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# Kinetic analysis of drug cleavage of closed-circular DNA

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**Abstract**

Various cleavage agents interact with circular double-stranded DNA molecules to convert the closed-circular form (form-I) to the open-circular (form-II) and linear (form-III) forms, and ultimately to small DNA fragments. The various cutting processes which take place in the DNA pool are here analyzed kinetically, and, by solving the kinetic equations, expressions are derived for the amounts of the closed-circular, open-circular, and linear forms of DNA as a function of reaction time and concentration of cleavage agent. Conversions between subspecies of forms II and III, differing in numbers of internal cuts, are taken into account. The only assumption required to solve the kinetic equations is that the concentration of cleavage agent obeys $[D] = D_0 f(t)$ where the function of time $f(t)$ is independent of $D_0$, the initial concentration of cleavage agent. By choosing parameters in the expressions for the calculated amounts of forms I, II and III to give the best fit to the measured amounts, one obtains information about the rates and rate constants for the conversions. The rate constants in turn give important information about the specificity and mechanism of action of the cleavage agents. The analysis is applied to the cleavage of pBR322, SV-40 and PM2 DNAs by DNase I, Fe–EDTA, and the antitumor agents calicheamicin and bleomycin. Cleavage rate constants are derived and discussed for these systems. © 1997 Elsevier Science B.V.

*Keywords:* Closed-circular; Cleavage; Kinetics; Rate constants; Specificity

## 1. Introduction

Most anticancer drugs exhibit cytotoxicity by interacting with cellular DNA [1]. The interactions range from equilibrium binding to covalent attachment or chemical change, and may be followed by cleavage of the sugar–phosphate backbone of DNA. Closed circular DNA molecules are ideal substrates for studying drugs which can cut DNA. If a drug produces a single break in the sugar–phosphate backbone, the molecule converts from the closed-circular form (form-I) to nicked-circular or open-circular DNA (form-II). If a second break is produced near the first cut but on the opposite strand, the DNA molecule is converted to the linear form (form-III). The maximum distance between the breaks for which this occurs is dependent on sequence, ionic strength, and temperature [2]. A drug which can simultaneously cut both DNA strands causes form-I to be converted directly to form-III DNA. From measurements of the amounts of the three forms of DNA before and after treatment with a DNA-cleaving drug, one can learn about the rates of these processes. The relative rates give information about cleavage mechanism and specificity.

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For example, if the cleavage agent can break only one strand at a time and cleavage is nonspecific, many breaks in form-II DNA will be necessary for its conversion into form-III. Then the rate of conversion of form-I DNA to form-II will be much greater than the rate of conversion of form-II to form-III and the apparent rate of conversion of form-I to form-III will be small. When this is the case, the agent is said to be a 'single strand' (ss) cleavage agent. On the other hand, the rate of conversion of form-I to form-III DNA will be significant if one molecule of cleavage agent possesses two DNA-damaging equivalents and can cut both strands of DNA in a single binding/cleavage event. Thus, for the enediyne antibiotics calicheamicin and esperamicin [3–5], extremely toxic drugs which cut DNA, a single activation event on the 'warhead' portion of the drug can result in cleavage of opposing DNA strands, i.e., a double-strand break. Since this type of lesion is more difficult to repair than a single-strand break, its presence jeopardizes the survival of the cell, contributing to the toxicity of these drugs.

A situation equivalent to double-strand cleavage arises when form-III is produced from form-I by multiple binding/cleavage events involving more than one drug molecule, but the rate constant for the second cleavage event is very large. For example, if the drug is highly sequence-specific, so that cutting is limited to a small number of sites on DNA, the probability that two breaks will occur on nearby sites on opposing strands will be high. This also holds when the cleavage agent has a high affinity for a nicked site, because the initial break by the drug creates a site which binds drug more effectively than an unbroken site. This allows efficient cleavage of the opposing strand by a second drug molecule, producing form III. Regardless of mechanism, a cleavage agent which appears to convert form I directly into form III is said to be a 'double-strand' (ds) cleavage agent.

It should be noted that, even if the first cut made on form-I DNA produces a site which has a higher affinity for the cleavage agent than an uncut site, so that a second cut near the first cut but on the opposite strand is more likely, the situation may still appear as single-strand cutting. Only if the rate constant for (form-II) → (form-III) is very large compared to the rate constant for (form-I) → (form-II) will the situation be indistinguishable kinetically from direct conversion of form-I to form-III by double-strand cleavage. To determine whether direct conversion, as opposed to two single-strand cleavage events, is occurring, one can plot the number of double-strand cleavages against the number of single-strand cleavages, as was done by Povirk et al. [6]. For double-strand cleavage, the dependence should be linear; if double-strand cleavage actually occurs by two independent random single-strand cleavages, the dependence should be quadratic. Unfortunately, if the nicked site has a high affinity for the cleavage agent, the two ss cleavages will not be independent. Also, this method does not provide quantitative information about rate constants, as can in principle be provided by the kinetic analysis presented here.

When a drug is known to be a single-strand cleavage agent, it should be possible to learn about site specificity. In this case, $R$, the ratio of the rates of conversion of form-I to form-II and conversion of form-II to form-III, should be largest when drug cutting is nonspecific. When there is complete randomness, $R$ should be equal to the number of sites in the DNA divided by the number of sites close enough to a break so that a cut at one of these sites leads to the II-III conversion. Increased sequence specificity should decrease $R$ because, by limiting the sites at which cleavage occurs, it makes it more likely that the second cut occurs near the first one.

Although closed-circular DNA molecules are widely used to study the kinetics of DNA cleavage by drugs, solutions to the kinetic equations for a number of important cases have not been published. In previous publications from this laboratory the kinetics corresponding to the transformation processes was analyzed by a simple model, in which no distinction was made between, say, form-II DNA molecules with one strand break and form-II DNA molecules with several strand breaks. By grouping together all form-II (and all form-III) molecules, we were able to reduce the kinetic equations to a very small number, and solve them explicitly. The price paid was that some of the rate constants whose values were derived were actually average rate constants for a number of processes, and interpreting these average constants may be misleading.

In the present paper, we explicitly take into ac-

count the varieties of forms II and III which differ in the numbers of internal nicks, and elucidate the different processes that convert one into another. For example, single-strand cleavage of a form-I molecule produces form II with a single internal cut. A second single-strand cleavage may then convert the molecule to form II with two internal cuts, or, if the second cleavage is close enough to the first and on the opposite strand, to form III with no internal cuts. A form-II molecule with two internal cuts is converted to form-III more readily (with a higher rate constant) than is a form-II molecule with one internal cut. A form-III molecule with no internal cuts cannot be converted into smaller fragments by a single-strand cleavage, but a form-III molecule produced from a form-II molecule with two internal cuts has itself one internal cut, and can be converted into small fragments by a single-strand cleavage. The different processes and subspecies of molecules are described in Section 2.

The kinetic equations are given, and are all second-order. They may be solved, to give the concentrations of all subspecies as a function of time $t$ and initial concentration of cleavage agent $[D]_0$, under a single assumption. It is assumed that the concentration of cleavage agent during the digest obeys $[D] = [D]_0 f(t)$ where $f(t)$ is independent of $[D]_0$. In fact, most cleavage agents or activated drugs can be degraded or deactivated by reactions other than DNA cleavage, so that the effective concentration of cleavage agent decreases during the digest in a way unrelated to the DNA cleavage. We will show that our analysis is still valid, although only ratios of rate constants, and not individual rate constants, can be determined.

The experimental data are measured amounts of the three forms of the DNA (as determined by quantitative agarose gel electrophoresis) as a function of drug concentration or digest time. From the solutions to the kinetic equations, the total amounts of forms I, II and III (the experimentally measured quantities) may be calculated. We analyze the data by seeking the values of rate-constant and other parameters which minimize the sum of the squared deviations of the calculated amounts of the three species from the measured amounts. From these parameters, one can calculate rate constants for conversion among the three forms of DNA, ascertain the relative amounts of single- and double-strand cleavage, and derive other interesting quantities.

The values of the rate constants are of course of greatest interest, but the ratios of rate constants are also important. For instance, one may compare the rate of cleavage of form I for different cleavage agents, or DNA molecules. Also, for elucidating the mechanism of the cleavage process, it is particularly important to determine whether an agent is a single-strand cleavage agent or whether it can cleave both strands of DNA simultaneously. Then the ratio of rate constants for the two processes is of interest. In addition, ratios of rate constants may give information about the sequence specificity of the cleavage agent. In order to convert form-II (open circular) DNA to form-III (linear) DNA by a cut on a single strand, this cut must be sufficiently close, say within $m$ base pairs, to a preexisting cut on the other strand. If a DNA fragment has $n$ base pairs, and all positions are equally likely for nicking by the cutter (random cutting), the probability that form-II DNA will be cut in such a way as to produce form-III DNA is $m/n$. If the ratio of rate constants for II $\rightarrow$ III and I $\rightarrow$ II is larger, it implies that cutting is not random with respect to position (specificity of the cleavage agent).

The kinetic analysis, given in Sections 2 and 3, is first applied to already published results involving the agents DNase I and calicheamicin. Afterwards, we present new experimental data involving the hydroxyl radical–generating complex, Fe–EDTA, and the antitumor agent bleomycin. We apply our analysis to these data to derive rate constants, which are discussed in terms of the specificity and cleavage mechanism of the drug.

## 2. Rate constants

The cleavage agent or drug, by nicking a molecule of DNA in the closed circular form, will convert it to the open circular form or, if cleavage occurs on both strands of DNA, to the linear form. A nick on one strand of an open circular DNA, if it is sufficiently near a pre-existing nick on the opposing strand, will convert the open circular DNA to linear DNA. A double-strand cleavage of open circular DNA also produces linear DNA. Nicking of linear DNA by the

drug may convert it to smaller fragments; as small fragments are not normally measured on the gel, this appears as a loss of DNA. All the processes are assumed to be second-order, first-order in the drug concentration and first-order in the concentration of the DNA species attacked.

We refer to the closed circular form of DNA as form-I, the open circular form as form-II, and the linear form as form-III. It is necessary to distinguish between different subspecies of forms II and III. We denote by 'II;$i$' form-II with $i$ internal cuts ($i$ must be at least 1) and by 'III;$i$' form-III with $i$ internal cuts (here $i$ can be 0, 1, 2, ...). Instead of having rate constants for conversion of form-I to form-II, form-I to form-III, etc., we have five series of rate constants $k_{ij}$ ($i = 1 \ldots 5$), with the second index referring to the number of nicks on the DNA molecule. The processes and the rate constants are shown in Fig. 1.

Single-strand cleavage of form I converts it to form II;1 with rate constant $k_{10}$. Single-strand cleavage of II;1 converts it to form II;2 with rate constant $k_{11}$ or, if the second cut is close to and opposite the first cut, to form III;0 with rate constant $k_{21}$. It seems that for this conversion to occur, the distance of the second cut from the first must be within about three base pairs in high ionic strength and 16 in low ionic strength at room temperature [2]. Thomas [7], studying the enzymatic degradation of linear DNA by viscosity and light-scattering measurements, analyzed this phenomenon to show that the DNA was double-stranded. Assuming that double-chain scission occurs whenever the two cuts are separated by less than $h$ base pairs, he showed the probability of forming two fragments was approximately $p^2(2h+1)$ where $p$ is the probability of a single cut. For his linear DNA at 25° Thomas deduced a value of about 2 for $h$.

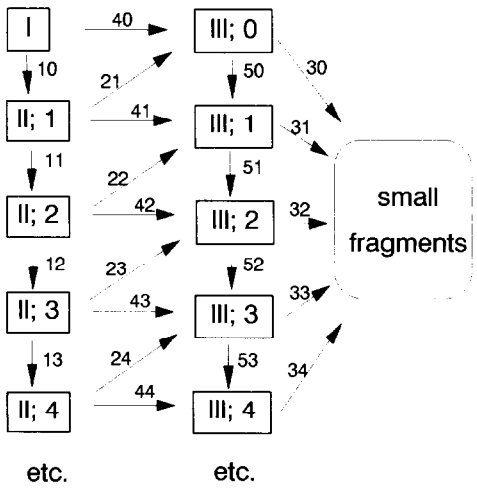


Fig. 1. Showing the different species and rate processes considered. I is closed-circular DNA; II:$i$ is open-circular DNA with $i$ internal nicks; III:$i$ is linear DNA with $i$ internal nicks. An arrow between two species shows a second-order rate process that converts one species to another, with rate constant $k_{ij}$; values of $i$ and $j$ are shown near the arrow.

For our open-circular DNA, II;$i$ is converted to II;$i+1$ or to III;$i-1$ by a single-strand cleavage. In the latter case, the break produced is opposite a pre-existing break, and the molecule of form-III has one fewer internal cut than the molecule of form-II because one of the cuts on II;$i$ is converted into the double-strand break. Double-strand cleavage of form I converts it to form III;0 with rate constant $k_{40}$ and double-strand cleavage of form II;$i$ converts it to form III;$i$ with rate constant $k_{4i}$. Cleavage of form-III;$i$ produces either form III;$i+1$, with rate constant $k_{5i}$, or small DNA fragments with rate constant $k_{3i}$. For $i > 0$, the latter process may involve a double-strand break or a single-strand break opposite a pre-existing single-strand break, so $k_{3i}$ should be related to $k_{2i}$ and $k_{4i}$. The small DNA fragments usually run too fast to be detected in the electrophoresis gel. The processes which convert one subspecies of form II to another (rate constants $k_{1i}$) or one species of form III to another (rate constants $k_{5i}$) were not considered in our previous model because it did not distinguish between subspecies of forms II or III.

Experimentally, one measures only the total amounts of each form, i.e., $[\mathrm{II}] = \Sigma_i[\mathrm{II};i]$ and $[\mathrm{III}] = \Sigma_i[\mathrm{III};i]$, and linear DNAs smaller than III are not detected in the gel. Thus, after, calculation of $[\mathrm{II};i]$ and $[\mathrm{III};i]$ from the solutions to the kinetic equations, we must calculate [II] and [III] for comparison with experiment. In some cases, the DNA before cleavage is not purely form-I. It will be assumed that, if some form-II is present, it is all II;1 and, if some form-III is present, it is all III;0 (the species with the lowest

number of internal cuts). Although this seems reasonable, there is no simple way to verify this.

Because data on the concentrations of the individual subspecies with different numbers of nicks is not available, it is impossible to determine all the rate constants. By making reasonable assumptions about the $k_{ij}$ we express them in terms of a small number of parameters which are later determined. We assume first that $k_{2i}$ $(i \geq 1)$ is equal to i times $k_2$; since $k_{2i}$ gives the rate at which cleavage takes place opposite a pre-existing cut, it should be proportional to the number of such cuts. The value of the parameter $k_2$ can be found by fitting the experimental data. The rate constant for single-strand cleavage of form-I, $k_{10}$, is set equal to the parameter $k_1$ and we assume this is equal to the rate constant for all single-strand cleavage of form II:$i$, i.e., $k_1 = k_{1i} + k_{2i}$. We assume further that all the $k_{4i}$ are equal to $k_4$ and that all the $k_{5i}$ are equal to the parameter $k_5$. Finally, we note that the conversion of form III to smaller fragments requires either a single-strand cut opposite an existing cut or a double-strand cut, so we assume $k_{3i} = k_{2i} + k_{4i}$. Since, in the cases considered, cleavage is terminated before significant amounts of fragments smaller than III are produced, this assumption is relatively unimportant.

With the assumptions thus given, there are only four parameters affecting the rate constants: $k_1$, $k_2$, $k_4$, and $k_5$. The rate constant $k_1$ describes single-strand cleavage, and the rate constant $k_2$ describes single-strand cleavage opposite a pre-existing cut. We generally expect $k_2$ to be much smaller than $k_1$, with the ratio $k_2{:}k_1$ giving information about how close the second nick must be to the first in order to produce a break. The rate constant $k_4$ describes double-strand cleavage, which produces form-III directly from form-I; the relative importance of single- and double-strand cleavage depends on the mechanism of DNA cleavage by the drug or cleavage agent.

## 3. Kinetic analysis

The differential equations are homogeneous, linear in DNA concentrations, and first-order, representing mixed second-order kinetics. For example, denoting concentrations by square brackets:

$$\frac{\mathrm{d}[\mathrm{I}]}{\mathrm{d}t} = -k_{10}[\mathrm{I}][D] - k_{40}[\mathrm{I}][D] \tag{1}$$

and:

$$\frac{\mathrm{d}[\mathrm{II{:}1}]}{\mathrm{d}t} = k_{10}[\mathrm{I}][D] - (k_{11} + k_{21} + k_{41})[\mathrm{II{:}1}][D] \tag{2}$$

where $[D]$ is the concentration of drug or cleavage agent. They can be solved explicitly under the assumption that $[D]/D_0$ is independent of $D_0$, so that $[D]$ obeys:

$$[D] = D_0 f(t) \tag{3}$$

where the function $f(t)$ is independent of the initial concentration of drug $D_0$.

Eq. (3) is likely to hold under a variety of conditions. If the drug or cleavage agent is destroyed or deactivated only by its interaction with DNA, its concentration varies with time according to:

$$\begin{aligned} -\frac{\mathrm{d}[D]}{\mathrm{d}t} &= (k_{10} + k_{40})[\mathrm{I}][D] \\ &+ (k_{11} + k_{21} + k_{41})[\mathrm{II{:}1}][D] \\ &+ (k_{12} + k_{22} + k_{42})[\mathrm{II{:}2}][D] \\ &+ \ldots + (k_{30} + k_{50})[\mathrm{III{:}1}][D]) \\ &+ (k_{31} + k_{51})[\mathrm{III{:}1}][D]) + \ldots \equiv \alpha[D] \end{aligned} \tag{4}$$

If the rate constants are about the same size, and if the loss of DNA to fragments shorter than form-III is small, $\alpha$ will be approximately constant. In addition, $\alpha$ will be approximately constant for early times, when the concentrations of the three forms of DNA have not changed much; then, $[D] = [D]_0(1 - ct)$ with $c$ a constant. If $\alpha$ is constant, Eq. (3) will be obeyed with $f(t) = \exp(-\alpha t)$. Clearly, $\alpha$ will be constant and equal to zero if $[D]$ does not change during the digest, which occurs when $[D]_0 = 0$ and also when $[D]_0$ is much larger than the concentration of DNA, so that only a small fraction of the cleavage agent is lost during digest. It may also be that the cleavage agent is not destroyed during cleavage, or that it is rapidly reactivated by radiation or reagents present in the reaction mixture, so that a single molecule of the agent can reload and be involved in

several cleavage events. Then $[D]$ is essentially constant and Eq. (3) would again obtain with $f(t) = 1$.

Let $A_i$ represent the concentration of the $i$th species. With the assumption of Eq. (3), the rate equations are of the form:

$$\frac{dA_i}{dt} = \sum_j c_{ij} A_j [D] = \sum_j c_{ij} A_j D_0 f(t) \tag{5}$$

where the $c_{ij}$ are combinations of rate constants. The $c_{ij}$ form a non-symmetric real matrix, whose eigenvalues may be found numerically. Let the $k$th eigenvalue be denoted by $\beta_k$ and the components of the corresponding eigenvector by $\alpha_{jk}$, so that:

$$\sum_j c_{ij} \alpha_{jk} = \beta_k \alpha_{ik} \tag{6}$$

The eigenvectors are assumed to be real and normalized according to:

$$\sum_j \alpha_{jk}^2 = 1 \tag{7}$$

Also, define $F(t)$ and $G(t)$ according to:

$$G(t) \equiv e^{F(t)} = \exp\left\{ \int_\phi^t f(t')\,dt' \right\}$$

so that $G(0) = 1$ and $dG/dt = G(t)\, f(t)$. Then it is easy to show by direct substitution that:

$$A_j(t) = \sum_k \gamma_k \alpha_{jk} G(t)^{\beta_k D_0} \tag{8}$$

is a solution to Eq. (5), where $\gamma_k$ are a set of constants, chosen so that $A_j(0)$ is equal to the initial concentration of species j. It is assumed that all the eigenvalues $\beta_k$ are distinct, and this can always be made the case by small changes in the values of the rate constants.

There is a simplification in that the matrix $c_{ij}$ contains many zero elements. Because of the unidirectional nature of the kinetic equations, whenever $c_{mn}$ is not zero for $m \neq n$, $c_{nm}$ vanishes. In fact, $c_{ij}$ is a tridiagonal matrix if the species are listed in the following order: I, II;1, II;2,... III;1, III;2,... The eigenvalues of a tridiagonal matrix are equal to the diagonal elements; it is not necessary to find them numerically. Then the eigenvector corresponding to a nondegenerate eigenvalue may be found easily.

The kinetic equations can be simplified if $[D]_0 t$ is small enough so that concentrations of cleavage agent and DNA forms are not much changed from their initial values. Then one can consider only initial rates in Eq. (5) and one has:

$$\frac{dA_i}{dt} = \sum_j c_{ij} A_j(0) D_0$$

which has as solution:

$$A_i(t) = A_i(0) + \sum_j c_{ij} A_j(0) D_0 t \tag{9}$$

All concentrations vary linearly with $D_0$ or with $t$. In particular, from Eq. (1) one gets:

$$[\mathrm{I}] = [\mathrm{I}]_0 - [\mathrm{I}]_0 (k_{10} + k_{40}) D_0 t \tag{10}$$

Similarly, one gets:

$$[\mathrm{II;1}] = [\mathrm{II;1}]_0 + [\mathrm{I}]_0 k_{10} D_0 t - [\mathrm{II;1}]_0 (k_{11} + k_{21} + k_{41}) D_0 t$$

and then, assuming form-II is initially all II;1 and noting that $k_{11}$ converts [II;1] to [II;2],

$$[\mathrm{II}] = [\mathrm{II}]_0 + [\mathrm{I}]_0 k_{10} D_0 t - [\mathrm{II}]_0 (k_{21} + k_{41}) D_0 t \tag{11}$$

Similarly, assuming form III is initially all III;1, one gets:

$$[\mathrm{III}] = [\mathrm{III}]_0 + [\mathrm{I}]_0 k_{40} D_0 t + [\mathrm{II}]_0 (k_{21} + k_{41}) D_0 t - [\mathrm{III}]_0 k_{30} D_0 t \tag{12}$$

Eqs. (10)–(12) show that, if plots of the concentrations of all DNA species vs. $t$ ($[D]_0$ fixed), or of DNA species concentrations vs. $[D]_0$ ($t$ fixed) are linear, one can obtain information about rate constants from the slopes of these plots. Since only three species of DNA are observed, however, only three slopes can be determined, so that one cannot obtain values for all the constants $k_{10}$, $k_{21}$, $k_{40}$, $k_{41}$ and $k_{31}$. Furthermore, when $k_{31}[\mathrm{III}]_0$ is small enough to neglect, the sum of the three slopes equals zero, and there are only two independent rate parameters. Thus, if the data are such that [I], [II] and [III] as functions of $D_0$ or $t$ are fitted as well by straight lines as by curves, only two or three rate constant parameters can be determined.

There are two kinds of experiments to be analyzed, either a series of digests with the same digest time but different initial concentrations of cleavage agent $D_0$, or a series of digests with the same initial cleavage-agent concentration but different digest times $t$. In the former case, spot intensities for DNA forms I, II and III are measured as a function of $D_0$; in the latter case, spot intensities are measured as a function of $t$. The measured spot intensity for any form of DNA is proportional to the concentration of that form, but the constant of proportionality depends on the efficiency with which ethidium bromide, the stain usually used to visualize DNA in a gel, stains the form, so it may be different for the different forms.

It has been shown [8] that, in solution, form-I (supercoiled) stains better than forms II and III for certain ethidium bromide concentrations, and less well for others. The ratio of staining efficiencies depends on the species of DNA involved. For DNA in an agarose gel, we have shown recently [9] that the ratio of staining efficiencies is very close to unity for two DNAs, and about 1.2 for a third. It does not seem possible to predict the ratio for an arbitrary DNA, and numerical experiments show that the value used for the ratio has only a small effect on calculated rate constants. Therefore, we assume equal staining efficiencies for all species in all cases. If the ratio of the intensity of the spot for species $j$ to the concentration of species $j$ is the same for all $j$, we can take $A_j$ in Eq. (5) to represent the spot intensity. The parameters $\gamma_j$ can then be calculated in terms of the initial spot intensities (for $D_0 = 0$ or for $t = 0$).

For several reasons, it is preferable not to evaluate the $\gamma_j$ from the initial spot intensities. First, this would give excessive weight to the measurements for one value of $D_0$ or $t$. Second, the data set may not include intensities for $D_0 = 0$ or $t = 0$. Finally, it is necessary to assume values for individual $A_j$, since only the sum of intensities for all species of form-II, and the sum for all species of form-III, are measured. As stated above, we assume all initial concentrations are zero except for forms I, II;1 and III;1. We take the initial intensities for these forms as parameters to be determined together with the rate-constant parameters (sometimes, the initial concentration of form-III is obviously zero).

Consider an experiment in which the digest time is constant and $D_0$ is varied. Intensities of forms I, II and III are measured for several values of $D_0$, perhaps including 0. Because the function $f(t)$ is not known, the actual rate constants cannot be determined, but *ratios* of rate constants can be found. Define:

$$k_i^* = k_i \int_0^t f(t')\,dt', \qquad k_{ij}^* = k_{ij} \int_0^t f(t')\,dt',$$

$$c_{ij}^* = c_{ij} \int_0^t f(t')\,dt', \qquad \beta_k^* = \beta_k \int_0^t f(t')\,dt'$$

The parameters $k_i^*$ and the initial intensities of forms I, II;1 and III;1 can be found by seeking the values which minimize the sum of the squared deviations of calculated intensities of forms I, II and III from the measured intensities. The procedure is as follows: given a set of values for four rate parameters ($k_1^*$, $k_2^*$, $k_4^*$, $k_5^*$) one can evaluate $k_{ij}^*$, the matrix $(c_{ij}^*)$, the eigenvalues $\beta_j^*$, and the eigenvectors $\alpha_{jk}$. Then one can find $\gamma_j$ from:

$$\gamma_j = \sum_j (\alpha^{-1})_{jk} A_k(0) \tag{13}$$

where values are assumed for I(0), II;1(0), and III;1(0) and $A_k(0)$ is taken equal to 0 for other species.

One can then calculate all $A_j$ for each value of $D_0$ according to:

$$A_j = \sum_k \gamma_k \alpha_{jk} \exp\{D_o \beta_k^*\} \tag{14}$$

and combine them to get theoretical values of the intensities of forms I, II and III for each $D_0$. These are subtracted from the measured intensities, and the differences squared and added together. The Simplex or other search method is employed to find the values of the seven parameters which minimize the sum of the square deviations. The rate-constant parameters determined are $k_i^*$, so $k_{ij}$ are not determined, but only $k_{ij}^*$. However, ratios of rate constants can be found without ambiguity, since $k_{ij}$ differs from $k_{ij}^*$ by a factor of $F$, and this factor is the same for all $i$ and $j$. If one is willing to assume $f(t) = 1$ or $F(t) = t$ ($t$ = digest time), one can derive values for the rate constants themselves.

For experiments with constant $D_0$ but variable digest time, one cannot derive values for rate constants or their ratios without some assumption about

$F$. The assumption would normally be that $f(t)$ equals $\tau$: conditions for which this is reasonable have been discussed above. The solutions to the rate equations, Eq. (8), become:

$$A_j(t) = \sum_k \gamma_k \alpha_{jk} \exp\{D_0 \beta_k t\} \qquad (15)$$

where $\beta_k$ and $\alpha_{jk}$ are obtained as the eigenvalues and eigenvectors of the matrix $c_{ij}$, and $\gamma_k$ are obtained from Eq. (9). The procedure is to assume values for the parameters [$k_1$, $k_2$, $k_4$, $k_5$, I(0), II;1(0), and III;1(0)], calculate $c_{ij}$, find $\beta_k$, $\alpha_{jk}$ and $\gamma_k$, and construct $A_j(t)$ for all $j$ and $t$. The deviations between measured spot intensities and $A_j(t)$ are squared, and one uses the Simplex or other search method to find the values of the parameters that make the sum of the squared deviations as small as possible.

In some cases, the cleavage agent must be activated by some means to produce breaks in DNA. Thus, cleavage is performed by an activated species, which we refer to here as $D^*$. The concentration of $D^*$ is not known, only the concentration of $D$, from which $D^*$ is produced. However, if one can assume $-\mathrm{d}[D^*]/\mathrm{d}t$ is small compared with $a$, the rate of activation (production of $D^*$), and $b$, the rate of deactivation (destruction of $D^*$), one can invoke the steady-state approximation, $a \simeq b$. Since $a$ is proportional to $[D]$ and $b$ is proportional to $[D^*]$, $a \simeq b$ implies that $[D^*]$ is proportional to $[D]$, say $[D^*] = \kappa[D]$, while $[D]$ is changing during the digest. The kinetic analysis is still valid, provided that we substitute $\kappa[D]$ for $[D]$ everywhere. Values of rate constants determined will be true rate constants multiplied by $\kappa$. Ratios of rate constants will be correctly determined.

Sometimes, such as when the cleavage agent may have degraded in storage, one does not know the actual concentrations, but, since all reaction mixtures are made up from the same stock solution, only relative values of $D_0$. The situation is equivalent to that of the preceding paragraph: $D_0$ is proportional to the nominal concentration. If the constant of proportionality is not known, one cannot get actual values of rate constants, but ratios of rate constants are obtainable from a series of experiments in which $t$ is fixed and $D_0$ changes.

## 4. Applications

We illustrate this analysis first using previously published data sets for calicheamicin and DNase I. Then we present new experimental results on Fe–EDTA and bleomycin and analyze them using the present model. Experimental details on the last two are given afterwards.

### 4.1. DNase I and calicheamicin

As a first illustration, we analyze previously published data for DNase I and the enediyne antibiotics calicheamicin and esperamicin acting on PM2 DNA at $9.45 \times 10^{-5}$ M [10]. Note that PM2 contains $10{,}900 \pm 60$ base pairs [11]. For the enzyme and for the drugs, we measured concentrations of the three forms of DNA as a function of enzyme or drug concentration, with fixed digest time of 10 min. Fourteen concentrations of DNase I were used, from 0 to 1.1 units/$\mu$l, and nine concentrations of calicheamicin, from 0 to 1.85 $\mu$M. Since we were particularly interested in determining whether these agents were single-strand or double-strand cutters, we analyzed the data for each according to simplified models, in which either the single-strand cutting rate constant or the double-strand cutting rate constant was set equal to zero. We compared the values of $S$, the sum of the squared deviations between experimental and calculated concentrations, produced by the two models. In the case of DNase I, $S$ was more than three times bigger for the double-strand cutting model than for the single-strand cutting model, and we concluded that DNase I is a single-strand cleavage agent. For calicheamicin, $S$ was about the same for both models, but the value of the rate constant for conversion of form-II to form-III in the single-strand cutting model was extremely (and impossibly) large, seven times the value of the rate constant for conversion of I to II. This means that, when form-II DNA is produced from form-I, it immediately undergoes a second cleavage to produce form-III, which is effectively double-strand cutting. The kinetic analysis can not distinguish between this mechanism (successive cleavages by two drugs) and true double-strand cutting (one drug cutting both

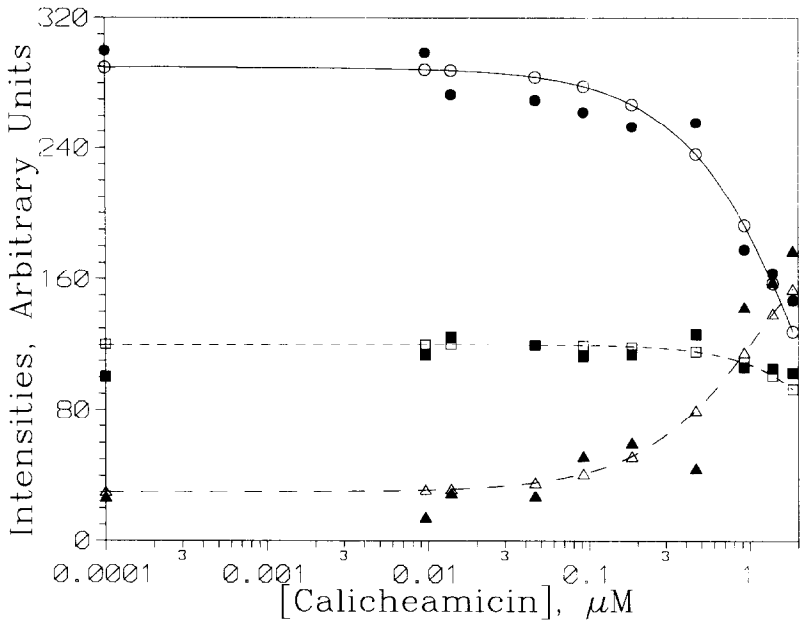


Fig. 2. Cleavage by calicheamicin. Symbols show measured intensities of three forms of PM2 DNA, in arbitrary units, as a function of initial concentration of calicheamicin (logarithmic scale): filled circles = form I, filled squares = form II, filled triangles = form III. Open symbols and smooth curves are intensities of three forms of DNA calculated using the kinetic model, parametrized to get best fit to measured intensities.

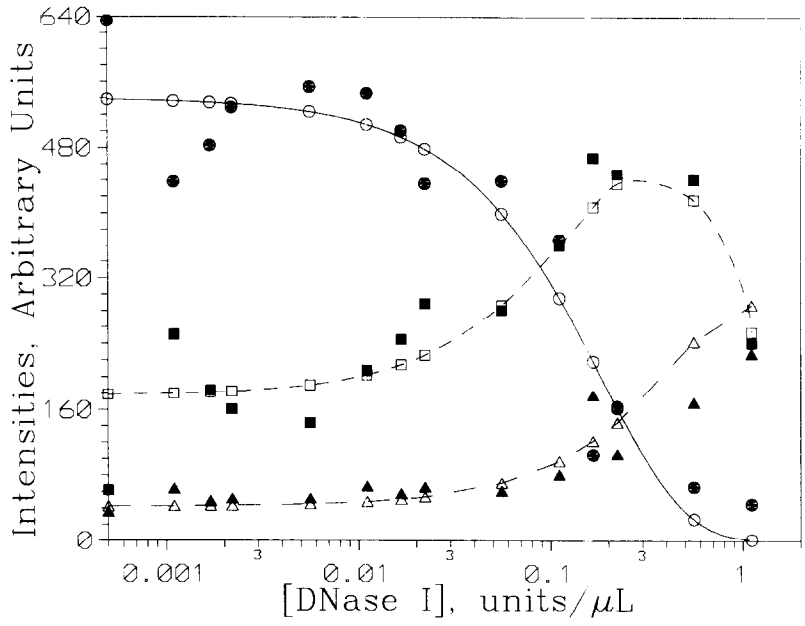


Fig. 3. Cleavage by DNase I. Symbols show measured intensities of three forms of PM2 DNA, in arbitrary units, as a function of initial concentration of DNase I (logarithmic scale): filled circles = form I, filled squares = form II, filled triangles = form III. Open symbols and smooth curves are intensities of three forms of DNA calculated using the kinetic model, parameterized to get best fit to measured intensities.

Table 1
Derived rate constants for cleavage by calicheamicin and DNase I[a]

| Drug | $S$[b] | $[I]_0$ | $[II]_0$ | $[III]_0$ | $k_{10}G(t)$[c] | $k_{21}G(t)$ | $k_{40}G(t)$ |
|---|---|---|---|---|---|---|---|
| Calicheamicin | 6322 | 289.9 | 120.7 | 31.0 | $0.13 \pm 0.04$ | $0.03 \pm 0.03$ | $0.31 \pm 0.03$ |
| DNase I | 95006 | 539.9 | 177.6 | 42.3 | $4.64 \pm 0.79$ | $0.006 \pm 0.006$ | $0.77 \pm 0.19$ |

[a]Data obtained from [10].
[b]$S$: sum of squares of deviations of calculated from experimental intensities.
[c]These quantities are in units of $\mu M^{-1}$ $min^{-1}$.

strands). We concluded that calicheamicin is a double-strand cleavage agent.

Applying the new model (Eqs. (5)–(8)) to these systems, we obtain the results shown in Figs. 2 and 3 for calicheamicin and DNase I respectively. The measured concentrations of the three forms of DNA are shown together with the values calculated from the model. Table 1 gives the resulting values of the parameters, as well as the best values of $S$, for both systems. The uncertainty given for each kinetic constant is the deviation from the best value required to increase $S$ by 5%. Values for the second-order rate constants would be obtained if one could assume that $[D]$ remains essentially constant during the digest, so that $f(t)$ is equal to the digest time (10 min), and that absolute drug concentrations were known. In fact, only relative concentrations were known, so absolute values of rate constants cannot be determined.

According to Table 1 $k_{40}$ is several times greater than $k_{10}$ for calicheamicin. This shows that calicheamicin is overwhelmingly a double-strand cleavage agent. The ratio of $k_{10}$ to $k_{21}$ seems very small, but the large value of $k_{21}$ probably reflects the inability of the model to distinguish between double-strand cutting and single-strand cutting followed immediately by a second cut. Previously, using an oversimplified double-strand cutting model, we found [10] that addition of the parameter $k_{10}$ did not improve the fit of theory to experiment, confirming our conclusion that calicheamicin is a double-strand cleavage agent. For DNase I, Table 1 shows that $k_{10}$ is about six times as big as $k_{40}$, so that DNase I is mostly, but not completely, a single-strand cutter. This agrees with what we concluded in our previous work. Experiments with simplified models showed that the value of $S$ could be decreased by including double-strand as well as single-strand cutting, suggesting a small amount of double-strand cutting was present. This is confirmed by the present results.

The intensities for calicheamicin are shown in Fig. 2 with a logarithmic scale for concentration. On a linear scale, they can be seen to be almost linear as a function of drug concentration. Therefore, we have fitted the intensities to lines and obtained the slopes, to use with Eqs. (10)–(12). As shown in Table 2, the three slopes add to zero, implying that there is no loss of form-III to small fragments ($k_{31} = 0$). From the data for form-I, $(k_{10} + k_{40})t = 0.29 \pm 0.03$. From the form-II data, $k_{10}t(280.2) - (k_{21} + k_{41})t(116.0) = -6.7$ so that, assuming that $k_{21}$ is much less than $k_{41}$, $k_{10}{:}k_{41} = 0.41 - 0.024/k_{41}t$. Assuming $k_{41} \approx k_{40}$ gives an equation relating $k_{10}t$ and $k_{40}\tau$; combining it with the equation from the data for form-I allows one to find $k_{40}t = 0.22 \pm 0.08$ and $k_{10}t = 0.07 \pm 0.07$. From the data for form-III, one gets no new information.

Applying the initial-slope analysis to DNase I, using the first 10 points (concentrations $\leq 0.11$ units/$\mu$l), we find results in qualitative agreement with those of Table 1. The sum, $(k_{10} + k_{40})t$, is calculated as $2.88 \pm 1.04$ from Eq. (10). Since DNase I seems to be a single-strand cutter, we assume

Table 2
Linear fits to calicheamicin (8-point) and DNAse I (10-point) intensities

| Species | Correlation coefficient | Intercept | Slope (95% confidence limits) |
|---|---|---|---|
| I (calich.) | 0.958 | $280.2 \pm 6.8$ | $-80.9 \pm 8.5$ |
| II (calich.) | 0.494 | $116.0 \pm 3.4$ | $-6.7 \pm 4.2$ |
| III (calich.) | 0.911 | $30.9 \pm 7.3$ | $87.6 \pm 9.1$ |
| I (DNase I) | 0.698 | $527.5 \pm 22.3$ | $-1520.3 \pm 550.7$ |
| II (DNase I) | 0.757 | $176.9 \pm 22.6$ | $1828.6 \pm 558.8$ |
| III (DNase I) | 0.719 | $52.9 \pm 3.5$ | $250.2 \pm 85.4$ |

$k_{10} \gg k_{40}$ so we have $k_{10}t = 2.88$; using this in Eq. (11), we find $(k_{21} + k_{41})t = 1.75 \pm 3.16$. The uncertainty is too large to conclude anything about $k_{21}$ or $k_{41}$. The sum of the slopes is $558 \pm 789$; the large uncertainty here makes it impossible to estimate $k_{31}$.

### 4.2. Fe–EDTA

The complex $[Fe(EDTA)]^{2-}$ was utilized by Tullius and Dombroski [12] as a footprinting reagent to detect protein–DNA contacts. It proved to be an ideal agent for this purpose, since it showed very little sequence specificity. The hydroxyl radical, produced via Fenton chemistry, is generated by Fe(II)-promoted reduction of dioxygen; it targets the deoxyribose sugars of DNA, which leads eventually to breakage of the sugar–phosphate backbone. Fe–EDTA is necessarily a single-strand cleavage agent ($k_4 = 0$). The lack of sequence preference is explained by the fact that both the Fe–EDTA and the DNA backbone possess negative charges, which severely hinders the ability of the cleavage agent to bind to DNA. Because there is little or no base specificity, the rate constant $k_2$ should be several orders of magnitude smaller than $k_1$. With $k_4$ and $k_2$ both small, there should be very little production of form-III DNA.

In two series of experiments, SV-40 DNA was cleaved by Fe–EDTA and the amounts of forms I and II were followed as a function of time. The total concentration of plasmid was 3.82 nM in molecules and that of Fe–EDTA ($= [Fe^{++}] = [EDTA]$) was either 100 $\mu$M or 300 $\mu$M. Initially, the plasmid was mostly form-I, with some form-II present. The amount of form-I decreased with time while that of form-II increased, but no form-III was observed, confirming that $k_2$ is much smaller than $k_1$. Since the sum of [I] and [II] must be constant, we divide each intensity by the intensity sum of the two forms to obtain fractional populations. Fig. 4 shows the fraction of DNA which is form-I, for 100 $\mu$M Fe–EDTA (squares) and for 300 $\mu$M (circles).

Because of the large excess of cleavage agent over DNA, $[D]$ is not much affected by the DNA cleavage process; if there were no other processes degrading Fe–EDTA, we would have $[D] = [D]_0$, and $F(\tau)$ would be equal to $\tau$, the digest time. Then the amount of form I should decrease exponentially

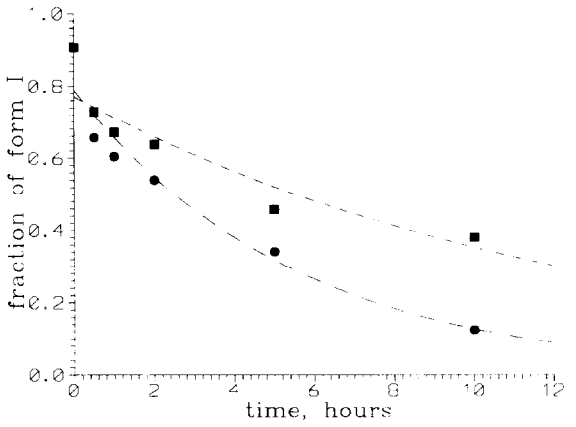


Fig. 4. Fractional population of form I DNA after cleavage by Fe–EDTA, as a function of digest time. Squares are for 100 $\mu$M Fe–EDTA, circles for 300 $\mu$M Fe–EDTA. Smooth curves are best-fit exponentials.

with time. The curves in Fig. 4 are the best-fit exponentials, and yield $k_{10}[D]_0 = 0.182 \pm 0.012$ h$^{-1}$ for 300 $\mu$M and $0.078 \pm 0.014$ h$^{-1}$ for 100 $\mu$M. The ratio, which should be 3, is $2.33 \pm 0.45$. It appears from the figure that the fit is poor, primarily due to what looks like a large experimental error in the measured fraction of form I at $t = 0$, $f_1(0)$. The exponential fits predict $f_1(0) = 0.789$ (from 300 $\mu$M) or 0.771 (from 100 $\mu$M). If the $t = 0$ point is eliminated, the fit for each Fe–EDTA concentration to an exponential is considerably improved. The 100 $\mu$M and 300 $\mu$M data give $f_1(0) = 0.720$ and 0.744 respectively, with $k_{10}[D]_0 = 0.174 \pm 0.008$ h$^{-1}$ and $0.069 \pm 0.010$ h$^{-1}$. The ratio is $2.52 \pm 0.37$. The values of $k_{10}$ from the two runs are $580 \pm 27$ M$^{-1}$ h$^{-1}$ and $690 \pm 100$ M$^{-1}$ h$^{-1}$. It is possible, of course, that the problem is not with the point at $t = 0$, but with the assumption that $[D]$ is constant so that $f(t) = t$. Because of processes other than DNA cleavage, $[D]$ may vary with time. There is no way to show this from the time-data alone.

Experiments were also performed on SV-40 DNA at 14 nM with variable Fe–EDTA concentration and fixed digest time $\tau$ of 30 min. The values of [Fe–EDTA] used were, in $\mu$M: 300, 150, 100, 75, 60, 50, 42.9, 37.5, 33.3, 30, and 0. The populations are shown in Fig. 5. Because no form-III was detected, the data was renormalized so that the sum of the intensities of forms I and II was constant with con-

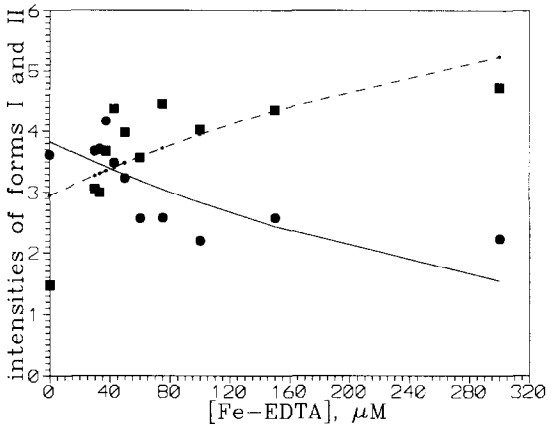


Fig. 5. Cleavage of 14 nM SV-40 DNA by Fe–EDTA as a function of Fe–EDTA concentration, with digest time fixed at 30 min. Intensities corresponding to populations of form I (circles) and form II (squares) are shown, with calculated (best-fit) values (curves).

centration and, in the analysis of the data according to our model, it was assumed that $k_2$ and $k_4$ vanished. The only rate parameter which could be determined was $k_{10}f(t)$; the value found was $3.03 \times 10^{-3}$ $\mu M^{-1}$. If $f(t) = \tau = 30$ min, $k_{10} = 101$ $M^{-1}$ $min^{-1}$, 10 times larger than what was determined from the variable-time data. The fractions of forms I and II are close to linear as a function of concentration (see Fig. 5), so that one should really use the linearized form (Eqs. (10)–(12)) of our solutions. Fitting fractions of forms I and II to linear functions of the concentration of cleavage agent, we get a slope and intercept of $-0.00155 \pm 0.00035$ and $0.550 \pm 0.039$ for form I, and $0.00150 \pm 0.00035$ and $0.453 \pm 0.039$ for form II. Applying Eqs. (16) and (17), we find $k_{10}\tau = (2.82 \pm 0.66) \times 10^3$ $M^{-1}$ or $k_{10} = (94 \pm 22) \times 10^2$ $M^{-1}$ $min^{-1}$, in agreement with the result from the full model.

The discrepancy between the values of $k_{10}$ from the variable-time results and the variable-concentration results implies that $f(t)$ is very different from $t$. Since

$$G(t) \equiv \exp\left\{\int_0^t f(t')\mathrm{d}t'\right\}$$

this means that $f(t) = D(t)/D_0$ deviates from unity. Thus one cannot assume $[\text{Fe–EDTA}] \simeq [D]_0$ because [Fe–EDTA] decreases due to processes other than DNA cleavage. The plots of Fig. 4 should not be exponentials. Because of the degradation, $f(t)$ and $F(t)$ are unknown, and the analysis of the variable-concentration data gives simply $k_{10}F(\tau) = (2.82 \pm 0.66) \times 10^3$ $M^{-1}$. If Fe–EDTA reacts primarily with something other than closed-circular DNA, and the reaction is first-order in Fe–EDTA, $[D] = [D]_0 e^{-\alpha t}$ for all $[D]_0$, where $\alpha$ is an (unknown) rate constant for the DNA-independent degradation of the cleavage agent. Then $F(t) = (1 - e^{-\alpha t})/\alpha$ and:

$$[\mathrm{I}] = [\mathrm{I}]_0 \exp\{-k_{10}[D]_0(1 - e^{-\alpha t})/\alpha\} \qquad (16)$$

The amount of data for each $D_0$ (Fig. 4) is too small to get reliable values for the three parameters $[\mathrm{I}]_0$ $k_1$ and $\alpha$. Assuming the same values of $\mathrm{I}_0$ and $\alpha$ for both runs, so only four parameters are to be determined, we find $[\mathrm{I}]_0 = 0.85 \pm 0.03$, $\alpha = 0.19 \pm 0.08$ $h^{-1}$, and $k_{10}D_0 = 0.34 \pm 0.07$ and $0.19 \pm 0.05$ $h^{-1}$ for the two values of $D_0$. The disagreements between $[\mathrm{I}]_0$ and the measured intensity at $t = 0$, and between the two values of $k_{10}$, suggests that the degradation is not first-order in [Fe–EDTA]. It is possible that some of the complex is converted to M-oxo dimers, which are incapable of cleaving DNA.

Experiments with Fe–EDTA were also carried out on pBR-322 DNA at a concentration of 11.5 nM. Intensity corresponding to form III was not observed,

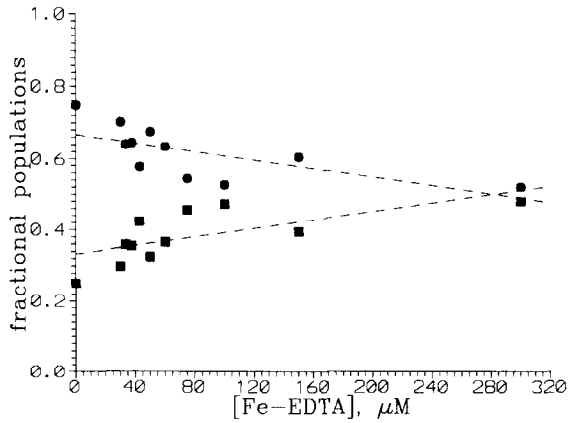


Fig. 6. Cleavage of 11.5 nM pBR-322 DNA by Fe–EDTA as a function of Fe–EDTA concentration, with digest time fixed at 30 min. Fractional populations of form I (circles) and form II (squares) are shown, with best-fit straight lines.

confirming that Fe–EDTA is a single-strand cutter, and that $k_2$ is very small compared to $k_1$, as expected. The fractional populations are shown in Fig. 6. A linear fit of the fraction of form-I vs. concentration (shown in Fig. 6) leads to an intercept of $0.667 \pm 0.024$ and a slope of $(0.00059 \pm 0.00022)$. Since the slope should be equal to $-f_{10}$ (the intercept) times the digest time (30 min) times the rate constant $k_{10}$, this gives $k_{10} = (29.5 \pm 11.0)\ \mathrm{M}^{-1}\ \mathrm{min}^{-1}$. Because we are using fractional populations and no form III is present, the same results are obtained from linear fits of fraction of form II as a function of concentration.

Since no form-III DNA is observed in these experiments, one cannot obtain a value for $k_{21}$. An upper limit on $k_{21}$ can be obtained by noting that, in the linear approximation (Eq. (10)), [III] should be equal to $k_2[\mathrm{II}]_0[D]_0\tau$. Since $[D]_0 = 300\ \mu\mathrm{M}$, $[\mathrm{II}]_0 = 11.5 \times 0.33 = 3.8$ nM (from the linear fit) and $\tau = 30$ min, [III] should equal $(3.4 \times 10^{-11}\ \mathrm{M}^2\ \mathrm{min})k_{21}$. The smallest reliably measurable spot intensity corresponds to a DNA concentration of about 0.1 nM, so:

$$k_{21} < \frac{0.1 \times 10^{-9}\ \mathrm{M}}{3.4 \times 10^{-11}\ \mathrm{M}^2\ \mathrm{min}} = 2.9\ \mathrm{M}^{-1}\ \mathrm{min}^{-1}$$

Thus $k_{10}$ is at least an order of magnitude larger than $k_{21}$. Applying the same reasoning to the time-dependent (SV-40) experiments, we have:

$$k_{21} < \frac{0.1 \times 10^{-9}\mathrm{M}}{(3.82\,\mathrm{nM} \times 0.35)(300\ \mu\mathrm{M})(10h)} = 0.42\mathrm{M}^{-1}\mathrm{min}^{-1}$$

where the largest values of $t$ and $[D]_0$ have been used. Since $k_{10}$ is about 11 $\mathrm{M}^{-1}\ \mathrm{min}^{-1}$ in this case, we conclude that $k_{10}$ is at least 1.5 orders of magnitude larger than $k_{21}$.

### 4.3. Bleomycin

Bleomycin is a naturally occurring glycopeptide antibiotic, produced by *Streptomyces verticillus* [13]. It is used clinically against certain malignant lymphomas and squamous cell carcinomas [14]. It has three structural domains: a bithiazole region involved in binding to DNA [15], a pyrimidine–imidazole portion involved in metal ion binding [16–18], and two sugar residues (gulose and mannose) involved in biological transport in vivo . For bleomycin to cut DNA, it requires the presence of oxygen, a metal ion, usually Fe(II) [16–18], and a reducing agent, usually a thiol such as DTT. Typically, bleomycin cuts at GC and GT sequences [19,20], yielding both single-strand and double-strand breaks [6].

Povirk et al. [6] have studied the cleavage of linear and supercoiled DNA by bleomycin. From the amounts of open circular and linear DNA produced by cleavage of supercoiled Col E1 DNA by various concentrations of bleomycin, they derived the numbers of single- and double-strand breaks per DNA molecule. That the number of double-strand breaks was proportional to the number of single-strand breaks indicated that both strands were being cleaved in a single event (or that there were two correlated single-strand cleavages, as discussed in Section 1). If the double-strand break had actually been produced by a break on one strand followed by an independent single-strand break on the opposite strand, the number of double-strand breaks would have been proportional to the square of the number of single-strand breaks.

The ratio of single- to double-strand breaks was about 6 to 1, comparable to what was found by other workers. The same linear relation between the numbers of single- and double-strand breaks was found for cleavage of linear T2 DNA, except that the ratio was $9 \pm 0.4$ to 1. Povirk et al. [6] also found that exposure of Col E1 DNA to alkaline solution after treatment with bleomycin increased the number of strand breaks (single- and double-) as much as fourfold. Thus, in addition to causing single-strand breaks, bleomycin induces alkali-labile bonds. In later experiments [21], bleomycin-treated Col E1 DNA was allowed to react with various endonucleases and polyamines in order to learn more about the chemical structures of bleomycin-induced sites.

Table 3
Rate constants in $\mu\mathrm{M}^{-1}\ \mathrm{min}^{-1}$ for bleomycin cleavage of PM2 DNA

| $k_{10}$ | $k_{21}$ | $k_{40}$ |
|---|---|---|
| $0.139 \pm 0.069$[a] | $0.00020 \pm 0.00015$ | $0.0101 \pm 0.016$ |
| 0.024 (from this work) | 0.0026 | 0.0032 |

[a] Lloyd et al. [22].

To study the kinetics of strand breakage by bleomycin, Lloyd et al. [22] carried out a series of digests of bleomycin with PM2 DNA Table 3. Populations of the three forms of DNA after digest were reported. The original bleomycin concentration was 0.40 $\mu$M (0.62 $\mu$g/ml) in all cases, and different digest times were used, up to 45 min. There were $2.6 \times 10^{-3}$ bleomycin molecules present per DNA phosphate; since PM2 DNA has about 10,900 base pairs, this implies a DNA concentration of 0.0075 $\mu$M in molecules. From Fig. 2 in Lloyd et al. [22], we have taken values for the fractional populations of the three forms of DNA as a function of digest time for our analysis. Using fractional populations implies that all the DNA is form-I, form-II, or form-III, so that there is no loss to smaller fragments and is essentially zero. Fig. 7 shows the fractional populations as a function of time, with the results of a fit to our model (Eqs. (1)–(8)).

We have performed bleomycin cleavage experiments on several DNAs (SV-40, pBR-322, and PM2), with the concentration of bleomycin in the reaction mixture varied and the digest time being held constant. For pBR-322, the DNA concentration was 8.6 nM in molecules. Two sets of experiments were done on SV-40 DNA, with [SV-40] = 7.21 nM. One set, labeled 'high' in the Tables, used 11 $D_0$-values from 0 to 484 nM; the other, labeled 'low', used 11 $D_0$-values from 0 to 242 nM. The experiments on

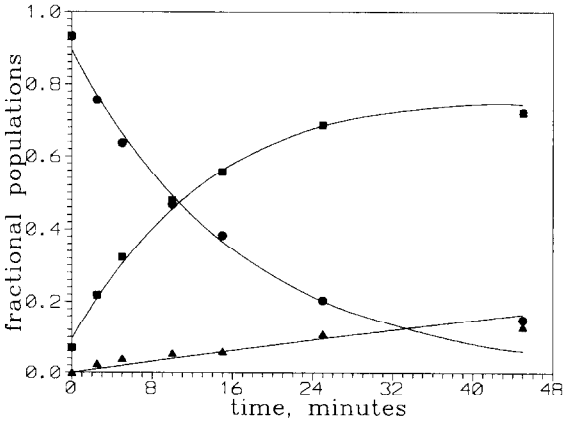


Fig. 7. Fractional populations of three forms of DNA after cleavage by bleomycin (data from Lloyd et al. [22]): solid circles, squares, and triangles represent forms I, II, and III respectively. Smooth curves represent percentage populations calculated from our kinetic model.

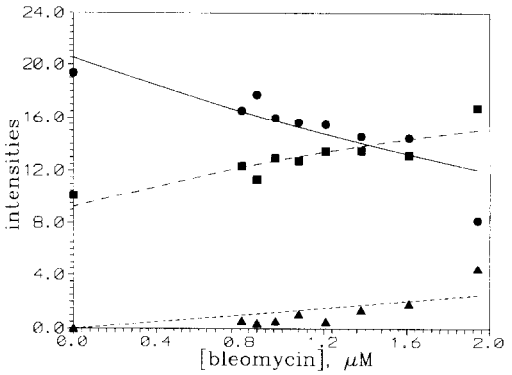


Fig. 8. Measured and calculated intensities (arbitrary units) of forms I, II, and III of PM2 DNA (circles, squares, and triangles respectively) after cleavage by bleomycin at various concentrations. Calculated intensities, using best-fit values of the parameters, are also shown.

PM2 DNA used [PM2] = 4 nM. The bleomycin concentrations $[B]$ are given in Section 5.2 below. The cleavage agent was present in large excess over the DNA in every case except for the controls ($[B] = 0$). It was found that the sum of the amounts of the three forms of DNA was essentially constant as a function of $[B]$. An example of experimental results (for cleavage of 4 nM PM2 DNA) is shown in Fig. 8, together with populations from the analysis according to Eqs. (1)–(8).

Since bleomycin is present in large excess over DNA in all experiments, we analyzed them assuming $F(\tau)$ is equal to $\tau$ in Eq. (6) et seq, i.e., $[D] = [D]_0$. Fig. 7 shows the best fit of Eqs. (1)–(8) to the time-dependent data of Lloyd et al. [22]. The fit is very good, except for the last point, which may reflect the breakdown in the assumption $F(\tau) = \tau$. Note that the determined quantities include $ak_{10}[D]_0$, $ak_{21}[D]_0$, $ak_{30}[D]_0$ ($k_{40}$ assumed equal to $k_{30}$), and $ak_{50}[D]_0$, where $[D]_0 = 0.40$ $\mu$M and $a$ is the fraction of the bleomycin which is activated for cleav-

Table 4
Linear fits to intensities for bleomycin cleavage of PM2 DNA[a]

| DNA species | Intercept | Slope | Corr. coeff. |
|---|---|---|---|
| Form I | $0.865 \pm 0.044$ | $-0.0354 \pm 0.0052$ | 0.9696 |
| Form II | $0.123 \pm 0.036$ | $0.0317 \pm 0.0043$ | 0.9736 |
| Form III | $0.012 \pm 0.007$ | $0.0037 \pm 0.0009$ | 0.8151 |

[a] Lloyd et al. [22]; first 5 points used.

Table 5
Results for bleomycin on various DNAs, fixed digest time

| DNA | $[I]_0$ | $[II]_0$ | $[III]_0$ | $k_{10}\tau$[a] | $k_{21}\tau$ | $k_{40}\tau$ |
|---|---|---|---|---|---|---|
| PM2 | 20.50 | 9.25 | $4.9\times10^{-5}$ | 0.239 | 0.025 | 0.033 |
| SV-40, high | 7.89 | 3.22 | $8.4\times10^{-5}$ | 0.460 | 0.031 | 0.041 |
| SV-40, low | 8.71 | 4.12 | $1.3\times10^{-4}$ | 0.286 | 0.021 | 0.0156 |
| pBR322 | 5.29 | 2.20 | $2.3\times10^{-5}$ | 0.810 | 0.0190 | 0.191 |

[a]Quantities $k_i\tau$ are in units of $\mu M^{-1}$.

age. Assuming $a = 1$ gives the rate constants in Table 4. The ratio of single-strand to double-strand breaks on form-I DNA, $k_{10}:k_{40}$, is $13.7 \pm 2.3$, and $k_{10}$ is about $7 \times 10^2$ times $k_{21}$, but the error on $k_{21}$ is extremely large.

We may also obtain rate constants from initial slopes, which we obtain by least-square fitting the first five points ($t \leq 15$ min) to the form $b + mt$. The results are shown in Table 4, the slopes adding to zero as expected. The correlation coefficients are higher than for calicheamicin and DNase I. From the form-I slope and intercept, $(k_{10} + k_{40})[D]_0 = 0.0409 \pm 0.0060$. Assuming bleomycin is mostly a single-strand cutter ($k_{40} \ll k_{10}$) gives an estimate of $k_{10}$ as $0.0409/0.40\ \mu M = 0.102\ \mu M^{-1}$; allowing for double-strand cutting leads to a smaller value. From the form-II slope, $0.0317 = 0.8649k_{10}[D]_0 - 0.1234(k_{21} + k_{41})[D]_0$, or $0.0916\ \mu M^{-1} = k_{10} - 0.143(k_{21} + k_{41})$. Since $k_{10}$ is about 10% less than 0.102, no useful estimate of $k_{21} + k_{41}$ can be obtained. From the form-III slope, neglecting $[III]_0k_{30}$, Eq. (12) leads to $k_{40}D_0 = 0.0043 - 0.1422(k_{21} + k_{41})D_0$, which means that $k_{40}$ is less than $0.0043/D_0 = 0.011\ \mu M^{-1}\ min^{-1}$.

In analyzing our data for bleomycin cleavage of PM2 DNA, we determine $ak_1\tau$, $ak_2\tau$ and $ak_4\tau$, where the digest time $\tau$ is 10 min. The experimental results, with the populations from the model, are shown in Fig. 8. The derived rate constants are given in Table 5; the errors, not given, are very large, probably because the concentrations used were not large enough to show much nonlinearity in populations as a function of concentration (compare Figs. 7 and 8). The discrepancy between rate constants from the time-dependent and the concentration-dependent experiments may be due to this, in part. However, it also indicates a problem with the assumption, $F(\tau) = \tau$. Note that, within the uncertainties in the rate constants, the ratio $k_{10}:k_{40}$ is the same from both experiments: $13.7 \pm 6.8$ from the time-dependent data and $7.5 \pm 2.1$ from our concentration-dependent data.

The results of linear fits to this data are given in Table 6. From the slope and intercept for form-I, we obtain $4.97 = 20.78(k_{10}\tau + k_{40}\tau)$. Assuming $k_{40}$ is small compared to $k_{10}$ yields $k_{10}\tau = 0.24$ and $k_{10} = 0.024\ \mu M^{-1}\ min^{-1}$. From the slope and intercept for form-II, $2.96 = 20.78k_{10}\tau - 9.69(k_{21}\tau + k_{41}\tau)$. Since the rate constant for double-strand cleavage is probably much larger than $k_{21}$, no estimate for $k_{21}$ can be obtained. From the form-III slope, assuming $[III]_0k_{30}\tau$ is negligible, $2.01 = 20.78k_{40}\tau + 9.69(k_{21} + k_{41})\tau$. If $k_{41}$ is equal to $k_{40}$ and much greater than $k_{21}$, the value of $k_{40}\tau$ is estimated as $2.01/30.47$, so $k_{40} \simeq 0.0066\ \mu M^{-1}\ min^{-1}$. The values of rate constants found in this way are very uncertain.

Table 7 shows the results of fitting the full model to our four sets of experimental data. Experimental results for cleavage of 7.21 nM SV-40 DNA are shown in Fig. 9, with intensities calculated from our model; corresponding results for PM2 DNA were given in Fig. 8. From Table 7, we conclude that bleomycin is predominantly a single-strand cleavage agent, but with some double-strand cutting. In all cases, intensities vary almost linearly with drug concentration, making the uncertainties in the parame-

Table 6
Linear-fit results for bleomycin on various DNAs, fixed digest time

| DNA | $[I]_0$ | $[II]_0$ | $[III]_0$ | $-d[I]/d[D]_0$ | $d[II]/d[D]_0$ | $d[III]/d[D]_0$ |
|---|---|---|---|---|---|---|
| PM2 | $20.78 \pm 1.19$ | $9.69 \pm 0.66$ | $-0.99 \pm 0.66$ | $4.97 \pm 0.98$ | $2.96 \pm 0.54$ | $2.01 \pm 0.54$ |
| SV-40, high | $7.94 \pm 0.51$ | $3.37 \pm 0.38$ | $-0.19 \pm 0.14$ | $3.49 \pm 0.90$ | $2.42 \pm 0.66$ | $0.87 \pm 0.25$ |
| SV-40, low | $8.86 \pm 0.63$ | $4.18 \pm 0.23$ | $-0.03 \pm 0.02$ | $2.74 \pm 2.20$ | $2.06 \pm 0.81$ | $0.44 \pm 0.06$ |
| pBR322 | $5.14 \pm 0.33$ | $2.25 \pm 0.30$ | $-0.03 \pm 0.08$ | $3.88 \pm 1.16$ | $2.80 \pm 1.04$ | $1.53 \pm 0.28$ |

[a]Quantities $k_i\tau$ are in units of $\mu M^{-1}$.

Table 7
Bleomycin rate constants from linearized model

| DNA | $r = k_{10}:k_{40}^{a}$ | $(k_{10}+k_{40})\tau^{b}$ | $k_{10}\tau$ | $(k_{21}+k_{41})\tau^{c}$ |
|---|---|---|---|---|
| PM2 | 7.2 | 0.24 | 0.21 | 0.14 |
| SV-40, high | 11.2 | 0.44 | 0.40 | 0.22 |
| SV-40, low | 18.3 | 0.31 | 0.29 | 0.12 |
| pBR322 | 4.2 | 0.75 | 0.60 | 0.13 |

[a]This estimate is obtained from the results of the nonlinear model.
[b]This quantity is obtained as $-\text{slope[I]}/[\text{I}]_0$. Units are $\mu M^{-1}$.
[c]See Eq. (17) for how this is calculated.

ters very large (in the case of $k_{21}$, the uncertainties are comparable to the values of the rate constants). On the other hand, the linear variation makes it possible to analyze all the data sets according to the linear model (Eqs. (10)–(12)).

In Table 6, we give the results of fitting to linear functions (intercepts and slopes). This gives a better picture of the inaccuracies in the determined parameters, although, as already seen, one cannot determine all the rate constants from the linear model. The values of $[\text{I}]_0$, $[\text{II}]_0$ and $[\text{III}]_0$ from Table 5 equal those given in Table 6 within the given uncertainties; $[\text{III}]_0$ is actually expected to be zero. The ratio $[\text{I}]_0$:$[\text{II}]_0$ should be the same from the two pBR-322 experiments, and we obtain $2.36 \pm 0.31$ and $2.12 \pm 0.23$.

The ratio of single-strand to double-strand cleavage, $r = k_{10}:k_{40}$, may be estimated using the rate constants in Table 5. For the four cases, we find $r = 7.2$, 11.2, 18.3 and 4.2, with large uncertainties because $k_{40}$ is not well determined. However, it seems clear that r is largest for SV-40 and smallest for PBR-322 DNA. According to Eq. (10), the slope of [I] should be $-[\text{I}]_0(k_{10}+k_{40})\tau$; combining this with the value of $r$, we determine values for $k_{10}$ and $k_{40}$ for each case (see Table 7). As noted previously, the three slopes must add to zero (this is confirmed by the data in Table 6), so there are only two pieces of information regarding rate constants available. The value of slope[I] having already been used, we use $\text{slope[II]} = \text{d[II]}/\text{d}D_0$ by rewriting Eq. (11) as:

$$\frac{[\text{I}]_0 k_{10}\tau - \text{slope[II]}}{[\text{II}]_0} = (k_{21}+k_{41})\tau \qquad (17)$$

Since $k_{41}$ is of the size of $k_{40}$, we expect $k_{21}+k_{41}$ to be dominated by $k_{41}$. By estimating $k_{41}\tau$ as $k_{40}\tau$, we could obtain an estimate of $k_{21}\tau$ but, because the values of $r$ and hence $k_{40}\tau$ are very uncertain, this estimate of $k_{21}\tau$ would be very rough. In fact, the values for $(k_{21}+k_{41})\tau$ (see Table 7) are very different from what the full model gives for $(k_{21}+k_{40})\tau$, suggesting the uncertainties are too large to take these seriously.

The analyses of all the experiments agree in indicating that bleomycin is mainly a single-strand cutter, with a small amount of double-strand cleavage. One can clearly see the production of form-III DNA, arising partly from double-strand cleavage, in Figs. 7–9. From the data of Lloyd et al. [22] we find $k_4:k_1 = 0.073$ for PM2 DNA, somewhat smaller than what we obtain from our own experiments on this plasmid (0.14). For cleavage of pBR-322, we find $k_{40}:k_{10} = 0.24$, and the SV-40 experiments give 0.089 and 0.055 for this ratio. Povirk et al. [6] found the ratio of double-strand breaks to single-strand breaks produced by bleomycin to be 0.2 on $T_2$ genome and 0.11 on Col E1 plasmid.

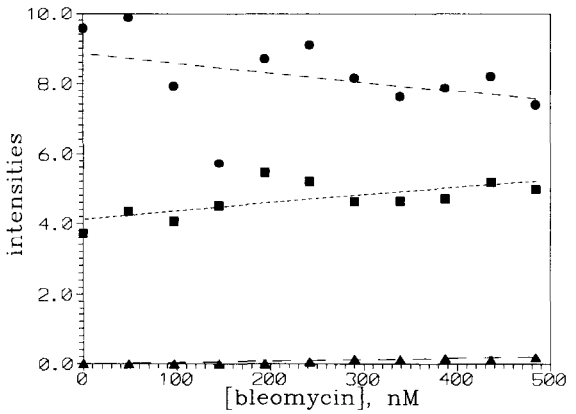


Fig. 9. Results of cleavage of 7.21 nM SV-40 DNA by bleomycin: intensities, proportional to populations of forms I, II, and III, are represented by circles, squares, and triangles respectively. Curves are the calculated populations, using best-fit values of the parameters.

## 5. Experimental methods

In the experiments involving the cleavage agents Fe–EDTA and bleomycin, the DNA, which is mostly in the closed-circular form, is digested by cleavage agent and the resulting mixture of different forms of DNA is loaded onto an agarose gel. Different lanes

on the gel correspond either to digests with different concentrations of cleavage agent, or to digests for different times with the same concentration of cleavage agent. After the different forms are separated by electrophoresis, the DNA is stained with ethidium bromide, so that one can measure the relative amounts of the different forms of DNA by fluorescence.

In order to visualize DNA, the stained gel is placed on a transluminator which emits UV light. The gel is photographed with a Polaroid camera using a film producing a positive and a negative, e.g., Polaroid Type 55, using one or more filters to eliminate background from the gel and transluminator. The photographic negative is then scanned using a Molecular Dynamics microdensitometer, and the volumes of the bands are determined utilizing Image Quant software [9].

Many transluminators do not produce uniform intensity over the surface being illuminated. There is also a vignetting effect due to the camera used to photograph the illuminated gel. In converting amount of fluorescence to amount of DNA for the various forms, one must correct for differences in loading, for the vignetting, and for uneven illumination of the lanes of the gel. If all the DNA after cleavage is measured (no conversion to small fragments which migrate too rapidly to be seen on the gel), one can correct for these effects by normalizing the sum of the populations to a constant. Equivalently, one can calculate fractional populations of the three forms.

To determine whether DNA is lost by conversion to small fragments, one can least-square fit the sum of the populations of forms I, II and III to a linear function of $x$, where $x$ is either time or concentration of cleavage agent, and examine the slope. A negative slope indicates loss of DNA. In this case, one can correct for differences in loading and illumination by fitting the sum of the populations to a decreasing function of $x$, $f(x)$ (usually linear or exponential). Then all three populations for a given value of $x$ are multiplied by the ratio of $f(x)$ to the actual population sum for this value of $x$. Sutherland et al. [23] have developed a method for normalizing spot intensities to concentrations, in which a distinct linear DNA is added to the supercoiled DNA stock before cleavage. In their experiments on cleavage by ionizing radiation, they found use of this mass standard superior to other correction methods.

It is known that the staining efficiency of closed-circular DNA in solution depends on the concentration of ethidium to which the DNA is exposed [7,24]. At low ethidium concentration, supercoiled DNA binds ethidium more readily than do the other forms, because ethidium intercalation releases the energy stored in the supercoiling. This enhances the binding affinity, but, at some point before the DNA lattice is saturated with ethidium, binding is inhibited. For the studies described here, an ethidium concentration of 0.5 $\mu$g/ml was used to soak the gel. It was found for several DNAs [9] that, under these conditions, forms I and III stain equally well. Furthermore, numerical experiments showed that a 10% change in the value used for the conversion factor changes the values of rate constants derived from the data by only a few percent. Therefore, no correction was made for differential staining.

### 5.1. Fe–EDTA

All reactions were carried out in a buffer of 50 mM tris–nitrate and 100 mM NaCl. Before addition of the DNA to the buffer solution, the EDTA (as $Na_2$ salt) and Fe (as ferrous ammonium sulfate) were added and metallation was allowed to proceed for 15 min. After DNA addition, 1 $\mu$L of a 50 mM DTT solution was added to all tubes, and the reaction allowed to proceed for the desired time, after which samples were electrophoresed on a 1% agarose gel and visualized by staining the gel in an ethidium bromide solution (0.5 $\mu$g/ml).

### 5.2. Bleomycin

In the bleomycin cleavage experiments on various DNAs (SV-40, pBR-322, and PM2), the concentration of bleomycin in the reaction mixture was varied with the digest time being held constant. An ampoule of Blenoxane (Bristol Labs) containing 15 units (7.5 mg) was dissolved in 1 ml of distilled water, for a stock concentration of 4.84 mM BLM. Aliquots of identical concentrations of bleomycin were added to a solution 5 mM in tris–HCl and containing the DNA. Next, DTT (8 mM) was added to all tubes, and the reaction allowed to proceed for 20 min, after which electrophoresis and subsequent steps were performed as detailed above for Fe–EDTA. For

pBR-322, the DNA concentration was 8.6 nM in molecules and the final bleomycin concentrations were 0, 48.4, 96.8, 145, 194, 242, 290, 339, 287, 436, or 484 nM. The same bleomycin concentrations were used for one set of SV-40 experiments with [SV-40] = 7.21 nM. A second set of experiments on SV-40 also used [SV-40] = 7.21 nM and [BLM] = 0, 96.8, 194, 290, 388, 484, 580, 678, 774, 872, and 968 nM. The experiments on PM2 DNA used [PM2] = 4 nM and [BLM] = 0, 0.807, 0.880, 0.968, 1.08, 1.21, 1.38, 1.61 and 1.94 $\mu$M.

Thus, the cleavage agent was present in large excess over the plasmid in every case except for the controls (bleomycin concentration = 0). It was found that the sum of the amounts of the three forms of DNA (intensities corrected for differential staining by ethidium) was essentially constant, so that we assumed $k_3 = 0$ in our analysis and converted amounts to fractional populations. An example of experimental results (for cleavage of 8.6 nM SV-40 DNA) is shown in Fig. 9, together with calculated populations from the analysis according to Eqs. (10)–(12).

## 6. Summary and conclusions

Our most general kinetic model for the interaction of a cleavage agent with closed-circular DNA includes the transformation of the closed-circular form to the open-circular and linear forms, the transformation of the open-circular to the linear form, and the cleavage of the linear form to smaller fragments. It also takes into account cleavage reactions which nick DNA but do not produce observable transformations from one form to another. We have assumed that the cleavage reactions are all second order, with rates proportional to the concentration of cleavage agent and the concentration of the DNA species being attacked. The second-order rate constants contain valuable information about the interaction of the cleavage agent with DNA.

The kinetic equations can be solved in closed form under the assumption that the fractional concentration of cleavage agent decreases with time in a manner independent of the initial concentration, i.e.:

$$\frac{1}{[D]}\frac{\mathrm{d}[D]}{\mathrm{d}t} = -f(t)$$

This includes the important situation where the cleavage agent degrades with time independently of its interaction with DNA, and also the case of $[D]$ remaining essentially constant during digest, i.e., $f(t) \simeq 0$. The solutions to the kinetic equations give concentrations of closed-circular (form-I), open-circular (form-II), and linear (form-III) DNA as a function of the initial concentration of cleavage agent and digest time. By choosing parameters in the model to minimize the sum of the squares of the deviations between actual and calculated DNA concentrations, information about rate constants is obtained, since the parameters include rate constants multiplied by:

$$G(\tau) = \int_0^\tau e^{-F(t)}\mathrm{d}t$$

where $F(t) = \int_0^t f(s)\,\mathrm{d}s$ and $\tau$ is the digest time. If $f(t)$ vanishes, so $[D]$ is independent of time, $G(\tau) = \tau$. Satisfactory fits to the model have been obtained in every case we have examined. Where data consisted of DNA concentrations as a function of time, the fits were satisfactory with $G(\tau) = \tau$, but this does not prove that $[D]$ is independent of time.

In the most favorable case, four rate constants can be determined: $k_{10}$ for single-strand conversion of form-I to form-II, $k_{21}$ for single-strand cleavage of form-II to yield form-III, $k_{30}$ for single-strand cleavage of form-III to smaller DNA fragments, and $k_{40}$ for double-strand (ds) conversion of form-I to form-III. Usually, however, the precision of the data does not permit precise determination of all four constants, or one or more of them are too small to be derived. When the measured concentrations of forms I, II and III DNA are linear functions of cleavage-agent concentration or digest time, one can obtain only two or three rate-constant parameters. In this case, the kinetic equations have been discussed and the solutions to the linearized equations compared with the general closed-form solutions.

Unless $f(t)$ is known (sometimes it may be inferred from the experiments themselves), only ratios of rate constants may be determined, since the parameters are rate constants multiplied by $G(\tau)$ with $G(\tau)$ being an unknown function of digest time $\tau$.

Further, if the cleavage agent must be activated to react with DNA, the concentration of the active species will be only a fraction of the nominal concentration at any time, and apparent values for rate constants will be too low by the same fraction. Again, ratios of rate constants will still be correctly determined. As discussed, these ratios are themselves of considerable interest.

Variable-concentration and variable-time experiments were performed for the agent Fe–EDTA, which was expected to be the simplest case: it was expected to perform single-strand (ss) cleavage only, and without base specificity (no preferred cleavage sites). The lack of observable form III when Fe–EDTA cleaved a mixture of forms I and II DNA implied the absence of double-strand (ds) cleavage. In addition, it indicated that $k_{21}$ (for conversion of form II to form III by ss cleavage) is much smaller than $k_{10}$ (for ss cleavage of form I), as should be true when there is no base specificity and no preference for attack near the site of cleavage which converts form-I into form-II. The ratio of $k_{10}$ to $k_{21}$ should be about $10^3$ if the two nicks must be within 10 base pairs to produce form III, but the experimental data allowed only the derivation of a lower limit of about 30 for this ratio. Comparison of rate constants derived from variable-time and variable-concentration data showed that the assumption $f(t) \simeq 0$ was not valid.

Our comparison of DNase I and calicheamicin showed clearly how the analysis distinguishes between single-strand and double-strand cleavage agents. For DNase I, it was not possible to get a good fit of the model to the data by ignoring ss cleavage (setting $k_{10}$ to zero), but ignoring ds cleavage (setting $k_{40}$ to zero) led to almost as good a fit as when both ss and ds cleavage were included. The full analysis suggests that a small amount of ds cleavage occurs, with $k_{10}$:$k_{40}$ being about 6 for DNase I. The reverse was true for calicheamicin, which was shown to act mostly as a ds cleavage agent ($k_{40}$ about 2.4 times $k_{10}$). The ratio of $k_{10}$ to $k_{21}$ for DNase I, while it could not be determined precisely, appeared to be much smaller than $10^3$. This implies the enzyme shows some base specificity and/or sequence preference, which is known to be the case [25].

For bleomycin, we analyzed both cleavage data in the literature (digest-time dependence of three forms of DNA from Lloyd et al. [22]) and the results of new experiments (concentration dependence). The values of rate constants found from the two data sets were not in good agreement, probably due to the breakdown of the assumption $f(t) = 1$. However, ratios of rate constants agreed to within the (rather large) probable errors. In particular, $k_{10}$:$k_{40}$ was found to be 20 from the literature data and 19 from our concentration-dependent data, while the values of $k_{10}/k_{21}$ were 13 and 3 respectively. We conclude that bleomycin is mostly, but not exclusively, a single-strand cleavage agent, and that it shows substantial site specificity, as is known to be the case [15].

For most of the cases discussed here, only ratios of rate constants were obtained, even though our analysis permits determination of values of the individual rate constants themselves. This is because, in order to obtain rate constants reliably from experiments with fixed digest time and variable $[D]_0$ (initial concentration of cleavage agent), one requires knowledge of the way $[D]$ varies with time during digest, or assurance that $[D]$ is constant. The latter may be gained from experiments with fixed $[D]_0$ and variable digest time. If activation of the cleavage agent is required, one cannot obtain actual rate constants without ascertaining the relation between $[D]$, the concentration of the unactivated cleavage agent, and the concentration of the active species. Also, in order to obtain the most information about a cleavage agent from measurements of cleavage of closed-circular DNA, one must ensure that the concentrations of the forms of DNA deviate from linear functions of the variable experimental parameter (time or $[D]_0$). This requires going to large digest times and/or concentrations, preferably large enough so that the sum of forms I, II and III DNAs decreases. Then one would learn about the rate constant $k_{30}$ and obtain reliable values for $k_{21}$ for discussion of specificity.

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