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Mechanism of Daunorubicin Inhibition of Hog Spleen Acid Deoxyribonuclease

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SUMMARY

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The inhibitory effect of daunorubicin on the enzymatic degradation of DNA was studied *in vitro*. The initial kinetics of acid deoxyribonuclease (deoxyribonucleate 3'-nucleotidohydrolase, EC 3.1.4.6) degradation was investigated using analytical ultracentrifugation to determine the ratio of single-strand to double-strand breaks. The late phase of the degradation was followed by hyperchromicity. Evidence is presented that the mechanism of inhibition of DNase activity involves nonproductive binding of daunorubicin-intercalated DNA. Calculations show that total inhibition occurs when 1 molecule of the antibiotic binds per 10 nucleotides.

INTRODUCTION

The antibiotic daunorubicin is a very active antimitotic agent which induces important remissions in acute leukemia, especially in acute promyelocytic leukemia and in acute myeloblastic leukemia (1–3). The drug interacts specifically with DNA, and two types of binding have been described: strong binding for the intercalation of molecules of daunorubicin between the base pairs of DNA, and weak binding for the interaction of phosphate groups of DNA with amino groups of the drug (ionic binding). The first seems to play an important part in modification of the biological and structural properties of DNA (4–6). Daunorubicin inhibits DNA-directed DNA and RNA synthesis (7) and deoxyribonuclease (8), presumably by interaction with the DNA substrate. The mechanism of inhibition of the enzyme by dyes or antibiotics intercalated in DNA has been studied by a number of authors (9–12).

Apart from the importance of DNases for the degradation of DNA *in vivo* (13), it has been shown that a correlation exists between DNase activity, frequency of some chromosomal aberrations, and, more generally, nucleic acid synthesis (14). Thus some carcinogens could induce an accelerated synthesis of acid DNase in rat liver (15). During some periods of the development of sea urchin embryos (*Paracentrotus lividus*), actinomycin and puromycin could induce an increase of DNase activity (16). In some mutants of T4 bacteriophage, which differ from the wild type in their ratios of 3'-exonuclease to polymerase activities, a consistent correlation was ob-

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served between nuclease to polymerase ratios and sensitivity to inhibition by some anticancer drugs (adriamycin and daunorubicin) (17).

In this paper we describe the kinetics of daunorubicin inhibition of hog spleen DNase (acid DNase, DNAse II). Analytical ultracentrifugation provided information for the initial kinetics of enzymatic degradation, and hyperchromicity, for the late phase. Some results have been compared with those obtained with beef pancreatic DNase. The results are of interest in understanding the enzymatic inhibition by some antimitotic anthracyclines.

MATERIALS AND METHODS

We are indebted to Rhône-Poulenc S. A. for the generous gift of daunorubicin (Rubidomycine or 13057 R.P.). The procedure of utilization has been described (6). Beef pancreatic DNase was a purified product of Worthington (code DPFF OED) with an activity of 2100 Kunitz units/mg. Hog spleen acid DNase was prepared by P. J. Sicard in our laboratory, using a previously described method (18). Calf thymus DNA and *Micrococcus luteus* DNA were extracted and purified in our laboratory (19). *Clostridium perfringens* DNA was obtained from Worthington and further purified by repeated extractions with phenol. Except when specified, all experiments used calf thymus DNA.

Physicochemical Methods

Spectrophotometric mesurements were carried out with a Unicam SP 800 instrument equipped with an *xy* recorder and a scale expansion of 10 or 20.

The sedimentaton coefficients of native and degraded DNA were determined at 130,000 × *g* with a Beckman model E ultracentrifuge equipped with an ultraviolet light source and a monochromator, using cells with Kel-F centerpieces. The decrease in molecular weight as a function of degradation was estimated using Studier's equations (20):

$$s^0_{20,w} = 0.0882\ M^{0.346} \text{ in 1 M NaCl}$$

$$s^0_{20,w} = 0.0528\ M^{0.400} \text{ in 0.9 M NaCl–0.1 M NaOH}$$

The weight average number of single-strand (SW) and double-strand (DW) breaks was calculated according to Sicard *et al.* (19). In neutral medium

$$DW = 2\left[\left(\frac{(s^0_{20,w})_0}{(s^0_{20,w})_t}\right)^{2.89}\right] - 1$$

In alkaline medium

$$SW = 4\left[\left(\frac{(s^0_{20,w})_0}{(s^0_{20,w})_t}\right)^{2.50}\right] - 1$$

where $(s^0_{20,w})_0$ and $(s^0_{20,w})_t$ are sedimentation coefficients at times 0 and t, respectively.

The ratio R of single-strand to double-strand breaks, which was used to determine the relative importance of haplotomic (single-strand breaks on either strand) and diplotomic (simultaneous scission of both strands) mechanisms, is given by R = SW/DW. In purely haplotomic degradation there is an interdependence of haplotomic and diplotomic mechanisms, the latter being a consequence of the former; R has been calculated and is equal to 133. When there is purely diplotomic degradation, R is very much lower and equal to 4 (19).

Gel Filtration on Sephadex G-75

The DNA-daunorubicin complex was equilibrated by dialysis against the same solution of daunorubicin in order to obtain r equal to 0.200 (6), where r is the ratio of concentration of bound daunorubicin to concentration of DNA (nucleotides per liter). This value of r was chosen because it corresponds to the maximum level of complexation which can be obtained without DNA precipitation. Acid DNase was assayed by measuring the liberation of acid-soluble oligonucleotides according to Bernardi *et al.* (21).

Samples containing mixtures of DNase, and DNA-daunorubicin complex were loaded on a column of Sephadex G-75 (45 × 1.6 cm) equilibrated with 0.1 SSC,[1] 1 mM EDTA at pH 5.0, and 10 μM daunorubicin. Fractions of 3.6 ml were collected at a flow rate of 15 ml/hr. All operations were carried out at 4°.

[1] The abbreviation used is: SSC, 0.15 M NaCl–0.015 M trisodium citrate.

Enzymatic Degradation of DNA

All enzymatic degradations were carried out at 25°.

Inital kinetics. Assays of DNase II were performed in SSC–1 mM EDTA, pH 5.0; concentrations were 100 μg/ml for DNA and 10–50 ng/ml for DNase II. After incubation for various times, the reaction was stopped by increasing either the ionic strength (final concentration, 1 M NaCl) or the pH (final medium, 0.9 M NaCl–0.1 M NaOH); in both cases the enzyme was completely inactivated.

Hyperchromicity. For DNase II the reaction medium contained 1.2 ml of SSC–1 mM EDTA, pH 5.0, with various concentrations of DNA (20–100 μg/ml) and daunorubicin (0–50 μM) and 2 μg of enzyme. For DNase I the reaction medium contained 1.2 ml of 0.15 M acetate buffer (pH 5.5), 50 mM $MgCl_2$, various concentrations of DNA (20–60 μg/ml) and daunorubicin (0–60 μM), and 2 μg of enzyme.

Hyperchromicity curves show a lag period, then a linear variation followed by a plateau. The reaction rate is calculated from the linear change and is defined as the absorbance increase at 260 nm per unit of time. Under our experimental conditions, hyperchromicity at 260 nm resulted only from DNA degradation and absorbance due to released daunorubicin could be neglected; there was no hyperchromicity at 480 nm (6).

Analysis of Kinetics of Enzymatic Degradation

Inhibition by inhibitor-substrate complexes was studied by Reiner (22), who demonstrated that it is suitable for investigating the function $I = f[i/(1 - i)]$, where i = (rate without inhibitor − rate with inhibitor) divided by rate without inhibitor. When $i/(1 - i)$ is small, the initial portion of the curve is linear, with slope y_1. As $(i/(1 - i)$ becomes infinite, there is a linear asymptote, with slope y_2 smaller than the initial slope. If we extend the asymptote back to the axis of I, it intercepts it at the value S_T, the apparent concentration of substrate in the medium.

This type of graph can be obtained for two mechanisms of inhibition of DNA degradation by inhibitor-substrate complexation.

1. The substrate concentration decreases by partial complexation of DNA by daunorubicin:

$$S + E \underset{}{\overset{K_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

$$S + I \overset{K_2}{\rightleftharpoons} SI$$

From Reiner's theory, it can be demonstrated that in this case $(y_2 - K_3)$ is always positive (9).

2. The substrate concentration decreases and in addition there occurs inactive binding of the enzyme to the DNA-daunorubicin complex:

$$S + E \overset{K_1}{\rightleftharpoons} ES \xrightarrow{K_2} E + P$$

$$S + I \overset{K_4}{\rightleftharpoons} SI$$

$$SI + E \overset{k_5}{\rightleftharpoons} ESI$$

In this case, $(y_1 - K_3)$ can be positive or negative (9). E, S, I, P, ES, SI, and ESI are the concentrations of enzyme, substrate, inhibitor, product of reaction, enzyme-substrate complex, inhibitor-substrate complex, and enzyme-substrate-inhibitor complex, respectively. Values of y_1 and y_2 are

$$y_1 = (K_3 + S_T)\left(1 + \frac{S_T}{K_1}\right)$$

$$y_2 = K_3\left(1 + \frac{S_T}{K_1}\right)$$

The dissociation constant of the complex K_3 can be calculated from the ratio $y_1/y_2 = S_T/K_3 + 1$.

RESULTS

Hyperchromic Study of Enzymatic Degradation

Figure 1 shows inhibition of acid DNase by daunorubicin. These data can be explained by complexation of the inhibitor to the DNA substrate, a theory which has been verified in some cases, for example, inhibition by ethidium bromide (9) and by acriflavine (23). A graph of $1/v = f(I)$ for inhibition by complexation of the inhibitor with the enzyme would give a straight line.

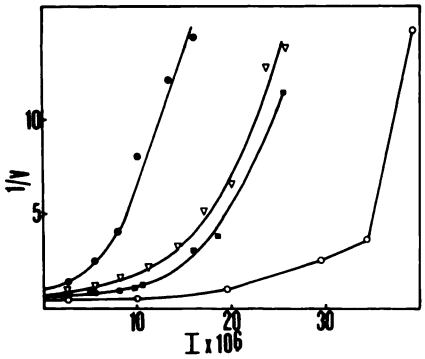

FIG. 1. *Inhibition of acid DNase by daunorubicin*

The ordinate shows reciprocal values of degradation rate, $1/v$, expressed as minutes per change in absorbance (min ΔA^{-1}). The abscissa shows daunorubicin concentration. Calf thymus DNA concentrations: ●——●, 23 μg/ml; Δ——Δ, 36 μg/ml; ■——■, 48 μg/ml; ○——○, 98 μg/ml.

Figure 2 shows the results obtained with different concentrations of DNA (A, 23 μg/ml; B, 36 μg/ml; C, 48 μg/ml; D, 98 μg/ml), following Reiner's representation (22). They are in good agreement with inhibition by substrate complexation. The same graphs could be obtained in some cases of inhibition by enzyme-inhibitor complexation, but the intercept of the asymptote with the y axis would be E_t (total enzyme concentration), independent of substrate concentration and usually very much smaller (22).

The dissociation constant of the complex, K_3, was calculated from y_1 and y_2 (see MATERIALS AND METHODS). The values of y_1, y_2, and K_3 are given in the legend to fig. 2. The mean value of K_3 presented in this work is equal to 1.1 μM. It should be noted that for the highest concentration of DNA the values of y_1 and y_2 are very inaccurate; therefore the corresponding calculated K_3 is meaningless and has been omitted for the determination of the mean value. $(y_2 - K_3)$ being negative, the inhibition of enzymatic degradation is due to the formation of the inactive enzyme-substrate-inhibitor complex (mechanism 2).

The apparent molecular weight M_0 of the substrate is calculated from the mean value of S_T:

$$M_0 \times S_T = C_{DNA} \times M_N$$

where C_{DNA} is the total concentration of DNA (moles of nucleotides per liter), and M_N is the nucleotide average molecular weight (326). We obtain M_0 equal to 3250, i.e., 10 nucleotides.

Two explanations are possible. Either 1 molecule of daunorubicin intercalated per 5 base pairs inhibits the formation of active binding of the enzyme, or there is only one enzymatic binding site for every 10 nucleotides. The second mechanism involves specificity of the enzyme related to the base composition of the DNA. With DNAs of different base composition (*C. perfringens*, 70% A-T; *M. luteus*, 28% A-T) M_0 is respectively 2800 (9 nucleotides) and 3150 (10 nucleotides), values in good agreement with the value previously determined with calf thymus DNA (56% A-T). Therefore it is reasonable to assume that the first is the right explanation.

K_3 is a constant related to the apparent molecular weight of the substrate. It is of

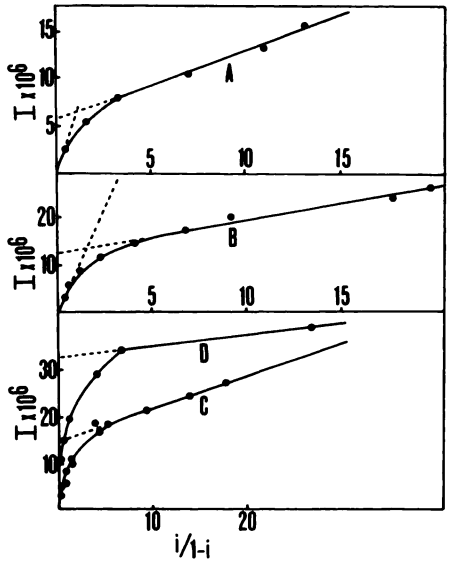

FIG. 2. *Inhibition of acid DNase by daunorubicin, plotted according to Reiner (22)*

The ordinate shows daunorubicin concentration, and the abscissa, $i/(1 - i)$, where i = (rate without inhibitor − rate with inhibitor) divided by rate without inhibitor. A. DNA, 23 μg/ml; S_T = 5.8 μM; M_0 = 3800; y_1 = 6.0 μM; y_2 = 0.73 μM; K_3 = 0.8 μM. B. DNA, 36 μg/ml; S_T = 12.8 μM; M_0 = 2840; y_1 = 8.5 μM; y_2 = 0.66 μM; K_3 = 1.07 μM. C. DNA, 48 μg/ml; S_T = 14.6 μM; M_0 = 3320; y_1 = 13 μM; y_2 = 0.87 μM; K_3 = 1.04 μM. D. DNA, 98 μg/ml; S_T = 32.3 μM; M_0 = 3030; y_1 = 36 μM; y_2 = 0.26 μM; K_3 = 0.25 μM.

the same order of magnitude as the constant determined by equilibrium dialysis (6).

The same type of plot is obtained with pancreatic DNase (Fig. 3). The mean value for M_0 is 3800, i.e., 12 nucleotides, but K_3 is equal to 5.5 μM. This discrepancy from the K_3 obtained with acid DNase can be attributed to the presence of Mg^{2+} in the reaction medium, which decreases the affinity of daunorubicin for DNA (4). As y_2 (7.8 μM) is larger than K_3, it is not possible to discriminate between the two mechanisms of inhibition.

Initial Kinetics of Enzymatic Degradation

The initial kinetics of DNA degradation was investigated by analytical ultracentrifugation. Fixation of daunorubicin between base pairs of double-helical DNA induces a decrease in the sedimentation coefficients. Therefore experimental values of sedimentation coefficients were corrected according to a coefficient α, which is equal to $s^0_{20,w}/s'^0_{20,w}$, where $s^0_{20,w}$ is the sedimentation coefficient of native DNA without daunorubicin and $s'^0_{20,w}$ is the sedimentation coefficient of the native DNA–daunorubicin complex. α was determined for various concentrations of daunorubicin. This factor depends only on the antibiotic concentration and can be applied whatever the DNA molecular weight may be.

The data obtained for the initial kinetics of degradation of DNA by DNase II in the presence of daunorubicin are presented in Fig. 4; they show the variation of $1/v$ with

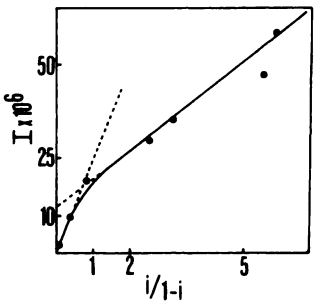

FIG. 3. *Inhibition of pancreatic DNase by daunorubicin, plotted according to Reiner (22)*

See the legend to Fig. 2 for explanation. DNA, 52 μg/ml; S_T = 12 μM; M_0 = 4333; y_1 = 25 μM; y_2 = 7.8 μM; K_3 = 5.5 μM.

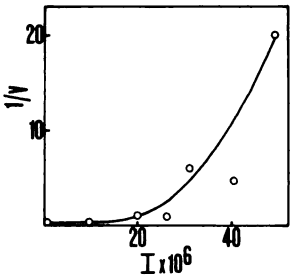

FIG. 4. *Inhibition of acid DNase by daunorubicin: initial kinetic*

The ordinate shows reciprocal values of degradation rate, expressed as minutes per single-strand break, SW. The abscissa shows daunorubicin concentration. DNA concentration, 100 μg/ml; DNase, 10 ng/ml.

increasing inhibitor concentration, where v is the number of single-strand breaks per unit of time. The curve looks like those plotted in Fig. 1; therefore the inhibition of both initial degradation and the late hyperchromic phase can be explained by complexation of the drug with the DNA substrate.

We know that hog spleen DNase degrades native DNA according to diplotomic and haplotomic mechanisms, but that there is a predominance of the first one (19). We obtain values of R = SW/DW of about 45, values larger than for a theoretical diplotomic mechanism but much smaller than for a pure haplotomic mechanism. Enzyme concentrations were calculated in the presence of daunorubicin in order to obtain the same level of degradation whatever inhibitor concentration was used; values of R were comparable only under these conditions. Binding of daunorubicin caused only a slight increase in R. This indicates that the mechanism of DNA degradation is not strongly modified, but that the diplotomic mechanism could be inhibited to a greater extent than the haplotomic one.

Evidence of a DNA–Daunorubicin–Acid DNase Ternary Complex by Gel Filtration

In order to demonstrate the formation of the ternary complex, we used gel filtration on Sephadex G-75. The elution volumes of the DNA-daunorubicin complex and DNase were quite different (Fig. 5A and

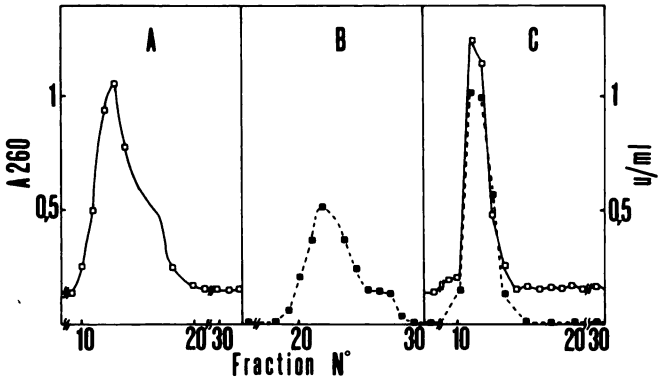

FIG. 5. *Association of acid DNase with daunorubicin-DNA complex*

Elution of the daunorubicin-DNA complex (A), acid DNase (B), or a mixture of the two (C) was carried out on a Sephadex G-75 column equilibrated with 0.1 SSC, 1 mM EDTA, and 10 μM daunorubicin at pH 5.0 (see MATERIALS AND METHODS). □——□, absorbance at 260 nm; ■- - -■, DNase activity.

B). If a mixture of acid DNase and DNA-daunorubicin complex was incubated for 24 hr at 4°, there was no enzymatic degradation; when this mixture was loaded on the column, all components were eluted at the same position (Fig. 5C). Therefore it is obvious that DNase II is bound to the DNA-daunorubicin complex and that the resulting ternary complex is inactive.

With pancreatic DNase, it was not possible to establish conditions under which binding of the enzyme would occur without any degradation of the DNA substrate. Increases in the daunorubicin concentration were limited by precipitation of the complex.

DISCUSSION

Evidence is presented in this paper which shows that at every phase of DNA degradation acid DNase inhibition is due only to fixation of the antibiotic to the DNA. During the initial phase of degradation there is only a slight modification of the kinetics, suggesting that the haplotomic mechanism might be less inhibited than the diplotomic one. Kinetic experiments as well as gel filtration clearly demonstrated the formation of an inactive ternary DNA–daunorubicin–acid DNase complex. Therefore daunorubicin does not prevent fixation of the enzyme to the substrate but inhibits the mechanism of subsequent degradation. After complexation of daunorubicin the mean value of the apparent molecular weight of the DNA degraded by hog spleen or pancreatic DNase is equal to 10 nucleotides. This result suggests that 1 molecule of daunorubicin intercalated per 5 base pairs inhibits the formation of active binding of the enzyme. With the same enzymes and ethidium bromide as inhibitor, Le Pecq (9) obtained an average value of 6 nucleotides. Using pancreatic DNase, Leith (23) calculated an average value of 8 nucleotides in the presence of acridine orange and 3.8 nucleotides in the presence of acriflavine. Therefore it is highly probable that the apparent molecular weight of the substrate is dependent on the nature of the intercalated drug. It is known that daunorubicin amino sugar interacts strongly with DNA phosphate groups away from the intercalation site (24).

From our present data we can assume that the inhibitory site (10 nucleotides) is larger than the intercalation site [5 nucleotides (6)]. On the other hand, there is formation of an inactive DNA–daunorubicin–acid DNase complex. These two observations may explain the potent inhibition of the enzymatic degradation of DNA, even at the low concentrations of daunorubicin used *in vivo*.

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