.0), 1 mM dithiothreitol, 5 mM ATP, 0.1 M NaCl, 4 nmol of E. coli DNA, 0.4 unit of enzyme, and divalent cation as indicated. Duplex DNase and ATPase activities were assayed as described (3).

**FIG. 2.** Sedimentation of phage T7 DNA in neutral sucrose after treatment with recBC enzyme. Reaction mixtures were as described in the legend to Fig. 1, except that phage fd DNA was replaced by 2 nmol of phage T7 DNA. Reactions were stopped by adding 10 \(\mu\)l of 0.2 M EDTA, layered onto gradients of neutral sucrose containing 2.5 M NaCl, and centrifuged for 2 h. A, sedimentation of untreated native (■) and denatured Ti DNA (○). B, sedimentation profiles after recBC treatment with either 1 mM Mg\(^{2+}\), 1 mM Ca\(^{2+}\) (■), or 20 mM Mg\(^{2+}\) (○). C, the effect of adding 12 \(\mu\)g of DNA binding protein to recBC reactions with either 1 mM Mg\(^{2+}\), and 1 mM Ca\(^{2+}\) (■), or 20 mM Mg\(^{2+}\) (○) present.

**TABLE II**

Variation of recBC enzyme activities with divalent cations at physiological ATP concentration

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Form I</th>
<th>Form II</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>90</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>+1.8 units of enzyme</td>
<td>60</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+1.8 units of enzyme + 3.2 pg of DBP</td>
<td>90</td>
<td>8</td>
<td>2</td>
</tr>
</tbody>
</table>
as full length, wholly duplex molecules (Fig. 3A; cf. Fig. 2).

When T7 DNA was first incubated with recBC enzyme in the absence of DBP and then DBP added to the ongoing reactions, short, single-stranded “bubbles” were observed in otherwise duplex DNA (Fig. 3B). With DBP present for 20 s, recBC enzyme formed “bubbles” by unwinding up to 1600 base pairs. Measurement of nine bubbles showed the enzyme to have an apparent unwinding rate of 65 ± 20 nucleotides/s. This value compares closely to 40 nucleotides/s which was the rate of release of nucleotides from duplex DNA by recBC enzyme in the absence of DBP, the T7 DNA remained as full length, wholly duplex molecules (Fig. 3A; cf. Fig. 2).

The reaction mixture in Fig. 3B contained 100 μl: 5 mM glycylglycine-NaOH (pH 7.0), 1 mM MgCl₂, 1 mM CaCl₂, 1 mM dithiothreitol, 5 mM ATP, 0.1 M NaCl, 2 nmoles of T7 DNA, and 1.6 units of recBC enzyme. After incubating at 37°C for 30 min, the reaction was stopped by adding 10 μl of 0.2 M EDTA and the DNA prepared for electron microscopy. The representative molecule shown is wholly duplex and has a contour length of 12.7 μm (essentially unit length); the bar represents 0.25 μm. B, reaction mixtures, as described in A, were incubated for 2 min at 37°C when 3.6 μg of DNA binding protein was added. The reaction mixture was incubated for an additional 20 s, stopped with 5 μl of 0.2 M EDTA, and prepared for electron microscopy. In the molecule shown, the single-stranded “bubble” has been formed by unwinding about 1,550 nucleotides. The bar represents 0.25 μm. C to E, reaction mixtures were as described under “Experimental Procedures.”

Characterization of the Ca²⁺-mediated Unwinding Reaction—Requirements for the unwinding of duplex DNA by recBC enzyme were studied by monitoring the conversion of duplex DNA to a form that was sensitive to the single strand specific DNase, E. coli exonuclease I (17). Using the reaction scheme described under “Experimental Procedures,” the amount of DNA made sensitive to exonuclease I was proportional to the amount of recBC enzyme present in the initial incubation (Table IV). However, the ultimate formation of acid-soluble material was dependent not only on the sequential actions of recBC enzyme and E. coli exonuclease I, but also on the presence of DBP in the initial recBC reaction mixture. This is consistent with the behavior of recBC-treated T7 DNA during sucrose gradient sedimentation (Fig. 2) and implies that in the absence of DBP the unwinding of duplex DNA by recBC enzyme is transient and may be localized in the region of the enzyme molecule.

Using electron microscopy to examine the unwinding of duplex DNA, we have found that the appearance of single-stranded DNA was dependent on the presence of both recBC enzyme and DBP. Even after prolonged incubation with recBC enzyme in the absence of DBP, the T7 DNA remained as full length, wholly duplex molecules (Fig. 3A; cf. Fig. 2).

When T7 DNA was first incubated with recBC enzyme in the absence of DBP and then DBP added to the on-going reactions, short, single-stranded “bubbles” were observed in otherwise duplex DNA (Fig. 3B). With DBP present for 20 s, recBC enzyme formed “bubbles” by unwinding up to 1600 base pairs. Measurement of nine bubbles showed the enzyme to have an apparent unwinding rate of 65 ± 20 nucleotides/s. This value compares closely to 40 nucleotides/s which was the rate of release of nucleotides from duplex DNA by recBC enzyme in the absence of DBP, the T7 DNA remained as full length, wholly duplex molecules (Fig. 3A; cf. Fig. 2).

Table IV

**Requirements for converting T7 duplex DNA to a form susceptible to E. coli exonuclease I**

<table>
<thead>
<tr>
<th>Reaction conditions in first incubation</th>
<th>Incubation with alkaline phosphatase</th>
<th>Incubation with exonuclease I + Mg²⁺</th>
<th>% DNA made acid soluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBP + 0.54 unit of recBC</td>
<td>+</td>
<td>+</td>
<td>36</td>
</tr>
<tr>
<td>+ 1.08 units of recBC</td>
<td>+</td>
<td>+</td>
<td>36</td>
</tr>
<tr>
<td>+ 1.62 units of recBC</td>
<td>+</td>
<td>+</td>
<td>89</td>
</tr>
<tr>
<td>DBP + recBC</td>
<td>+</td>
<td>-</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>+ 1.08 units of recBC</td>
<td>+</td>
<td>-</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>+ 1.62 units of recBC</td>
<td>+</td>
<td>-</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>+ 2.00 units of recBC</td>
<td>+</td>
<td>+</td>
<td>62</td>
</tr>
<tr>
<td>-DBP + 1.08 units of recBC</td>
<td>-</td>
<td>+</td>
<td>7</td>
</tr>
</tbody>
</table>

With Ca²⁺ and Mg²⁺, we observed duplex DNA molecules with two single-stranded tails at the same terminus at both early (Fig. 3C) and late (Fig. 3D) times of the reaction. Such
Modulation of recBC Enzyme Activities by Ca\(^{2+}\)

When T7 DNA was allowed to unwind in the presence of Mg\(^{2+}\) and Ca\(^{2+}\), it was observed that the single-stranded tails frequently differed in length, and other intermediates had only one single-stranded tail attached to the DNA molecule (Fig. 3E). Thus, a limited amount of nicking appeared to accompany the DNA unwinding reaction.

Characterization of the DNA Nicking Activity Associated with the Unwinding Reaction—When T7 DNA was allowed to unwind by recBC enzyme with DBP and Ca\(^{2+}\) and Mg\(^{2+}\) present, the DNA was found as single-stranded fragments of 5,000 to 35,000 nucleotides. Thus, it would seem that the recBC enzyme makes a limited number of nicks in each strand of the T7 DNA molecule as it unwinds the duplex. However, the size distribution of the single-stranded molecules produced in this way is shown by the broken line.

FIG. 4. Histogram of DNA fragment size after complete unwinding of T7 DNA by recBC enzyme with Ca\(^{2+}\) and Mg\(^{2+}\) present. Reaction mixtures contained in 50 μl of 5 mM glycylglycine-NaOH (pH 7.0), 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), 1 mM dithiothreitol, 5 mM ATP, 0.1 mM NaCl, 4 μg of DBP, 0.9 nmol of T7 DNA, and 1.6 units of enzyme which is equivalent to approximately 3 enzyme molecules/duplex terminus. Reactions were incubated for 30 min at 37°C and immediately prepared for electron microscopy. The size distribution of the fragments observed after this treatment is shown by the solid line. As a control, T7 DNA was denatured by adding formamide to 75% and heating at 60°C for 5 min. The size distribution of the single-stranded molecules produced in this way is shown by the broken line.

molecules have never been observed when only Mg\(^{2+}\) was included as a divalent cation. However, the two single-stranded tails frequently differed in length, and other intermediates had only one single-stranded tail attached to duplex DNA or were wholly single stranded (Fig. 3E). Thus, a limited amount of nicking appeared to accompany the DNA unwinding reaction.

Effect of temperature and ionic strength on the nicking activity associated with recBC-catalyzed unwinding

Reaction mixtures, as described in the legend to Table V, with 0.75 unit of recBC enzyme and 1.6 μg of DBP were incubated for 30 min at varying temperatures between 0°C and 42°C or were supplemented with different amounts of NaCl and incubated for 30 min at 37°C. All subsequent operations were as described in the legend to Table V.

To determine more exactly the amount of endonuclease activity associated with the unwinding reaction, T7 DNA was incubated with recBC enzyme and then sedimented in alkaline sucrose. With DRP present, complete unwinding of the DNA by recBC enzyme (as monitored separately by electron microscopy) was accomplished by the introduction of about five nicks per DNA molecule; increasing the amount of recBC enzyme did not increase the amount of endonuclease activity significantly (Table V). When DRP was omitted from the reaction, the number of nicks introduced per DNA molecule increased in proportion to the amount of recBC enzyme present (Table V), perhaps because more than one enzyme molecule is able to track along each of the DNA molecules which remain duplex under these conditions.

Other factors have been found to influence the amount of nicking activity associated with the Ca\(^{2+}\)-mediated unwinding. Increasing the ionic strength of the reaction mixture or decreasing the incubation temperature leads to a reduction in the average number of nicks introduced per DNA molecule (Table VI). As with the electron microscopy, sedimentation of the DNA fragments in alkaline sucrose gave no evidence for endonuclease activity at preferential sites with any of the reaction conditions.

Model for recBC Enzyme Activity with Ca\(^{2+}\) and Mg\(^{2+}\) Present—In the presence of Mg\(^{2+}\) and Ca\(^{2+}\), we propose that the recBC enzyme acts in the manner shown in Fig. 5. After binding to the terminus of the duplex DNA, the enzyme tracks along the DNA, nicking the DNA at about every 150 nucleotides, as shown by the solid line. As a control, T7 DNA was denatured by adding formamide to 75% and heating at 60°C for 5 min. The size distribution of the single-stranded molecules produced in this way is shown by the broken line.

FIG. 5. Model for the action of the recBC enzyme with Ca\(^{2+}\) and Mg\(^{2+}\) present. A detailed explanation of this model is given in the text.

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(Additional details and figures related to the text are not transcribed here due to the constraint of reproducing the document accurately.)
along the DNA, locally unwinding the double helix as it does so. Around the enzyme molecule a region of probably less than 50 nucleotides is unwound, and the DNA renatures after the enzyme has moved past that point. If DBP is present, renaturation is prevented and the DNA is converted to the single-stranded form. (When DBP is added after tracking has begun stable single-stranded DNA is found only in that portion of the DNA unwound after the addition (see Fig. 3B).) During unwinding with DBP present throughout the reaction, about five nicks are introduced into each T7 DNA molecule, apparently at random locations in each strand of the molecule. If DBP is omitted the number of nicks introduced is similar, but cannot be precisely determined because more than one enzyme molecule can pass through each DNA duplex.

**DISCUSSION**

We have studied the recBC enzyme with Ca\textsuperscript{2+} and Mg\textsuperscript{2+} present under conditions which suppress its DNase activities but not ATPase and have confirmed the previous hypothesis that at least part of the energy derived from ATP hydrolysis can be coupled to unwinding of the helix as the enzyme tracks along the DNA (6). Under these conditions, the stable formation of single-stranded DNA requires the presence of DBP. During this unwinding reaction we have observed duplex intermediates with single-stranded tails up to 32,000 nucleotides long (Fig. 3), while after complete unwinding, we found stable single-stranded fragments from 5,000 to 35,000 nucleotides long (Fig. 4). In addition, two tails were often observed at the same terminus (Fig. 3). We did not observe any single-stranded material less than 500 nucleotides long at any stage of the reaction. These observations clearly contrast with the situation found previously (6) with only Mg\textsuperscript{2+} present under conditions that favor the formation of intermediates. In that case, the formation of single-stranded DNA did not require DBP, and intermediates were observed to be either duplex molecules with single-stranded tails up to 6,000 nucleotides long or short single-stranded fragments of 100 to 500 nucleotides. Two single-stranded tails were never found at the same terminus, and, when DBP was absent, all of the DNA was ultimately degraded to acid-soluble oligonucleotides (9).

The various structures observed after enzyme activity with Ca\textsuperscript{2+} present are incompatible with the mechanism of action described for enzyme activity in the absence of Ca\textsuperscript{2+} and vice versa. Consequently, not only are the various enzyme activities differentially sensitive to reaction conditions, but the mechanism of action of the enzyme also appears to change. This variation of mechanism might be explained in terms of the diverse biological functions of the enzyme, since the action of the recBC enzyme during recombination may differ from the way the enzyme acts to degrade restricted DNA in vivo (28). It is then to "recklessly" degrade DNA after irradiation of a recBC bacterium (29). In fact, there is no reason to suppose that the recBC enzyme acts by only one mechanism during recombination.

During unwinding, recBC enzyme makes a small number of nicks in each strand of the T7 DNA molecule, although at apparently random sites. If the nicking activity of the enzyme is significant with respect to the function of the enzyme in vivo, it may be that other factors act to impart some specificity to this reaction. We have shown that the number of nicks introduced per DNA molecule varies somewhat with the reaction conditions. Changes in conditions, such as increased temperature or decreased ionic strength, which might be expected to allow the enzyme to track along the DNA more quickly, also appear to increase the amount of concurrent endonuclease activity (Table VI). Since a nick may be introduced in response to changes in the environment of the enzyme that alter the rate at which the enzyme is tracking, a particular region of the DNA may be cleaved if certain nucleotide sequences were able to change the rate of enzyme tracking.

Mutations which stimulate recBC-mediated recombination of phage \(\lambda\)-DNA occur at only a few places on the phage chromosome (31) and, apparently, form sites that are identical or similar to those that occur naturally in E. coli DNA (32). These are termed Chi sites (genetically designated chi\textsuperscript{L}). Although the nucleotide sequence of a DNA fragment containing Chi has been determined, the boundaries of the Chi site within this sequence are unknown (33). The component of the recombination system which recognizes the Chi sequence has not yet been identified and may well be the recBC enzyme itself. In this context one might speculate that the role of Chi could be to change the rate of recBC enzyme tracking, thereby inducing the enzyme to nick the DNA in the Chi region. However, we have not observed conditions under which the enzyme appears specifically to nick at Chi sites.

Finally, our results highlight the difficulty of attempting to establish the mechanism of action of an enzyme in vivo by studies with purified enzyme in vitro. Nonetheless, the mechanism that we have proposed for recBC activity with Ca\textsuperscript{2+} present has obvious relationship to current models for genetic recombination and, in particular, the uptake of single-stranded fragments by superhelical DNA (34). The actual mechanism by which the recBC enzyme promotes these events remains to be established.

**Acknowledgments**—We thank Ms. Alice Taylor for instruction and assistance with the electron microscopy, Dr. Arthur Kornberg, Stanford University, for providing DNA binding protein, and Dr. Gerry Smith, University of Oregon, for communicating results prior to publication.

**REFERENCES**


\* Alternatively, it is interesting to note that in vitro recBC enzyme might consist of three subunits (30), one of which might be lost during our enzyme purification (3). Perhaps this third subunit could impart some degree of specificity to this endonuclease reaction.
Modulation of recBC Enzyme Activities by Ca$^{2+}$

Modulation of the action of the recBC enzyme of Escherichia coli K-12 by Ca\textsuperscript{2+}.

J Rosamond, K M Telander and S Linn


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