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The Reactions of the *Eco*RI and other Restriction Endonucleases

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The reaction of the *Eco*RI restriction endonuclease was studied with both the plasmid pMB9 and DNA from bacteriophage λ as the substrates. With both circular and linear DNA molecules, the only reaction catalysed by the *Eco*RI restriction endonuclease was the hydrolysis of the phosphodiester bond within one strand of the recognition site on the DNA duplex. The cleavage of both strands of the duplex was achieved only after two independent reactions, each involving a single-strand scission. The reactivity of the enzyme for single-strand scissions was the same for both the first and the second cleavage within its recognition site. No differences were observed between the mechanism of action on supercoiled and linear DNA substrates. Other restriction endonucleases were tested against plasmid pMB9. The *Hin*dIII restriction endonuclease cleaved DNA in the same manner as the *Eco*RI enzyme. However, in contrast with *Eco*RI, the *Sal*I and the *Bam*HI restriction endonucleases appeared to cleave both strands of the DNA duplex almost simultaneously. The function of symmetrical DNA sequences and the conformation of the DNA involved in these DNA–protein interactions are discussed in the light of these observations. The fact that the same reactions were observed on both supercoiled and linear DNA substrates implies that these interactions do not involve the unwinding of the duplex before catalysis.

Restriction endonucleases provide a system for the study of the interactions between proteins and specific nucleotide sequences on DNA. With a few exceptions (Brown & Smith, 1977), the DNA sequences that are recognized by restriction enzymes possess 2-fold rotational symmetry (Roberts, 1976). Symmetry is also a common feature of the DNA sequences that are recognized by proteins involved in the control of gene expression (Dickerson *et al.*, 1975). A number of suggestions have been made about the significance of DNA symmetry in DNA–protein interactions (Jovin, 1976), and the experiments described in the present paper demonstrate an additional role for symmetry in the binding of an enzyme to DNA.

We have examined the reaction of the *Eco*RI restriction endonuclease on DNA. [This paper is concerned with only class-II restriction enzymes. These cleave double-stranded DNA at a fixed location relative to their recognition site and require only Mg^{2+} as their cofactor (Arber, 1974). The nomenclature of Smith & Nathans (1973) is employed for restriction enzymes and their recognition sites. The restriction sites for the *Eco*RI restriction endonuclease on the DNA from wild-type phage λ are numbered *srI*1 to *srI*5 from left to right (Thomas & Davis, 1975).] The *Eco*RI restriction enzyme cleaves both strands of double-stranded DNA at the recognition site:

the bonds cleaved being indicated by the arrows (Hedgepeth *et al.*, 1972). However, it is possible that both strands of the recognition site are cleaved together or, alternatively, the two strands may be cleaved by successive single-strand scissions. The *Eco*RI restriction endonuclease is a dimer of two protein subunits (Roulland-Dussoix *et al.*, 1974; Modrich & Zabel, 1976). Thus an attractive model for the interaction of this protein with its recognition site involves duplicated symmetry between the protein subunits and the DNA sequence (Kelly & Smith, 1970). The advantage of such an interaction would be maximized if the protein subunits acted co-operatively to cleave both strands of the DNA within a single enzyme–substrate complex. But some cleavage reactions must involve single-strand scissions, because it has been reported that the reaction of the *Eco*RI restriction enzyme with supercoiled DNA molecules yields some nicked open circles before the linear DNA products (Mulder & Delius, 1972; Modrich & Zabel, 1976; Ruben *et al.*, 1977).

The experiments described here confirm and extend these observations. In addition, a kinetic analysis of the reaction catalysed by the *Eco*RI enzyme is given which yields the relative rates of the first- and the second-strand scissions within a single recognition site. These experiments have been carried out with both supercoiled and linear DNA molecules in order to determine whether the conformation of the DNA affects its interaction with the protein. The hydrolysis of a single phosphodiester bond within supercoiled DNA will result in a different free-energy change from that in linear DNA on account of the relaxation of the supercoil (Bauer, 1978). We have also studied the reactions of some other restriction endonucleases in order to find out whether or not these cleave double-stranded DNA by the same mechanism as the *Eco*RI enzyme. The enzymes examined, and their recognition sequences, are: *Hin*dIII, 5′-A-A-G-C-T-T-3′ (Old *et al.*, 1975); *Sal*I, 5′-G-T-C-G-A-C-3′ (Arrand *et al.*, 1978); *Bam*HI, 5′-G-G-A-T-C-C-3′ (Roberts *et al.*, 1977).

Materials and Methods

DNA preparations

Escherichia coli strain UB5201 (*pro*, *met*, *nal*A, *rec*A-56) was transformed with the monomeric form of the plasmid pMB9 (Tc^r, *col*$E1^{imm}$; Rodriguez *et al.*, 1976). The plasmid was prepared from a transformant (Grinsted *et al.*, 1978) and further purified by centrifugation to equilibrium (32h at 45000rev./min, 15°C, in the 10×10ml rotor of the MSE Prepsin 50) in 10mM-Tris/1mM-EDTA, pH7.5, to which was added, per ml, 1.0g of CsCl and 0.2mg of ethidium bromide. The band of plasmid DNA was located in the gradient by near-u.v. illumination, and the ethidium bromide extracted from the DNA with CsCl-saturated propan-2-ol. The DNA was dialysed against 10mM-Tris/0.1mM-EDTA, pH7.5, and stored at 4°C. Plasmid pMB9 labelled with [^{3}H]thymidine was prepared in the same manner, except that 10μCi of [*methyl*-^{3}H]thymidine (The Radiochemical Centre, Amersham, Bucks., U.K.) and 0.5mg of 2′-deoxyadenosine (Sigma Chemical Co., Kingston upon Thames, Surrey, U.K.) were added per ml of growth medium. In electrophoreses of the plasmid pMB9 directly after preparation, more than 90% of the DNA possessed the mobility characteristic of monomeric supercoiled plasmid pMB9, less than 5% was located in the band due to either the open-circle form or multimeric forms, and less than 3% was linear plasmid pMB9. However, ^{3}H-labelled plasmid pMB9 (and to a lesser extent the unlabelled samples) relaxed over a period of weeks to yield the open circle. All the experiments described below were carried out with preparations of plasmid pMB9 containing more than 75% supercoiled DNA.

Wild-type bacteriophage λ was from this laboratory and phage λ_{421} from K. Murray (Department of Molecular Biology, University of Edinburgh). Phage λ_{421} carries the mutations *b*538 (▽*srI*1–2), *srI*3°, *srI*5°, *cI*857: this derivative is described as phage XI by Murray & Murray (1974). Preparations of either phage were made by the addition of the phage to an exponentially growing culture of *E. coli* C600 *thy*$^-$ (from this laboratory) in nutrient broth at 37°C made 10mM in $MgCl_2$. After the absorbance of the culture had fallen to a minimum, lysis was completed by the addition of chloroform (10ml/litre). Cell debris was removed by centrifugation (15min at 15000*g*). The lysate was then made 10% (w/v) poly(ethylene glycol) 6000 and 0.5M-NaCl by the addition of the solid reagents, and the solution left for more than 2h at 4°C (Yamamoto *et al.*, 1970). The phage were recovered by centrifugation (10min at 12000*g*), resuspended in 10mM-Tris/10mM-$MgCl_2$, pH7.5, and this resuspension was clarified by further centrifugation (10min at 12000*g*). To each ml of the supernatant was added 0.75g of CsCl and the solution was subjected to two cycles of equilibrium centrifugation (each for 16h as described above). The phage band from the gradient was dialysed against 10mM-Tris, pH7.5. Phage were deproteinized by two extractions with redistilled phenol equilibrated in 10mM-Tris/0.1mM-EDTA, pH7.5, and the phage-λ DNA was dialysed against this buffer before storage at 4°C over chloroform. The DNA from phage λ_{421} was labelled with [^{3}H]-thymidine by the addition of 20μCi of [*methyl*-^{3}H]thymidine per ml of culture of *E. coli* C600 *thy*$^-$ at the same time as the phage; the phage were then prepared in the same manner.

The concentration of all DNA solutions was estimated from their absorbance by using the value for $A_{260}^{0.1\%}$ of 20cm^{-1}. The molecular weight of the DNA from wild-type phage λ was taken as 30.8×10^6 (Davidson & Szybalski, 1971), and that from phage λ_{421} calculated from this value to be 25.8×10^6, given the size of the *b*538 deletion from Davidson & Szybalski (1971). The molecular weight of the DNA of plasmid pMB9 was found to be 3.6×10^6 from the electrophoretic mobility of the linear form (in close agreement with Rodriguez *et al.*, 1976). The molarities of DNA solutions are given in terms of DNA molecules; for both phage λ_{421} and plasmid pMB9, this is equal to the molarities of the *Eco*RI recognition sites.

Gel electrophoresis

Electrophoresis of DNA through agarose (Sigma, type 1) was carried out in slab gels on apparatus purchased from Raven Scientific Ltd. (Haverhill, Suffolk, U.K.) and modified for use with agarose gels 0.5cm thick. The running buffer was 40mM-Tris/acetate/5mM-sodium acetate/1mM-EDTA, pH8.0, also containing 1μg of ethidium bromide/ml

(Sharp *et al.*, 1973). The same conditions were employed for the electrophoresis of DNA denatured with alkali (Hayward, 1972). After electrophoresis, the bands of DNA were located by observation over a u.v.-transilluminator (U.V. Products Inc., San Gabriel, CA, U.S.A.). The amount of DNA in each band was quantified with [^{3}H]DNA by scintillation counting after excising an area of the gel enclosing the band. The gel slice (of volume between 0.2 and 0.7ml) was dissolved by incubation with 5ml of 5% (v/v) $HClO_4$ overnight at 67°C, and then 10ml of toluene/Triton X-100 (2:1, v/v) containing 6g of butyl-PBD [5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole]/litre (Fisons, Loughborough, Leics., U.K.) was added. Background values were obtained on gel slices adjacent to the slice containing the DNA.

The supercoil, open-circular and linear forms of plasmid pMB9 were separated from each other by electrophoresis through a 1% agarose gel at a voltage gradient of 5V/cm. Under these conditions, the supercoil (form I) had the fastest mobility, the open circle (form II) the slowest and the linear molecules (form III) a mobility intermediate between forms I and II (Johnson & Grossman, 1977). Separation of linear molecules of different molecular weights was by electrophoresis on agarose gels at 9V/cm, when the DNA samples were run in their native form, and at 1.2V/cm, when the DNA was denatured before electrophoresis. The two single strands obtained by the denaturation of a double-stranded molecule possess slightly different electrophoretic mobilities (Hayward, 1972): the gel slices excised for scintillation counting always included both bands in the doublet originating from the denaturation of one double-stranded molecule.

Enzymes

The *Eco*RI restriction endonuclease of *E. coli* RY 13 was obtained as a partially purified preparation from the Microbiological Research Establishment (Porton Down, Salisbury, Wilts., U.K.). The preparation was further purified by a method developed by A. Atkinson and A. Bingham of the Microbiological Research Establishment (personal communication). Before each stage, the enzyme was dialysed against the starting buffer for the next column. The partially purified preparation was first subjected to chromatography on cellulose phosphate (Whatman P11) with a linear gradient elution from 0.25 to 0.75M-NaCl, both buffers also containing 25mM-K_2HPO_4/10mM-β-mercaptoethanol/0.2% (v/v) Triton X-100, pH7.5. The enzyme was then applied to hydroxyapatite (Bio-Gel HTP, Bio-Rad, Richmond, CA, U.S.A.) and eluted with a linear gradient from 0.03 to 0.50M-K_2HPO_4, both buffers containing 0.50M-NaCl/10mM-β-mercaptoethanol/0.2% Triton X-100, pH7.0. Finally the preparation was filtered through Sephacryl S-200 (Pharmacia, Uppsala, Sweden) in 20mM-K_2HPO_4/400mM-NaCl/10mM-β-mercaptoethanol/0.2% Triton X-100, pH7.5. The enzyme at a concentration of 1.5×10^6 units/ml, was stored at −20°C in 10mM-K_2HPO_4/200mM-NaCl/10mM-β-mercaptoethanol/0.2% Triton X-100/50% (v/v) glycerol, pH7.5. Dilutions of the enzyme before assay were made in the storage buffer also containing 0.1mg of gelatin/ml (Greene *et al.*, 1974).

Assays to evaluate the concentration of the *Eco*RI enzyme were conducted by determining as 1 unit the amount of enzyme needed to complete the digestion of 0.5μg of phage-λ DNA in 20μl of 100mM-Tris/10mM-$MgCl_2$, pH7.5, within 30min at 37°C. The specific activity of the purified enzyme was found to be 1×10^7 units/mg. This value is in good agreement with that reported by Bingham & Atkinson (1978) for the homogeneous enzyme, if correction is made for the different assay conditions used here. Tests for contamination by non-specific nucleases in the preparation of the *Eco*RI enzyme were made by incubating various amounts of the enzyme with 0.8μg of the supercoiled replicative form of the DNA from bacteriophage ϕX174 *am*3 *cs*70 in 20μl of 50mM-Tris/100mM-NaCl/5mM-$MgCl_2$, pH7.5, for 1h at 37°C. The DNA (replicative form) of phage ϕX174 lacks an *Eco*RI recognition site (Sanger *et al.*, 1977); this DNA was a gift from Dr. N. L. Brown (Department of Biochemistry, University of Bristol). With 150 units of the *Eco*RI enzyme in this incubation, no degradation of the DNA from phage ϕX174 was detected; with 1500 units of enzyme, about half of the supercoiled DNA from phage ϕX174 had been converted into an open-circle form within 1h, though none of the linear form had been produced. The amount of the *Eco*RI preparation required to degrade a DNA molecule lacking an *Eco*RI recognition site was thus at least one thousand times in excess of that required to cleave a DNA molecule containing an *Eco*RI recognition site. We therefore suggest that the reactions described in this paper are due to the *Eco*RI restriction endonuclease rather than to non-specific nuclease impurities present in our preparation.

The *Hin*dIII restriction endonuclease of *Haemophilus influenzae* Rd was obtained as an exonuclease-free preparation from the Microbiological Research Establishment. The *Sal*I restriction endonuclease of *Streptomyces albus* G was partially purified (exonuclease-free) by the method of Arrand *et al.* (1978), except that the aminopentyl-Sepharose column from that procedure was omitted. A preparation of the *Bam*HI restriction endonuclease was obtained from *Bacillus amyloliquefaciens* H by the same procedures as that employed for the *Sal*I enzyme. All experiments with these three enzymes were carried out at 37°C under the following conditions: for *Hin*dIII, 10mM-Tris/50mM-NaCl/10mM-$MgCl_2$/7mM-β-mercaptoethanol, pH7.5; for *Sal*I, 50mM-Tris/100mM-NaCl/

5mM-$MgCl_2$, pH 7.5; for *Bam*H1, 20mM-Tris/7mM-$MgCl_2$/5mM-β-mercaptoethanol, pH 7.5.

The protocol for one kinetic experiment with a restriction endonuclease on a DNA substrate (that illustrated in Fig. 2) is given below. Other experiments, employing different restriction enzymes, different DNA substrates and altered reaction conditions, were carried out by minimal adjustment to this protocol. The reaction was initiated by the addition of 5μl of *Eco*RI restriction endonuclease (40 units) to 200μl (incubated at 22°C) containing 3.0μg of supercoiled plasmid pMB9 [^{3}H]DNA (4.3nM) in 50mM-Tris / 100mM-NaCl / 5mM-$MgCl_2$, pH 7.5. Samples (15μl) were removed at timed intervals after the addition of enzyme and added immediately to 20μl of 50mM-EDTA/5% Ficoll 400 (Pharmacia)/ 0.025% Bromophenol Blue, pH 8.0, to terminate the reaction within each sample (the zero-time point was withdrawn before the addition of enzyme). The samples were heated to 67°C and quenched on ice in order to dissociate loose complexes between DNA molecules. The entire volume of each sample was then loaded on one slot of an agarose slab gel. In experiments where the DNA products were analysed as single strands, 5μl of 1.0M-NaOH was added to each sample immediately before loading on the gel. When both double- and single-strand analyses on the products from the same reaction were carried out, the procedures were performed as above on a reaction mixture of twice the volume; samples taken at alternate time points were then either heated to 67°C or treated with alkali. The slab gel containing 12 or more time samples from one reaction was then subjected to electrophoresis, after which the bands containing the DNA were located and quantified as described above. The amount of DNA within each band was normalized against the total DNA added to each slot in order to correct for any variations in the volumes of the reaction mix loaded on to the gel. The extent of reaction (from 0 at zero time to 1.0 at completion) at each time point was calculated by reference to the concentrations of substrate and products present at 'zero' time and 'infinite' time. The volume of enzyme added to different reaction mixtures was always kept in the range 2–10μl, an appropriate pre-dilution of the enzyme being used to ensure that the reaction was sufficiently slow to permit analysis of the kinetic data yet fast enough to reach completion within 60min. Thus the reaction mixtures contained, in addition to the components specified above, the enzyme-storage buffer diluted by a factor of between 20 and 100 and also 50μM-EDTA from the buffers in which the DNA was stored. Tris buffers were adjusted to the requisite pH with HCl unless specified otherwise. The pH of all solutions refers to the value at room temperature.

Kinetic theory

It is shown below that the kinetics of the reaction of restriction enzymes with double-stranded DNA is compatible with the scheme where both steps are catalysed by the enzyme:

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C \qquad (1)$$

in which A represents the intact DNA duplex, B a DNA molecule in which one of the two strands has been cleaved at the recognition site for the restriction enzyme and C the DNA molecule (or molecules) in which both strands have been cleaved at this site: k_1 and k_2 are apparent first-order rate constants for the two steps in eqn. (1). Provided that $k_1 \neq k_2$ and that $[B_0] = [C_0] = 0$, the variations in the concentrations of the species in eqn. (1) with time are given by the equations:

$$[A] = [A_0]\,e^{-k_1 t} \qquad (2)$$

$$[B] = [A_0]\left(\frac{k_1}{k_2 - k_1}\right)(e^{-k_1 t} - e^{-k_2 t}) \qquad (3)$$

$$[C] = [A_0]\left[1 + \left(\frac{1}{k_1 - k_2}\right)(k_2 e^{-k_1 t} - k_1 e^{-k_2 t})\right] \qquad (4)$$

where [A] refers to the concentration of A at time t and $[A_0]$ to that at zero time (Gutfreund, 1972).

For reactions in which the initial substrate, A, is a supercoiled DNA molecule containing one recognition site for the restriction enzyme, the species B will then be a nicked open circle of this DNA and C the linear form. These three forms of the DNA may be separated from each other by agarose-gel electrophoresis (Johnson & Grossman, 1977) and thus the change in the concentration of all three forms may be monitored. Hence the rate constants k_1 and k_2 may be evaluated unambiguously by application of eqns. (2)–(4). Rate constants evaluated graphically by this procedure were subsequently tested by computer simulation of the reaction profile from eqn. (1) (Fig. 1*a*). The computer permitted the analysis of reactions in which the boundary conditions used here for the algebraic solutions (eqns. 2–4) were not held. For reactions with DNA preparations containing some open circle at zero time (i.e. $[B_0] \neq 0$), it was assumed that this had been formed by non-specific nicking during storage and was therefore treated separately from the open circles generated during the reaction.

For reactions following eqn. (1) in which the initial substrate, A, is a linear DNA molecule containing one recognition site for the restriction enzyme, the final product C will consist of two linear DNA molecules of lower molecular weight and thus separable from the substrate A by agarose-gel electrophoresis. However, the intermediate B, a linear molecule nicked in one strand at the recognition site, will possess the same electrophoretic

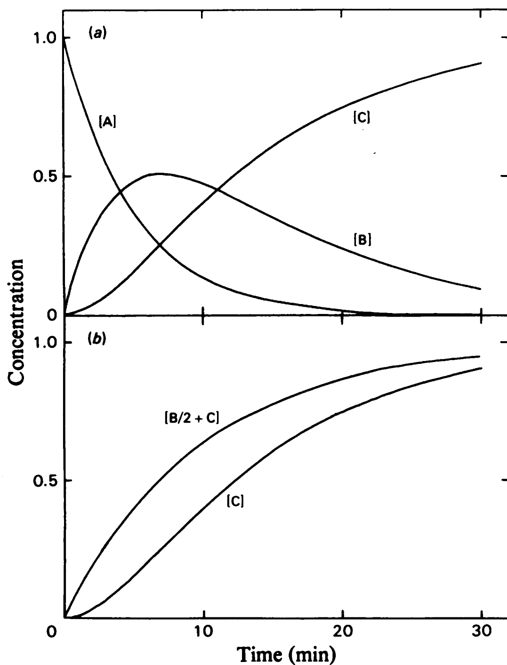

Fig. 1. *Theoretical curves from the reaction $A \rightarrow B \rightarrow C$*

(*a*) Theoretical curves from the reaction $A \xrightarrow{k_1} B \xrightarrow{k_2} C$ displaying changes in concentration with time for A, B and C (curves noted on Figure). Boundary conditions were set as $[A_0] = 1.0$, $[B_0] = [C_0] = 0$, $k_1 = 0.2\,\text{min}^{-1}$, $k_2 = 0.1\,\text{min}^{-1}$. (*b*) Theoretical curves from the reaction $A \xrightarrow{k_1} B \xrightarrow{k_2} C$ displaying the changes in concentration with time for B/2+C and C (curves noted on diagram). Boundary conditions were as in (*a*).

mobility as A when analysed under non-denaturing conditions. But if the DNA samples are denatured with alkali before electrophoresis and the samples run as single-stranded molecules (Hayward, 1972), one-half of the DNA in species B will migrate with the same mobility of the bands from the final product C. Thus in analyses under non-denaturing conditions, the appearance of product with time will follow the concentration of C in eqn. (1). In analyses under denaturing conditions, the appearance of product with time will follow the amount [B/2+C]. Theoretical curves from eqn. (1), showing the predicted data when product formation is monitored by either [C] or [B/2+C], are given in Fig. 1(*b*). Hence the existence of a nicked DNA intermediate may be detected during this reaction on a linear DNA substrate. But if the nicked intermediate B is either non-existent or present at very low concentrations, the two curves for the extent of reaction against time should be superimposable.

The reactions studied here are not formally the sequence of first-order reactions as indicated by eqn. (1), though the analysis in terms of successive exponentials is valid. For a reaction involving an enzyme that obeys Michaelis–Menten kinetics with a substrate that is present at an initial concentration below its K_m value, the overall appearance of product with time follows an exponential progress curve. But the rate constants, k_1 and k_2, evaluated by reference to eqn. (1), are apparent rate constants, where:

$$k_1 = \frac{V_{m1}}{K_{m1}} \text{ and } k_2 = \frac{V_{m2}}{K_{m2}} \quad (6)$$

in which V_{m1} and K_{m1} are the Michaelis parameters for the enzyme-catalysed conversion of A to B and likewise for k_2 in the conversion of B to C. The determination of the individual values for V_m and K_m has not proved possible to date because it requires the analysis of experiments carried out with substrate present in excess of the K_m values. Under those conditions, the equations that describe the rate of product formation are non-linear; in previous analyses of restriction enzyme kinetics (Modrich & Zabel, 1976; Ruben *et al.*, 1977; Rubin & Modrich, 1978), linear equations were employed for reactions that are non-linear under our conditions.

The integrated rate equations are applicable only under the following conditions: the initial substrate concentration must be less than the value of the K_m; the enzyme must retain its specific activity throughout the time course of the reaction; the product of the reaction must not be an inhibitor. We have not measured the value of the K_m of the *Eco*RI restriction endonuclease for either plasmid pMB9 or phage-λ_{421} DNA, but, for a series of reactions under the same conditions, we observed no alterations in the kinetic parameters, k_1 and k_2, upon altering the concentration of plasmid pMB9 DNA from 1.5 to 13.5nM. Hence, with $K_m > 13.5$nM, the condition $[S_0] < K_m$ has been met throughout our experiments. An enzyme concentration of 200 units/ml corresponds to about 0.4nM-*Eco*RI enzyme. Though enzyme and substrate concentrations are of the same order of magnitude, the concentrations of enzyme-bound intermediates will be negligible compared with those of the free substrate and products, because both reactants are present at concentrations below the K_m value. The stability of the enzyme during the time course of the reaction was proved by diluting the *Eco*RI enzyme from its storage buffer into 50mM-Tris/100mM-NaCl/5mM-$MgCl_2$, pH7.5, and incubating this solution for a period of time at 37°C before initiating a reaction by the addition of phage-λ_{421} DNA. Provided that the DNA was added to the enzyme after a preincubation of 60min or less, the rate of product formation in this reaction was indistinguishable from that in a parallel reaction,

where enzyme had been added to the DNA in the reaction buffer. Preincubation of the enzyme in the reaction buffer for longer than 60min caused a detectable fall in the activity of the enzyme. The absence of product inhibition (at the concentrations used here) was demonstrated by comparing the rate of product formation in two 200μl reaction mixtures containing the *Eco*RI enzyme (80 units) and 4.9μg of [^{3}H]DNA from phage λ_{421} in 50mM-Tris/100mM-NaCl/5mM-$MgCl_2$, pH7.5, at 22°C. One of these reaction mixtures also contained 4.5μg of [^{1}H]DNA from phage λ_{421} but no [^{3}H]DNA at the start of the reaction; sufficient time (30min) was allowed to elapse for the complete hydrolysis of the [^{1}H]DNA and then the [^{3}H]DNA was added to the mixture. The rate of the cleavage of the labelled DNA was the same in both reaction mixtures. Thus the products from the hydrolysis of the unlabelled DNA present in one of the reactions were not inhibitory.

Results and Discussion

EcoRI with supercoiled DNA

The products obtained at various times during the reaction of the *Eco*RI restriction endonuclease on the supercoiled form of the plasmid pMB9 were analysed by electrophoresis through agarose gels (Fig. 2*a*). The plasmid pMB9 contains a single *Eco*RI recognition site and thus the supercoiled form is cleaved by this enzyme to yield as the final reaction product the linear species (Rodriguez *et al.*, 1976). But during the reaction, the linear product appeared to be formed after the production of an open circular form of the DNA. Thus the open circle of plasmid pMB9 might be an intermediate during the conversion of supercoils to linear molecules. Observations of this nature have been made by others using different supercoiled-DNA substrates for the *Eco*RI restriction enzyme (Mulder & Delius, 1972; Modrich & Zabel, 1976; Ruben *et al.*, 1977). We now proceed to a kinetic analysis of this reaction.

The changes with time in the concentration of each species of DNA on the gel in Fig. 2(*a*) are shown in Fig. 2(*b*). The kinetics of this reaction are fully compatible with the sequential reaction mechanism of eqn. (1): good agreement exists between the experimental curves (Fig. 2*b*) and the theoretical curves for this mechanism (Fig. 1*a*). By itself, the existence of an open circle during this reaction (Fig. 2*a*) excludes the reaction mechanism in which the only process is the direct conversion of supercoiled DNA to the linear product. But the concentrations of these species during the reaction (Fig. 2*b*) prove that the open circle must lie on the direct pathway catalysed by the *Eco*RI enzyme between the supercoiled and linear forms, rather than on an alternative pathway. At the beginning of the reaction, the initial rate of formation of the open-circle form is equal to that of the disappearance of the supercoil, and a lag phase is observed in the generation of the linear product.

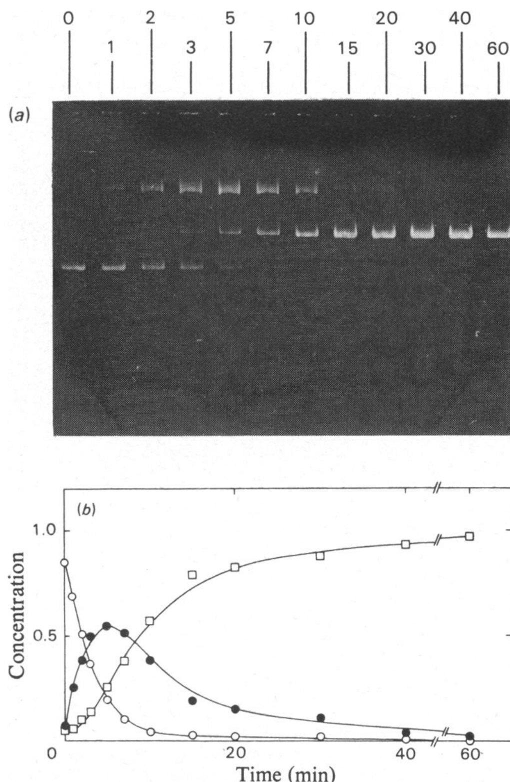

Fig. 2. *Agarose-gel electrophoresis (a) and quantitative determination (b) of the products of the reaction of the EcoRI restriction endonuclease on the supercoiled form of plasmid pMB9*

(*a*) Analysis by electrophoresis on a 1.0% agarose slab gel of samples withdrawn at the times (in min) specified by each slot on the gel from a reaction mixture of 200μl containing 40 units of *Eco*RI restriction endonuclease in 4.3nM-supercoiled plasmid pMB9 (^{3}H-labelled) and 50mM-Tris/100mM-NaCl/5mM-$MgCl_2$, pH7.5, at 22°C. Under the conditions employed for the electrophoresis, the three forms of plasmid pMB9 migrate towards the anode at the bottom of the gel in the following order: supercoiled DNA > linear DNA > open-circle DNA. (*b*) Determination of the concentration of the supercoiled (○), open-circle (●) and linear (□) forms of plasmid pMB9 (^{3}H-labelled) during the reaction in (*a*) by scintillation counting of radioactivity on slices taken from the above gel. The concentrations of each species of DNA are given relative to a total DNA concentration of 1.0.

By application of eqns. (2)–(4) to the data in Fig. 2(*b*), values for the apparent rate constants k_1

Table 1. *Apparent rate constants k_1 and k_2 for the reaction of EcoRI restriction endonuclease with plasmid pMB9*
Apparent rate constants k_1 and k_2 were evaluated (and their ratio k_1/k_2 calculated) from records of the reaction of the *Eco*RI restriction endonuclease on 4.3nM-supercoiled plasmid pMB9 in solutions at pH 7.5 containing the concentrations of Tris, NaCl and $MgCl_2$ indicated and at the specified temperature. The concentrations of the *Eco*RI enzyme were varied among the reactions, so that the kinetic analysis of each reaction could be conducted on the 0–60 min time scale; the experimental values of k_1 and k_2 were subsequently standardized against an enzyme concentration of 200 units/ml. The apparent rate constant k_1 refers to the conversion of the supercoiled DNA to the open-circle form and the apparent rate constant k_2 to the conversion of the open-circle DNA to the linear form.

	Reaction conditions				Apparent rate constants		
Reaction	Tris (mM)	NaCl (mM)	$MgCl_2$ (mM)	Temperature (°C)	k_1 (min^{-1})	k_2 (min^{-1})	Ratio k_1/k_2
A	50	100	5.0	22	0.32	0.16	2.0
B	50	100	5.0	37	1.05	0.42	2.5
C	50	100	0.5	22	0.046	0.020	2.3
D	50	50	5.0	22	0.42	0.20	2.1
E	50	150	5.0	22	0.14	0.06	2.3

and k_2 were obtained, which, in turn, yielded a value for the ratio k_1/k_2 of 2.0 (Table 1). In order to ascertain whether this 2:1 ratio for k_1/k_2 was a significant feature of the reaction of the *Eco*RI restriction enzyme with plasmid pMB9 or merely coincidental, a series of reactions similar to that in Fig. 2 was carried out under different reaction conditions. Alterations in reaction conditions are known to affect the activity of the *Eco*RI restriction enzyme (Roulland-Dussoix *et al.*, 1974). Under all conditions tested, reaction profiles similar to that in Fig. 2 were obtained; the cleavage of the supercoiled DNA to the linear product involved an open-circle intermediate and values for the apparent rate constants, k_1 and k_2, were determined (Table 1). The different conditions employed here resulted in the values of both k_1 and k_2 varying by a factor of 20 between the highest and the lowest rates. Yet throughout this range the ratio k_1/k_2 remained constant at close to 2.0. We therefore suggest that the 2:1 ratio for k_1/k_2 is an intrinsic feature of the reaction of the *Eco*RI restriction endonuclease.

The experiments with supercoiled plasmid pMB9 demonstrate that the *Eco*RI restriction endonuclease catalyses the hydrolysis of a susceptible phosphodiester bond on only one strand of this duplex at a time. Consequently the reaction of this enzyme on the supercoiled DNA molecule containing a single recognition site generates first an open-circle form of the DNA. Provided that the nick on the circular DNA is at the *Eco*RI recognition site, the subsequent hydrolysis of the susceptible phosphodiester bond within the second strand of the recognition site yields the linear product. We suggest that the 2:1 ratio between the apparent rate constants for the first- and the second-strand scissions originates on purely statistical grounds from the double-stranded nature of DNA. A supercoiled DNA molecule contains two strands; we assume that either can be nicked at the recognition site to produce an open circle. But an open circle produced in this manner contains only one strand that may be cleaved at the recognition site to yield the linear product. Thus, with two pathways for the conversion of supercoil to open circle but only one for the conversion of open circle to the linear product, the apparent rate constants for these reactions must differ by a ratio of two if all other factors are equal. The apparent rate constants are equal to the ratio V_m/K_m for each of the two reactions that are catalysed by this enzyme. Thus, after correction for the statistical factor, the ratio V_m/K_m is the same for both first- and second-strand scissions. Although it is possible that both V_m and K_m for the second-strand scission happen to differ by an identical factor from the values for the first strand, a simpler explanation is that both V_m and K_m remain unaltered. It follows from the likelihood that the K_m values are the same for the first- and second-strand scissions that the enzyme must dissociate freely from the nicked intermediate. If the enzyme remained bound to the intermediate, the K_m value for the second-strand scission would be infinitely low.

EcoRI with linear DNA

Further experiments have been carried out in order to determine whether the sequential mechanism by which the *Eco*RI restriction endonuclease cleaves the supercoiled DNA substrate also applies to linear DNA molecules. With a linear DNA molecule, it is possible to detect the presence or otherwise of an intermediate in which one of the two strands has been nicked at the recognition site, by analysing the products obtained during the time course of the reaction both with and without denaturation of the DNA before agarose-gel electrophoresis. One of the linear substrates employed in these studies was the DNA from phage λ_{421}. This phage carries mutations that have eliminated four out of the five *Eco*RI

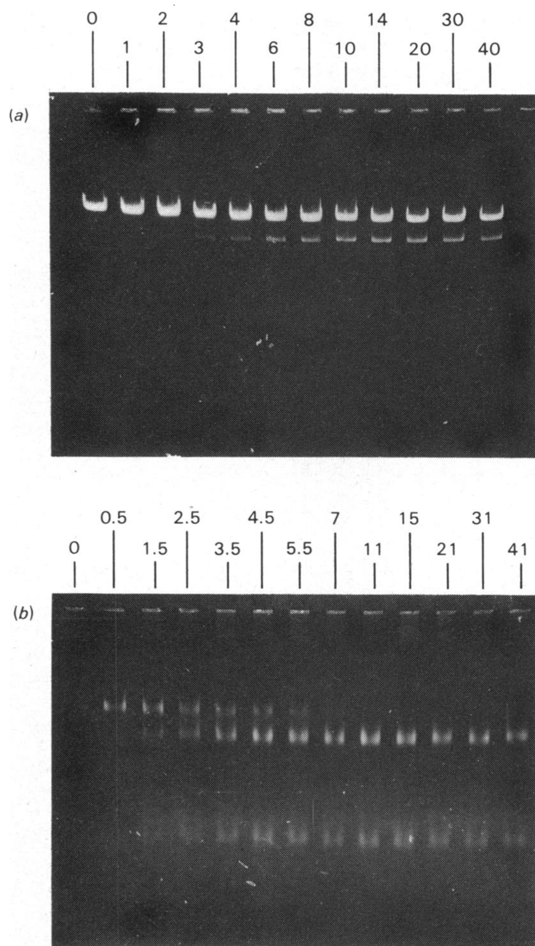

Fig. 3. *Agarose-gel electrophoresis of products of reaction of EcoRI restriction endonuclease with phage-λ_{421} DNA*
Analysis by electrophoresis on agarose slab gels of samples withdrawn at the times (in min) specified by each slot on the gel from a reaction mixture of 400 μl containing 80 units of *Eco*RI restriction endonuclease, 1.6 nM-[^{3}H]DNA from phage-λ_{421} and 50 mM-Tris/100 mM-NaCl/5 mM-$MgCl_2$, pH 7.5, at 22°C. (*a*) Samples of native DNA on a 1% gel. (*b*) Samples of DNA denatured with alkali on a 0.7% gel.

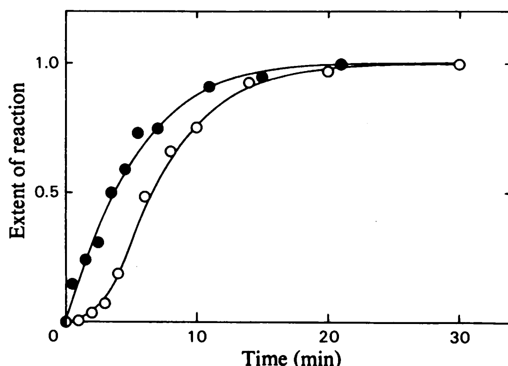

Fig. 4. *Extent of reaction with time from the reaction of the EcoRI restriction endonuclease on DNA from phage λ_{421} in Fig. 3*
The amount of [^{3}H]DNA in gel slices containing each of the bands observed on the gels in Fig. 3 was determined by scintillation counting of radioactivity and the extent of reaction at each time point calculated from the disappearance of substrate and/or the appearance of product. ○, Extent of reaction determined from samples of native DNA on the gel in Fig. 3(*a*); ●, extent of reaction determined from samples of denatured DNA on the gel in Fig. 3(*b*).

recognition sites present on the DNA from wild-type phage λ, the only site remaining being *srI*4 (Murray & Murray, 1974).

The products obtained during the reaction of the *Eco*RI restriction endonuclease on the DNA from phage λ_{421} are shown in Fig. 3. When the electrophoresis was carried out on samples of native DNA (Fig. 3*a*), one of the products of the reaction had a mol.wt. of 19.9×10^6 and was not separated from the substrate of mol.wt. 25.8×10^6; the extent of reaction was then calculated from the increase in the radioactivity of the gel slice containing the product of mol.wt. 5.9×10^6. When the DNA was denatured before electrophoresis (Fig. 3*b*), the single strands of the substrate were separated from both of the products in their single-strand forms, and thus the extent of reaction could be calculated from all three bands. The extent of reaction against time, from analyses of both double-stranded and single-stranded product formation out of a single reaction mixture, are shown in Fig. 4. A lag phase was noted when product formation was analysed as double-stranded DNA. However, the formation of product in the analysis of single-stranded DNA did not incur a lag phase and preceded the double-stranded product. Good agreement exists between the experimental curves (Fig. 4) and the theoretical curves predicted for the sequential reaction mechanism in eqn. (1) with a ratio for k_1/k_2 equal to 2.0 (Fig. 1*b*). The experiment illustrated in Fig. 3 was repeated at 37°C with very similar results to those in Fig. 4.

The linear form of plasmid pMB9 was also used as a substrate for the *Eco*RI restriction endonuclease. The plasmid contains a single recognition site for the *Sal*I restriction endonuclease located about 0.8 kilo bases from the *Eco*RI site (Rodriguez *et al.*, 1976). Thus digestion of plasmid pMB9 with the *Sal*I restriction enzyme produces a linear DNA molecule of mol.wt. 3.6×10^6, which may be cleaved by the

*Eco*RI restriction enzyme to two products of mol.wts. 3.0×10^6 and 0.6×10^6 respectively. Both products may be separated from the substrate by agarose-gel electrophoresis of either the native or the denatured DNA. The rate of formation of the products from the reaction of the *Eco*RI restriction enzyme on linear plasmid pMB9 was measured on samples taken from one reaction mixture, samples taken at alternate time points being analysed as native and as denatured DNA (Fig. 5*a*). Again it was found that product formation determined on samples of single-stranded DNA preceded that on the samples of double-stranded DNA.

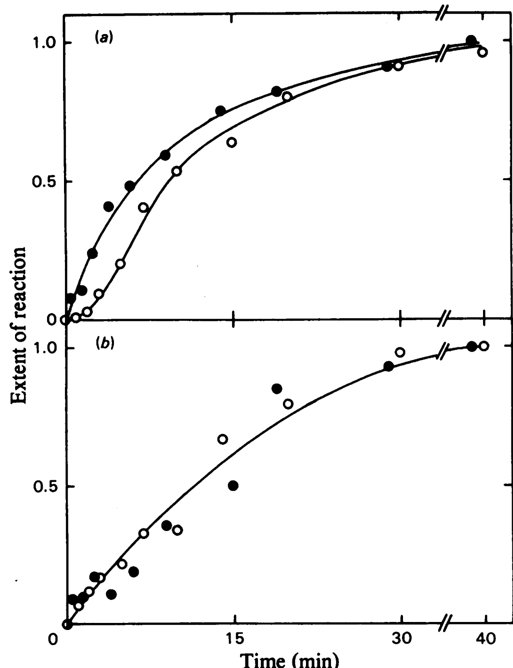

Fig. 5. *Extent of reaction with time from the reactions of restriction endonucleases on the linear form of the DNA of plasmid pMB9*

The linear form of plasmid pMB9 DNA was obtained by digesting, in 500μl, 8.3μg of ^{3}H-labelled supercoiled pMB9 (4.6nM) and 50mM-Tris/100mM-NaCl/5mM-$MgCl_2$, pH7.5, at 37°C with either the *Sal*I or the *Eco*RI restriction endonuclease. Upon completion of the linearization reaction, the solution was heated to 67°C for 30min to denature the restriction enzyme and then incubated at the requisite temperature for a second reaction with the other restriction enzyme from this pair. Samples withdrawn at timed intervals after the addition of the second restriction enzyme were analysed by electrophoresis either on 1.0% agarose gels as native DNA or on 1.4% agarose gels after denaturation of the DNA with alkali. The amount of [^{3}H]DNA in gel slices containing each of the bands observed on the gels was determined by scintillation counting, and the extent of reaction at each time point calculated from the disappearance of substrate and the appearance of product. ○, Extent of reaction determined from samples of native DNA; ●, extent of reaction determined from samples of denatured DNA. (*a*) Reaction of the *Eco*RI restriction endonuclease (160 units) at 22°C on plasmid pMB9 linearized by *Sal*I. (*b*) Reaction of the *Sal*I restriction endonuclease (25μl) at 37°C on plasmid pMB9 linearized by *Eco*RI.

The experiments with the DNA from both phage λ_{421} and linear plasmid pMB9 demonstrate that the reaction catalysed by the *Eco*RI restriction endonuclease on these linear DNA substrates is the cleavage of a single strand at its recognition site on the DNA duplex. A linear DNA molecule nicked in this manner contributes to the formation of products from this reaction when the DNA is denatured before electrophoresis, but not when the electrophoresis is carried out with native DNA. Product formation is observed with native DNA only after the restriction enzyme has cleaved the second strand of the nicked intermediate, thus accounting for the lag phase in these analyses. The single-strand scission that generated the nicked intermediate must have occurred at the *Eco*RI recognition site because random nicking of linear DNA would yield products of heterogeneous molecular weight when analysed under denaturing conditions. The results obtained with the supercoiled and the linear forms of plasmid pMB9, as substrates for the *Eco*RI enzyme (Figs. 2*b* and 5*a*) demonstrate that the mechanism of this enzyme is unaffected by gross changes in the conformation of the DNA. The concentration of the nicked intermediate during the cleavage of linear plasmid pMB9 was calculated from the gap between the two curves in Fig. 5(*a*), and it was found that the maximal concentration of the nicked linear molecule was equal to the maximal concentration of the open-circle form of plasmid pMB9 during the cleavage of the supercoiled substrate (Fig. 2*b*). The different reactivities of the *Eco*RI enzyme on the linear and the supercoiled forms of plasmid pMB9 may be due to protein impurities in the preparation of the *Sal*I enzyme, which are binding to the DNA and thus inhibiting the activity of the *Eco*RI enzyme on the linear substrate (Fig. 2*b*, 200 enzyme units/ml in reaction; Fig. 5*a*, 320 units/ml).

Similar results were obtained in this study with two different DNA substrates for the *Eco*RI restriction enzyme (Figs. 4 and 5*a*). However, Rubin & Modrich (1978) have proposed that the mechanism of this enzyme varies with the substrate. It appears that the supercoiled form of the plasmid *col*E1 interacts with the *Eco*RI enzyme in a different manner, because the open-circle form of this DNA accumulated when the reaction was carried out at temperatures below 15°C but not higher temperatures

(Modrich & Zabel, 1976). But our results cannot be compared directly with the previous observations on account of alterations in the reaction conditions (particularly the substrate concentrations), the enzyme preparation and the methods used for the analysis of the kinetic data (see the Materials and Methods section).

Other restriction enzymes

The plasmid pMB9 contains a single recognition site for the *Hin*dIII and the *Bam*HI restriction endonuclease as well as for the *Sal*I and the *Eco*RI enzymes (Rodriguez *et al.*, 1976). The reactions of these other restriction enzymes on the supercoiled DNA of plasmid pMB9 were examined. With the *Hin*dIII restriction enzyme, a reaction profile very similar to that recorded with the *Eco*RI enzyme was observed (results not shown); the supercoiled form of the substrate was converted into an open circle and subsequently the open circle was cleaved to yield the linear species as the final product of the reaction. The apparent rate constants for the two reactions catalysed by the *Hin*dIII restriction enzyme yielded a value for the ratio k_1/k_2 of approx. 2:1.

However, in the reaction of the *Sal*I restriction endonuclease on the supercoiled form of plasmid pMB9, an entirely different result was obtained. An analysis of the products formed during the reaction of the *Sal*I enzyme on plasmid pMB9 is shown in Fig. 6(*a*) and quantification of these data is given in Fig. 6(*b*). The direct conversion of the supercoiled DNA to the linear form was observed without detecting an open-circle intermediate. The preparation of plasmid pMB9 used here was about 12% open-circle form at zero time, but the generation of any of the open-circle form could not be detected during the reaction catalysed by the *Sal*I restriction enzyme. In this reaction (Fig. 6*b*) the rate of appearance of the final linear product did not involve a lag phase (as had been observed in the reaction with the *Eco*RI enzyme; Fig. 2*b*) and was equal throughout the reaction to the rate of disappearance of the supercoiled substrate.

The *Sal*I restriction endonuclease was also tested against a linear DNA substrate. The complete reaction of the *Eco*RI restriction enzyme on supercoiled plasmid pMB9 yielded a linear DNA molecule, which may be cleaved by the *Sal*I restriction enzyme. The time course of product formation during the reaction of the *Sal*I restriction enzyme on the linear DNA from plasmid pMB9 was determined by analysing samples withdrawn at various times from one reaction mixture as either native or denatured DNA (Fig. 5*b*). The two curves for the extent of reaction against time were found to be superimposable within the limits of experimental accuracy. The deviations from a single curve for Fig. 5(*b*) are random in that for approximately half of the pairs of time points (two samples being withdrawn from the reaction mixture for analyses as either native or denatured DNA at time intervals separated by 0.5 or 1.0min) the reaction has proceeded further when

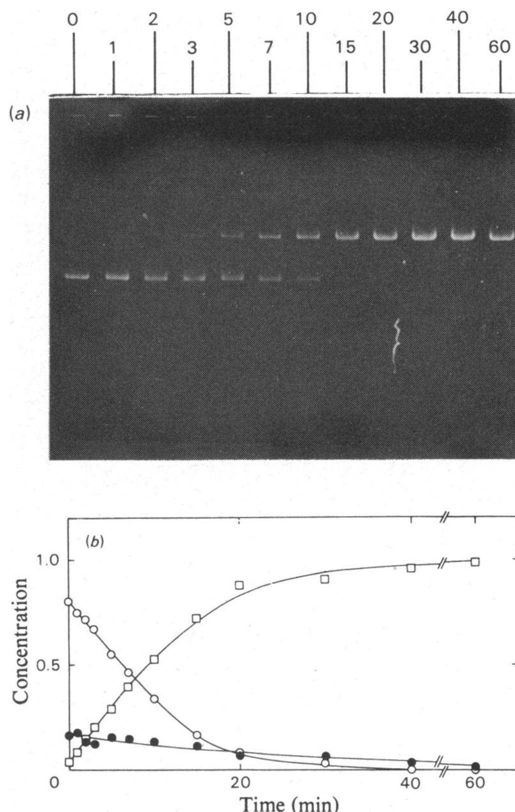

Fig. 6. *Agarose-gel electrophoresis (a) and quantitative determination (b) of the products of the reaction of the SalI restriction endonuclease on the supercoiled form of plasmid pMB9*

(*a*) Analysis by electrophoresis on a 1.0% agarose slab gel of samples withdrawn at the times (in min) specified by each slot on the gel from a reaction mixture of 200μl containing the *Sal*I restriction endonuclease (10μl) in 4.3nM-supercoiled plasmid pMB9 (^{3}H-labelled) and 50mM-Tris/100mM-NaCl/5mM-$MgCl_2$, pH7.5, at 37°C. Under the conditions employed for the electrophoresis, the supercoiled form of pMB9 migrates to the anode at the bottom of the gel faster than the linear form; the open-circle form would give rise to a band of lower mobility than the linear form. (*b*) Determination of the concentrations of the supercoiled (○), open-circle (●) and linear (□) forms of plasmid pMB9 (^{3}H-labelled) during the reaction in (*a*) by scintillation counting on gel slices taken from the above gel. The concentrations of each species of DNA are given relative to a total DNA concentration of 1.0.

monitored by the formation of single-stranded DNA as compared with double-strand product formation; for the remaining pairs of time points, the reverse was noted. In the reaction of the *Eco*RI restriction enzyme on linear plasmid pMB9 (Fig. 5*a*), the differences between native and denatured DNA product formation are systematic in that, for every pair of time points, the amount of single-stranded product was always greater than or equal to the amount of double-stranded product. Thus, in contrast with the *Eco*RI enzyme, an intermediate DNA structure in which one strand of the duplex has been nicked at the recognition site has not been detected before the cleavage of both strands during the reaction of the *Sal*I restriction enzyme on either linear or circular DNA substrates.

The action of the *Bam*HI restriction endonuclease on the supercoiled form of plasmid pMB9 yielded a reaction profile similar to that observed with the *Sal*I enzyme, except that, in the case of the *Bam*HI enzyme, a small amount of the open-circle form of plasmid pMB9 was generated during the conversion of the supercoil to the linear species (Fig. 7). At the maximum on the plot of the concentration of the open-circle form against time, about 10% of the total DNA in the reaction mixture was open circle that had been generated in the course of the *Bam*HI reaction. We therefore estimate the value for the ratio of the apparent rate constants, k_1/k_2, to be less than 0.2 for the reaction catalysed by *Bam*HI, in contrast with the value of 2.0 for this ratio with *Eco*RI. But, on account of the low concentration of the open-circle form (Fig. 7), it cannot be proved that this species is an intermediate in the reaction of the *Bam*HI restriction enzyme between the supercoiled and linear forms; it might be generated by a non-specific nuclease impurity.

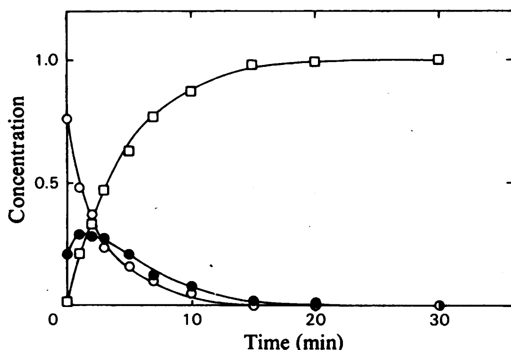

Fig. 7. *Determination of the concentrations of the supercoiled* (○), *open-circle* (●) *and linear* (□) *forms of plasmid pMB9 during the reaction of the BamHI restriction endonuclease on* 4.3nM-*supercoiled pMB9* (^{3}*H-labelled*) *in* 20mM-*Tris*/7mM-$MgCl_2$/5mM-β-*mercaptoethanol*, *pH*7.5, *at* 37°C

Samples withdrawn from the reaction mixture at various times were analysed by electrophoresis on a 1.0% agarose gel, and the concentrations of the three forms of the DNA determined by scintillation counting of radioactivity on slices from the gel. The concentrations of each species of DNA are given relative to a total DNA concentration of 1.0.

The results presented here demonstrate unequivocally that different restriction endonucleases isolated from various bacterial species do not possess a common mechanism of action, despite the fact that all class-II restriction enzymes appear to catalyse an equivalent reaction, namely the cleavage of both strands of the DNA at a fixed location relative to their respective recognition sites. The *Eco*RI restriction endonuclease cleaves the DNA substrates examined here by means of two separate reactions, the reaction catalysed by this enzyme being a single-strand scission, though the enzyme displays equal reactivity towards the first and second strands. The *Hin*dIII restriction enzyme operates in the same manner as the *Eco*RI enzyme, and it has been reported elsewhere that the *Hpa*II restriction endonuclease from *Haemophilus parainfluenzae* cleaves the supercoiled form of SV40 DNA through an open-circle intermediate (Ruben *et al.*, 1977). In contrast, the cleavage of a single strand within the recognition site for the *Sal*I restriction enzyme has not been detected with this enzyme, though the existence of a transient intermediate has not been eliminated. Thus, if the *Sal*I enzyme cleaves double-stranded DNA by means of two single-strand scissions, then either V_m must be much higher or K_m much lower for the second strand compared with the first. However, the possibility remains that with the *Sal*I enzyme both strands of the DNA duplex are cleaved within a single enzyme–substrate complex. If this possibility were to be proved, then the *Sal*I and the *Eco*RI restriction enzymes catalyse different reactions at the respective recognition sites. The *Bam*HI restriction enzyme also displays a higher reactivity towards the second strand of the DNA, once it has cleaved the first strand of its recognition site.

However, the *Sal*I and the *Eco*RI restriction endonucleases are similar in one respect. Though the enzymes display different mechanisms of action, each enzyme retains its own mechanism with both supercoiled and linear DNA substrates. Thus the mechanisms of both enzymes are unaffected by changes in the gross conformation of the DNA. In addition, the conformational change of the DNA that must follow the nicking of one strand in a supercoiled molecule does not affect the relative rates of the first- and second-strand scissions with either enzyme.

Symmetry in DNA–protein interaction

The DNA sequences recognized by many proteins that bind to specific locations on DNA molecules possess 2-fold rotational symmetry. These include the recognition sites for most restriction endonucleases. It has been suggested that a section of DNA possessing 2-fold rotational symmetry can loop out of the double-helical structure and form intra-strand base-pairs, the resulting cruciform arrangement being the site recognized by the protein (Gierer, 1966; Meselson *et al.*, 1972). However, a strong argument against the requirement of a cruciform to enable the *Eco*RI restriction enzyme to bind to DNA is the fact that a self-complementary octanucleotide containing the *Eco*RI recognition site can function as a substrate for the *Eco*RI enzyme only when present as a duplex (Greene *et al.*, 1975).

Yet there remains the possibility that the binding of the protein to the DNA might induce a conformational change in the DNA at the recognition site, which results in a cruciform being established only in the DNA–protein complex; other rearrangements of the DNA within the complex are also possible (Jovin, 1976). But the generation of a cruciform from duplex DNA will require unwinding of the helix. On account of the topological restraints that apply to covalently closed DNA (Bauer, 1978), the induction of such a conformation into a supercoiled DNA molecule by the binding of the protein will involve a different free-energy change from that on a linear DNA molecule, which in turn might be expected to affect the kinetic parameters of the DNA–protein interaction. The same argument applies to alternative rearrangements of the DNA–protein complex. However, both the *Eco*RI and the *Sal*I restriction endonucleases display the same mechanism of action on the supercoiled and the linear forms of one DNA molecule. Moreover, in the case of the *Sal*I enzyme, the reactions on the two conformations of the substrate proceed at very similar rates; values for V_m/K_m were calculated as $0.08\,min^{-1}$ with supercoiled plasmid pMB9 (Fig. 6*b*) and as $0.07\,min^{-1}$ with the linear form (Fig. 5*b*). Thus unwinding of the DNA, either before or after the binding of the protein, seems not to be part of the interactions between DNA and restriction enzymes. It had previously been shown that extensive unwinding of the DNA was also unnecessary for the binding of either the *lac* or the λ repressors to their respective operators (Wang *et al.*, 1974; Maniatis & Ptashne, 1973). In contrast, RNA polymerase binds more tightly to supercoiled DNA than to relaxed DNA (Warner & Schaller, 1977).

An alternative model for DNA–protein interactions proposes that, within the DNA–protein complex, the axis of symmetry through the DNA recognition sequence is superimposed by an axis of symmetry relating two or more protein subunits in an oligomeric protein (Kelly & Smith, 1970; Steitz *et al.*, 1974). This model remains an attractive working hypothesis from our data for a restriction endonuclease such as *Sal*I. We suggest that the simultaneous cleavage of both strands in the DNA duplex during the reaction catalysed by the *Sal*I enzyme is achieved by the binding of a symmetrical protein composed of two or more subunits at the symmetrical recognition site and that the protein subunits then function in a highly co-operative manner. We also suggest a similar mechanism for the *Bam*HI restriction enzyme, except that the co-operativity between the protein subunits may be lower. The *Bam*HI restriction enzyme is a dimer of two protein subunits (Smith & Chirikjian, 1977), but the subunit composition of the *Sal*I enzyme is not known at present. However, this model is less attractive for the *Eco*RI restriction enzyme. Though the *Eco*RI restriction enzyme exists as a dimer (Modrich & Zabel, 1976) and thus symmetry may be involved in the binding of the protein to its recognition site, the advantage of a symmetrical interaction is not realized because the protein subunits do not act co-operatively during the catalytic reaction.

An additional consideration that we propose for the role of symmetrical DNA sequences at recognition sites for proteins arises from the biological function of the protein. One function of a restriction endonuclease as part of a host-specified restriction/modification system is the degradation of any DNA molecules within the host that lack the appropriate modification markers. The degradation of the unmodified DNA is achieved by the restriction endonuclease cleaving both strands of the DNA duplex and thus creating substrates for exonucleases that break the restriction fragments down to their constituent mononucleotides (Arber, 1974). Yet the reaction catalysed by the *Eco*RI restriction endonuclease is the cleavage of only one strand of the duplex at its recognition site, a reaction that by itself fails to produce a substrate for many exonucleases. Thus the only procedure by which the biological function and the catalytic activity of this enzyme may be reconciled is for the recognition site to possess the same 5′–3′ sequence on both strands of the DNA. It is only due to the symmetrical DNA sequence that, after the cleavage of one strand on the duplex, the *Eco*RI restriction enzyme can return to the same location on the DNA for the cleavage of the second strand.

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