Yeast Mitochondrial Deoxyribonuclease Stimulated by Ethidium Bromide. 2. Mechanism of Enzyme Activation†

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ABSTRACT: An endonuclease (EtdBr DNase), which is more active in the presence of EtdBr, has been purified from yeast mitochondrial membrane (Jacquemin-Sablon, H., et al. (1979) *Biochemistry 18* (preceding paper in this issue)). This paper deals with the analysis of the mechanism of this activation. Determination of the enzyme activity in the presence of intercalating and nonintercalating agents showed that the enzyme does not recognize the DNA structure modification provoked by drug intercalation. Studies carried out with a series of phenanthridinium derivatives led to the following model. The EtdBr DNase activation would result from the formation of a ternary complex, DNA–drug–Triton X-100. The activation capacity of a drug depends on its ability to bind simultaneously to the DNA (not necessarily by intercalation) and the detergent. When this complex is formed, the DNA molecule is surrounded with Triton X-100 molecules which constitute an hydrophobic environment and make the substrate more prone to interaction with the enzyme. The implications of this model are discussed.

Ethidium bromide,[1] as well as many other DNA intercalating drugs, is known to inhibit most of the enzymes of the nucleic acid metabolism (Waring, 1975). However, an endonuclease, which can be stimulated up to 25-fold in the presence of intercalated EtdBr, has been purified from yeast isolated mitochondria and designated EtdBr DNase. In the preceding paper (Jacquemin-Sablon et al., 1979), it was shown that Triton X-100 is required for both the solubilization and the activity of the enzyme, indicating that the EtdBr DNase is tightly bound to the mitochondrial membrane. The stimulation of the enzyme activity depends on the amount of intercalated EtdBr and is maximal at saturation of the intercalation sites. In this paper, our study has been directed at determining the mechanism of this activation. Studies on the effects of a number of intercalating drugs (acridine, ellipticine, and phenanthridinium derivatives) demonstrated that intercalation by itself does not account for the stimulation of the enzyme. More detailed studies were carried out with a series of 20 phenanthridinium derivatives, indicating that the groups at positions 5 and 6 (Figure 1), lying outside the DNA helix, play a critical role in this process. Evidence presented here shows that these drugs have an important affinity for Triton X-100, which depends on the hydrophobicity of the 5,6 groups. Moreover, substitutions can be introduced at these positions, which do not affect either the affinity or the intercalation of the molecule into DNA but decrease its affinity for the detergent. Such substitutions drastically lower the ability of the drug to stimulate the enzyme activity. These results led us to propose the following model: EtdBr, or any other activating drug, could bind simultaneously to the DNA and the detergent, mediating the formation of a ternary complex, DNA–drug–Triton X-100. In this complex, the DNA surrounded by an hydrophobic environment would be more accessible to the enzyme micelles.

Materials and Methods

Enzyme and Substrate. EtdBr DNase (fraction IV) and tritium-labeled T7 DNA were prepared as described in the preceding paper (Jacquemin-Sablon et al., 1979).

Drugs. Phenanthridinium derivatives: the chemical structure and the code number of the compounds used in this work are summarized in Table I. The numbering scheme of the phenanthridinium ring is shown on Figure 1. Compounds I–V were obtained from the Boots Pure Drug Co. Compound VI (propidium) was purchased from Calbiochem. Compounds VII, VIII, IX, XV, XVII, and XVIII, acridine and phenanthridinium dimers, were gifts from Drs. Barbet and Roques. Compounds XI, XII, XIII, XVI, XIX, and XX were kindly provided by Dr. Waring. Compounds X, XIV, and XXI have been previously described (Le Bret & Chalvet, 1977). Actinomycin D was purchased from Calbiochem; proflavin and quinacrine were from Allied Chemical and Dye Corp. Ellipticine and 9-hydroxyellipticine were gifts from Dr. Dat-Xuong. Hydroxystilbamidine was purchased from May and Baker and berenil from Hoeschst.

Other Materials. Triton X-100 was purchased from Calbiochem, Brij 58 from Atlas Chemical Industries, and Tween 80 from S.P.I.A.

Enzyme Assay. EtdBr DNase activity was determined by measuring the release of acid-soluble products from T7 [^{3}H]DNA in the conditions of the standard assay previously described (Jacquemin-Sablon et al., 1979). The enzyme activation provoked by a drug is defined as the ratio of the rate of hydrolysis in the presence of the drug to that without drug. Therefore, the value of this ratio may vary from one experiment to another, mainly because the enzyme activity in absence of drug is very low and its determination somewhat inaccurate. In each table, the values obtained with the different drugs should be compared with the activation produced by EtdBr, measured in the same experiment. The concentrations of phenanthridinium and acridine derivatives were adjusted to give a r value of 0.16 (r is the number of intercalated molecules per nucleotide). As shown in the preceding paper (Jacquemin-Sablon et al., 1979), this r value corresponds to the maximal activation of the enzyme by EtdBr in these assay conditions. Experiments with acridine and ethidium dimers were also carried out at $r = 0.16$, r referring here to the number of intercalated monomers. In the case of actinomycin D and ellipticine, r cannot exceed a value of 0.1 (actinomycin D is known to bind only to GC base pairs, and at higher concentrations the DNA–ellipticine complex precipitates). The nonintercalating drugs (berenil, hydroxy-

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[1] Abbreviation used: EtdBr, ethidium bromide.

0006-2960/79/0418-0128$01.00/0

Table I: Chemical Structure of the Phenanthridinium Derivatives[a]

compounds	R_3	R_5	R_6	R_8	K_{app}[b] ((mol/L)$^{-1}$)	unwinding[c] angle (deg)	ref
I	NH_2	C_2H_5	C_6H_5	NH_2	5.0×10^5	26	*d*
II	NH_2	CH_3	C_6H_5	NH_2	5.0×10^5	26	*d*
III	NH_2	$CH(CH_3)_2$	C_6H_5	NH_2	5.0×10^5	26	*d*
IV	NH_2	$(CH_2)_4CH_3$	C_6H_5	NH_2	5.0×10^5	26	*d*
V	NH_2	$CH_2CH{=}CH_2$	C_6H_5	NH_2	5.0×10^5	26	*d*
VI	NH_2	$(CH_2)_3N^+(C_2H_5)_2CH_3$	C_6H_5	NH_2	5.0×10^5	26	*e*
VII	NH_2	$(CH_2)_3N^+H_3$	C_6H_5	NH_2	5.0×10^5	26	*d*
VIII	NH_2	$(CH_2)_3N^+H(C_2H_5)_2$	C_6H_5	NH_2	5.0×10^5	26	*d*
IX	NH_2	$(CH_2)_3N^+H(CH_2)_2N^+H_3$	C_6H_5	NH_2	5.0×10^5	26	*d*
X	NH_2	CH_2COO^-	C_6H_5	NH_2	1.0×10^4		*f*
XI	NH_2	CH_3	$C_6H_4COO^-(p)$	NH_2	1.0×10^5	25	*g*
XII	H	CH_3	$C_6H_4NH_2(p)$	NH_2	5.0×10^5	19	*g*
XIII	H	C_2H_5	$C_6H_4NH_2(p)$	NH_2	5.0×10^5	23	*g*
XIV	NH_2	C_2H_5	$C_6H_4NO_2(p)$	NH_2	5.0×10^5	26	*f*
XV	NH_2	C_2H_5	α-naphthyl	NH_2	5.0×10^5	26	*h*
XVI	NH_2	C_2H_5	β-naphthyl	NH_2	5.0×10^5	26	*h*
XVII	NH_2	CH_3	H	NH_2	5.0×10^5	18	*g*
XVIII	NH_2	C_2H_5	CH_3	NH_2	5.0×10^5	26	*h*
XIX	NH_2	C_2H_5	C_6H_5	$NHCOCH_3$	1.6×10^5	17	*g*
XX	$NHCOOC_2H_5$	C_2H_5	C_6H_5	$NHCOOC_2H_5$	3.6×10^4	11	*g*
XXI	$N(CH_3)_2$	C_2H_5	C_6H_5	$N(CH_3)_2$	1.0×10^4		*f*

[a] The numbering of carbon atoms in the phenanthridinium ring is given in Figure 1. [b] Association constants (K_{app}) measured in 0.1 M NaCl. [c] The values have been computed taking the unwinding angle of EtdBr equal to 26° (Wang, 1974). [d] Le Pecq (1965). [e] Waring (1970). [f] Le Bret & Chalvet (1977). [g] Wakelin & Waring (1974). [h] Le Bret (1977).

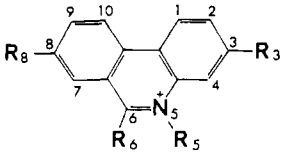


FIGURE 1: Numbering of carbon atoms in the phenanthridinium ring.

stilbamidine, and compounds X and XXI) were assayed at different concentrations ranging from 2×10^{-7} to 2×10^{-4} M.

Binding of Phenanthridinium Derivatives to Triton X-100. The fluorescence of these molecules was determined on a single photon counting apparatus built in this laboratory. Unless otherwise stated, excitation wavelength was 540 nm and emission wavelength was 640 nm. Fluorescence intensities were measured in 0.05 M Tris-HCl buffer, pH 7.6, 0.01 M $MgCl_2$, either in the absence of Triton X-100 (I_0) or in the presence of increasing concentrations of detergent (I). At low drug concentration, the following relationship can be used (Eisinger et al., 1965)

$$I_0/(I - I_0) = (1 + 1/KnT)/(V - 1)$$

where T is the total molar concentration of Triton X-100, K the affinity constant of the drug, n the number of drug molecules bound per detergent molecule, and V the ratio between the fluorescence intensity emitted by the bound and the free drug. When $I_0/(I - I_0)$ was plotted against $1/T$, a straight line was obtained for all the drugs. The intercept of this line at the origin gives the value of $1/(V - 1)$ and the slope yields Kn. When the fluorescence enhancement factor V was larger than 7, the binding parameters were determined with the Scatchard representation as previously described (Le Pecq & Paoletti, 1967). For smaller values of V, the data were not sufficiently precise for a valuable utilization of this method.

Results

Effects of Intercalating and Nonintercalating Drugs on the EtdBr DNase Activation. In an earlier work (Paoletti et al., 1972), we reported that the EtdBr DNase was activated by four intercalating drugs and inhibited by three nonintercalating ones. We concluded that there might be a correlation between the intercalation of a molecule and its ability to stimulate the enzyme activity. However, these experiments were carried out with a crude mitochondrial extract and in conditions where the level of enzyme activation (activity + drug/activity - drug) was rather low. The role of drug intercalation in the EtdBr DNase activation was reexamined using a purified enzyme, a more sensitive assay, and a greater number of intercalating or nonintercalating agents.

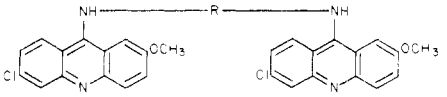
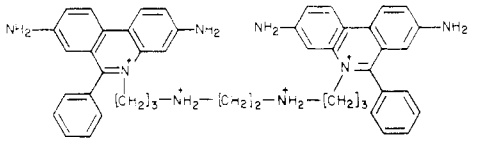
Table II: EtdBr DNase Activation in the Presence of Intercalating and Nonintercalating Drugs[a]

compounds	intercalation	K_{app}[b] ((mol/L)$^{-1}$)	unwinding[b] angle	ref	EtdBr DNase activation
I (EtdBr)[c]	+	5.0×10^5	26	*d*	17
VI	+	5.0×10^5	26	*e*	1.7
XI	+	1.0×10^5	26	*f*	2.5
XII	+	5.0×10^5	18.6	*f*	15
X	−	1.0×10^4		*g*	4.7
XXI	−	1.0×10^4		*g*	0.8
ellipticine	+	2.5×10^5	20	*h*	1.0
9-OH-ellipticine	+	2.3×10^6	25	*h*	1.0
actinomycin D	+	1.2×10^6	25	*e*	1.0
proflavin	+	1.2×10^6	19	*e*	4.5
quinacrine	+	1.2×10^6	19	*e*	7.0
berenil	−	0.7×10^6		*i*	0.5
hydroxystilbamidine	−	2.0×10^5		*i*	0.7

[a] EtdBr DNase activation was measured as described in Materials and Methods. [b] See legend to Table I. [c] Compounds listed in Table I. [d] Le Pecq (1965). [e] Waring (1970). [f] Wakelin & Waring (1974). [g] Le Bret & Chalvet (1977). [h] Le Pecq et al. (1974). [i] Festy et al. (1970).

Table II shows the results obtained with some phenanthridinium, ellipticine, and acridine derivatives. With the exception of compound X, a molecule from the phenanthridinium series (see structure in Table I), the nonintercalating drugs do not activate the EtdBr DNase. Intercalating drugs display variable effects on the enzyme activity. Some phenanthridinium and acridine derivatives significantly stimulate

Table III: EtdBr DNase Activation in the Presence of Dimers of Intercalating Drugs

drugs	intercalation	unwinding angle (deg)	DNase activation
diacridines			
			
diac I, R = $[CH_2]_3N^+H_2[CH_2]_4N^+H_2[CH_2]_3$	bis	38	1
diac II, R = $[CH_2]_3N^+H_2[CH_2]_4$	bis	38	1
diac III, R = $[CH_2]_3N^+H_2[CH_2]_3$	mono	17	4
diethidium			
	mono	26	1

the enzyme activity, while actinomycin D and ellipticines do not. The DNA binding of each intercalating drug can be characterized by its association constant for DNA and the unwinding angle of the DNA helix at the intercalation site. As shown in Table II, the effect of the drugs on the enzyme activity is not correlated to any of these parameters. For example, when compared with EtdBr, the phenanthridinium derivatives, compounds VI and XI, poorly activate the enzyme, although they have about the same association constant and unwinding angle. 9-OH-ellipticine, which has the same unwinding angle as EtdBr, but a higher affinity for DNA, does not activate the enzyme. On the other hand, compound XII stimulates quite efficiently the enzyme activity, although its unwinding angle is only 18°.

Dimers of acridine or ethidium have been synthetized (Le Pecq et al., 1975; Roques et al., 1976) in which the aromatic rings are linked by spermin-like chains of different lengths (Table III). These molecules are characterized by a very high affinity for DNA ($K > 10^9\ M^{-1}$). Depending on parameters such as the length of the chain, some of them (diac I, diac II) are bisintercalating, while others (diac III, diethidium) are monointercalating. Diethidium and bisintercalating derivatives of acridine did not stimulate EtdBr DNase activity, whereas a fourfold increase was observed with the monointercalating acridine dimer.

These results show that intercalation by itself does not account for the EtdBr DNase activation. One nonintercalating agent is able to stimulate the enzyme activity, while several intercalating drugs have no effect. Neither the unwinding angle nor the DNA affinity of the tested molecules could be correlated with the enzyme activation. Therefore other parameters must be considered. As shown in Table II, the best activation was produced by EtdBr. Attempts to establish a structure–activity relationship led us to examine whether some substitutions in the EtdBr molecule could either abolish or increase the enzyme stimulation.

Effects of Phenanthridinium Derivatives on the EtdBr DNase Activation. Recently, the structure of a EtdBr–dinucleotide complex was determined by X-ray crystallographic studies. From this work, a DNA–ethidium binding model was proposed (Sobell et al., 1977) in which the ethyl and phenyl groups, respectively, at positions 5 and 6 of the phenanthridinium ring are externally located in the narrow groove of the double helix. The amino groups at positions 3 and 8 lie inside the helix and interact with the sugar–phosphate backbone through hydrogen bonds. Therefore two series of compounds, carrying different substitutions, either at positions 3 and 8 or 5 and 6, were tested.

Table IV: EtdBr DNase Activation in the Presence of Phenanthridinium Derivatives Substituted at Positions 3 and 8

compounds	R_8	R_3	EtdBr DNase activation
I (EtdBr)	NH_2	NH_2	21
XIX	$NHCOCH_3$	NH_2	5
XX	$NHCOOC_2H_5$	$NHCOOC_2H_5$	1.0

Table V: EtdBr DNase Activation in the Presence of Phenanthridinium Derivatives Substituted at Positions 5 and 6

compounds	R_5	R_6	EtdBr DNase activation
I (EtdBr)	C_2H_5	C_6H_5	24
II	CH_3	C_6H_5	22
III	$CH(CH_3)_2$	C_6H_5	22
IV	$CH_2(CH_2)_3CH_3$	C_6H_5	21
V	$CH_2CH{=}CH_2$	C_6H_5	20
XII	CH_3	$C_6H_4NH_2(p)$	23
XIII	C_2H_5	$C_6H_4NH_2(p)$	25
XV	C_2H_5	α-naphthyl	14
XVI	C_2H_5	β-naphthyl	12
XVII	CH_3	H	14
XVIII	C_2H_5	CH_3	18

Removal of the 3-amino group (compounds XII and XIII, Table VI), which has no apparent effect on DNA intercalation other than a small reduction in the unwinding angle (Wakelin & Waring, 1974), does not modify the stimulation of the EtdBr DNase. In contrast, introduction of bulky substituents at positions 3 and 8 has important consequences (Table IV). When the 8-amino is acetylated (compound XIX), both the DNA affinity and the enzyme activation are significantly lowered. When both positions are blocked by carbethoxyamino groups (compound XX), the interaction of the molecule with DNA differs markedly from intercalation with EtdBr; according to Wakelin & Waring (1974), the DNA affinity and the unwinding angle fall drastically, while the number of binding sites is about two to three times higher. This drug was unable to stimulate the EtdBr DNase activity.

We then tested phenanthridinium derivatives carrying either charged or uncharged substituents at positions 5 and 6. All these molecules are able to intercalate, although some differences in the binding constants and the unwinding angles can be observed (Table I). Table V shows that uncharged substitutions at position 5 had no significant consequences on the enzyme activation. In contrast, the 6-phenyl ring appears to play a more critical role in the activation process. Its removal, or replacement by a naphthyl or a methyl group, led

Table VI: Effect of Charged Substitution at Positions 5 and 6 of the Phenanthridinium Ring on the EtdBr DNase Activation

compounds	R_5	R_6	EtdBr DNase activation
I (EtdBr)	C_2H_5	C_6H_5	24
VI	$(CH_2)_3N^+$-$(C_2H_5)_2CH_3$	C_6H_5	1.5
VII	$(CH_2)_3N^+H_3$	C_6H_5	6.5
VIII	$(CH_2)_3N^+H$-$(C_2H_5)_2$	C_6H_5	6.5
IX	$(CH_2)_3N^+H$-$(CH_2)_2N^+H_3$	C_6H_5	3
XI	CH_3	C_6H_5-COOH(*p*)	4

to a somewhat lowered activation. However, introduction of a *p*-amino group did not change the level of enzyme stimulation. The significance of these results will be discussed below.

The most drastic effects were observed in the presence of charged substituents at either positions. Although, the number of available molecules in this category is rather limited, Table VI shows that the presence of a positive charge at position V or a negative charge at position VI resulted in an important decrease of the enzyme activation.

These results show that the nature of the substituents at positions 5 and 6 has an important influence on the EtdBr DNase stimulation. As indicated earlier, these substituents are externally located in the narrow groove of the DNA helix. Therefore, when all the intercalation sites are occupied, the apolar 5-ethyl and 6-phenyl groups constitute an hydrophobic structure around the DNA molecule. Drug molecules, carrying substitutions which preserve this hydrophobic structure, activate the enzyme nearly at the same level as EtdBr. In contrast, the presence of a charged group on these substituents results in a drastic decrease of the activation. This suggested that, through their 5,6 substituents, these drugs create a hydrophobic environment, surrounding the substrate molecule and facilitating the interaction with the enzyme. Indeed, the EtdBr DNase, which is a membrane protein, is purified and stored in the presence of 1% Triton X-100. Therefore the enzyme molecules are included in detergent micelles. We next investigated the possible influence of the detergent on the enzyme activation.

Effect of Triton X-100 on the EtdBr DNase Activity. As shown in the preceding paper (Jacquemin-Sablon et al., 1979), and on Figure 2A (lower curve), when the EtdBr DNase activity was measured at different DNA concentrations, in the presence of a constant amount of intercalated EtdBr, the curve obtained was biphasic, exhibiting an hyperbolic region, followed after a maximum by a descending region. Usually this type of curve is interpreted as resulting from an inhibition by excess of substrate. However, Yedgar & Gatt (1976), who studied the effect of Triton X-100 on the hydrolysis of sphingomyelin by the sphingomyelinase from rat brain, showed that this type of curve may also be due to substrate detergent interactions. They demonstrated that the formation of mixed micelles of Triton X-100 and lipids makes the substrate more prone to interactions with the enzyme and that the optimal enzyme activity requires a proper ratio of detergent to substrate. This led us to wonder whether comparable detergent substrate interactions might also affect the EtdBr DNase activity. Indeed, in the standard assay conditions, the reaction mixture contains Triton X-100, brought in by the enzyme dilution, at a final concentration of about 0.03%, which is above the critical micellar concentration (about 0.01%; Helenius & Simons, 1975). Therefore, all the enzyme molecules should

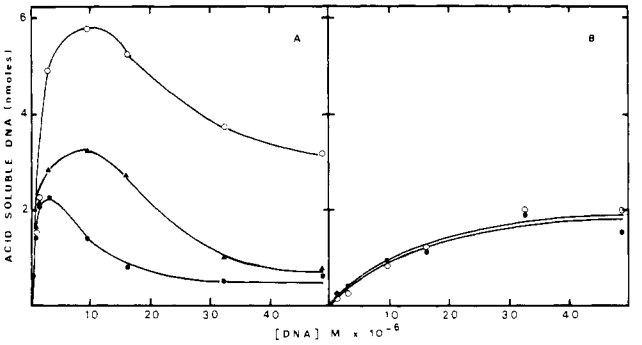


FIGURE 2: Effect of Triton X-100 on the EtdBr DNase activity at different substrate concentrations. EtdBr DNase activity (0.15 unit) was measured, in the standard assay conditions, at different substrate concentrations, either (A) in the presence or (B) in the absence of EtdBr (r = 0.17). (●—●) With 0.03% Triton X-100; (▲—▲) 1% Triton X-100; (○—○) 5% Triton X-100.

be in the micellar form. In these conditions, an interaction between the substrate and the detergent could account for our results, according to the following model: at low DNA concentrations, there would be enough Triton X-100 able to interact with the EtdBr–DNA complex, thus facilitating the interaction with the enzyme. In the range of DNA concentrations lower than 3×10^{-6} M, the rate of hydrolysis first increases with increasing substrate concentrations (Figure 2A, lower curve, left part) until a maximum is reached. Beyond this point, the enzyme activity decreases: when the DNA concentration further increases, the ratio of Triton X-100 to DNA decreases and so does the availability of the substrate to the enzyme. In agreement with this model is the fact that, when the detergent is added to the reaction mixture at a final concentration of 1% or 5%, both the maximal rate of the reaction and the corresponding DNA concentrations are increased (Figure 2A, upper curves). In contrast, the addition of 5% Triton X-100 has no effect on the enzyme activity measured in absence of EtdBr (Figure 2B).

Should EtdBr be required for the substrate–detergent interaction, the drug would have some affinity for Triton X-100. The binding of EtdBr to Triton X-100 was examined by measuring the fluorescence of the dye in the presence of increasing concentrations of detergent. The fluorescence enhancement factor V, calculated from the plot shown on Figure 3, is equal to 10. The Scatchard representation could then be used to accurately determine the binding parameters (Figure 4). The association constant K is equal to 2.4×10^4 M^{-1} and the number of detergent molecules per drug molecule is about 150. This value, which is comparable to the results obtained for other dyes (Rubalcava et al., 1969), is compatible with the presence of one EtdBr molecule per detergent micelle.

Although the affinity of the EtdBr–DNA complex for Triton X-100 was not determined experimentally, we may assume from the previous results that the activation of the EtdBr DNase by EtdBr results from the formation of a ternary complex: DNA–EtdBr–Triton X-100. Hydrophobic groups, at position 5 and 6 of the EtdBr molecule, lying outside the DNA helix, should be involved in the formation of such a complex. Therefore modifications of these groups, resulting in changes in the affinity of the drug for the detergent, might explain the variations previously observed (Tables V and VI) in the ability of the different phenanthridinium derivatives to stimulate the enzyme. The detergent affinities of some of these derivatives were determined by measuring the enhancement of fluorescence of these dyes in the presence of Triton X-100. For each drug, the ratio V was determined as described for EtdBr (Figure 3). This representation also allows the de-

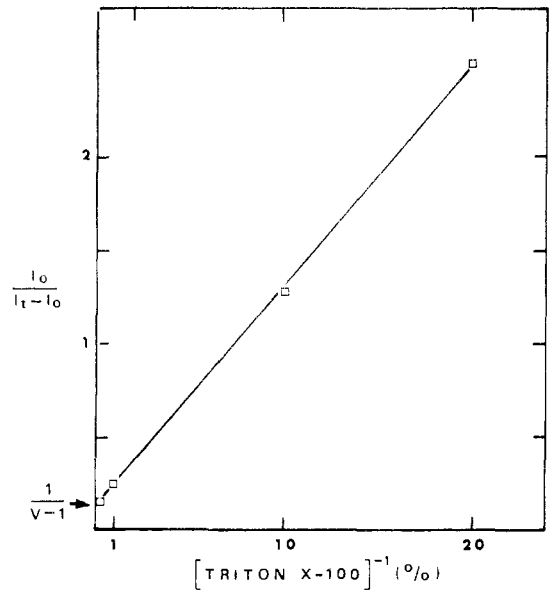


FIGURE 3: Determination of V (ratio between the fluorescence intensity emitted by the bound and the free EtdBr). Fluorescence measurements were carried out as described in Materials and Methods: EtdBr concentration is 10^{-5} M. V is calculated from the intercept of the curve with the Y axis.

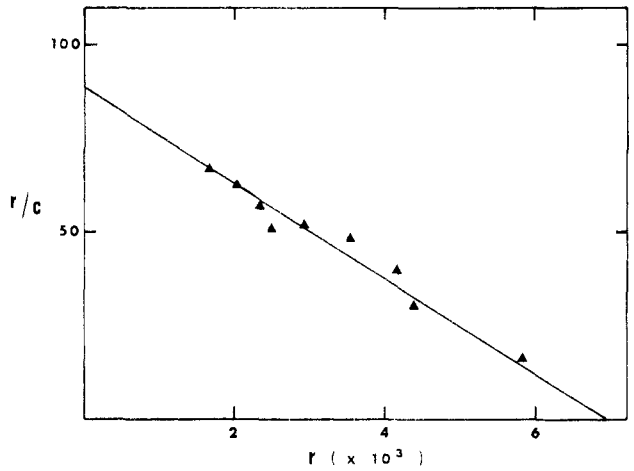


FIGURE 4: Scatchard plot of EtdBr binding to Triton X-100. Dye fluorescence was measured in the presence of 1% Triton X-100. r is the ratio of EtdBr bound per Triton X-100 molecule. c is the free dye concentration (M). r and c were computed as described by Le Pecq & Paoletti (1967), using the V value determined in Figure 3.

termination of Kn (K = association constant; n = number of detergent molecules per drug molecule). When the value of V was larger than 7, K and n were determined from the Scatchard representation. In all possible cases, very similar values of n were obtained. Therefore, the variations of the product Kn, shown on Table VII for the homogeneity of the presentation, parallel those of K. Table VII shows that changes in the substituents at positions 5 and 6 of the phenanthridinium ring result in important modifications of the values of Kn and V. Introduction of a charged group at either position (compounds VI and XI) or replacement of the 6-phenyl by a 6-methyl (compound XVIII) decreases the affinity of the dye for the detergent. On the other hand, a naphthyl group at position 6 (compound XVI) does not change the Kn value, while the increase in the length of the aliphatic chain at position 5 (compound IV) is associated with a very high Kn value. These results are in agreement with the hypothesis of a hydrophobic interaction of the drug with Triton X-100 through the R_5 and R_6 substituents.

Table VII: Binding of Phenanthridinium Derivatives to Triton X-100[a]

compounds	V	Kn ((mol/L)$^{-1}$)
I	10	81
II	7.2	51
III	7.3	344
IV	15	625
VI	2.7	5
VII	11	14
XI	6	16
XII[b]	8	23
XVI	11	85
XVIII[c]	4	32

[a] V and Kn were determined as described in Materials and Methods. [b] λ_{ex}, 460 nm; λ_{em}, 640 nm. [c] λ_{ex}, 500 nm; λ_{em}, 600 nm.

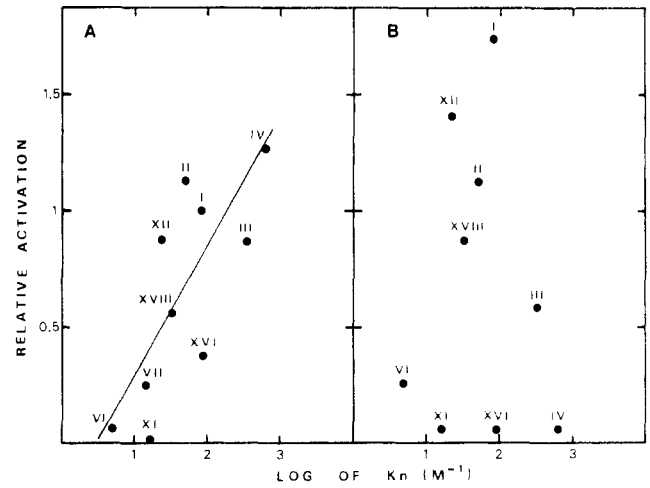


FIGURE 5: EtdBr DNase activation as a function of phenanthridinium derivatives affinity for Triton X-100. Roman numbers refer to Table I listing. Kn was determined as described in Materials and Methods. Enzyme activity was measured under the standard assay conditions (A) or in the presence of 5% Triton X-100 (B). Activation is expressed as percent of the activation produced by EtdBr in the standard assay conditions.

Figure 5A shows that a linear correlation (correlation coefficient = 0.8; F ratio, 12) is observed between the apparent affinities of the phenanthridinium derivatives for Triton X-100 and their ability to stimulate the EtdBr DNase activity, under the conditions of the standard assay (0.03% Triton X-100). At high detergent concentration (5%), this correlation is lost and variable effects are observed, depending on the affinity of each drug for the detergent (Figure 5B). In these conditions, the enzyme activation by those molecules which have a moderate affinity for Triton X-100 ($Kn < 85$ M^{-1}), with the exception of compound XVI, is slightly increased. In contrast, drugs which display a very high affinity for the detergent (compounds III and IV) have a much lower ability to stimulate the EtdBr DNase. At high concentration, the detergent could compete with the DNA for the binding of these compounds in such a way that the concentration of DNA intercalated drug would drastically decrease, thus decreasing the concentration of ternary complex.

This competition between DNA and Triton X-100 for the binding of the drugs makes the interpretation of the results obtained beyond the standard assay conditions very difficult at high concentrations of DNA and Triton X-100.

Effect of Other Detergents on the EtdBr DNase. We wondered how specific is the role of Triton X-100 in the EtdBr DNase activation. For example, one might imagine that a specific interaction between the phenyl group of the detergent

and the R_6 substituent of the phenanthridinium molecule could occur. Therefore other nonionic detergents, Brij 48 and Tween 80, very similar to Triton X-100 but having no aromatic part, were tested. Both stimulate the EtdBr DNase, in the presence of EtdBr, at the same level as Triton X-100. This indicates that the hydrophobicity of the detergent, rather than its particular chemical structure, is required for the EtdBr DNase activation.

Discussion

The preceding paper (Jacquemin-Sablon et al., 1979) describes the purification and some properties of an endonuclease (EtdBr DNase), of which activity, in contrast to all other known deoxyribonucleases, is increased in the presence of DNA-intercalated EtdBr. The present work is concerned with more detailed analysis of this activation mechanism.

Intercalative binding of EtdBr to DNA is known to result in modifications of the DNA secondary structure. A detailed model for ethidium–DNA binding based on information afforded by crystallographic studies of ethidium–dinucleoside monophosphates complexes has been recently described (Sobell et al., 1977). Since it was shown in the preceding paper that the level of EtdBr DNase stimulation depends on the amount of intercalated EtdBr, we first attempted to correlate the enzyme activation with the drug induced conformational change of the DNA molecule. For this purpose, the effects of intercalating and nonintercalating agents on the enzyme activity were compared. This study demonstrated that intercalation by itself does not account for the EtdBr DNase activation: one nonintercalating compound (compound X) was able to stimulate significantly the enzyme activity, whereas some intercalating drugs, which induce the same DNA conformational changes as EtdBr, had no effect. This indicated that, in addition to its intercalative capacity, other properties of the EtdBr molecule are involved in the enzyme activation process.

The phenanthridinium ring (Figure 1) carries substituents which, in the intercalated molecules, are either located inside the DNA helix (R_3 and R_8) or lying outside the helix in the narrow groove (R_5 and R_6). A study dealing with 20 phenanthridinium derivatives showed that different modifications of these substituents have important consequences on the enzyme activation and led us to propose the following model: the enzyme activation would depend on the formation of a ternary complex DNA–drug–Triton X-100. The formation of this complex would be mediated by the drug which could simultaneously intercalate into the DNA molecule and interact with the detergent through the R_5 and R_6 substituents. When this complex is formed, the DNA molecule is surrounded by Triton X-100 molecules which constitute a hydrophobic environment, and the enzyme, which itself is included in detergent micelles, would have more affinity for the complex than for the free DNA.

According to this model, the capacity of a drug to stimulate the enzyme relies on its capacity to induce the formation of such a ternary complex. For each drug, the complex formation will depend on the experimental conditions (DNA and Triton X-100 concentrations) and on its respective affinities for the DNA and the detergent. For example, compound XIX, which carries an 8-acetylamino group, displays a DNA affinity about 4-fold lower than that of EtdBr and has an enhanced affinity for Triton X-100 (Kn = 102 M^{-1}). When both 3 and 8 positions are blocked (compound XX), the DNA affinity is about 14-fold lower than that of EtdBr, while the detergent affinity is one of the highest in the phenanthridinium series (Kn = 486 M^{-1}). EtdBr DNase activation was only 4-fold in the presence of compound XIX and undetectable with compound XX. The decreased DNA affinity of these molecules, associated with a high Triton X-100 affinity, might result in a preferential binding of the drug to the detergent and therefore a partial or complete loss of the activation capacity.

All the tested compounds, which differ from one another by their 5,6 substituents, had about the same DNA affinity. In contrast, their Triton X-100 affinities were markedly different with Kn values ranging from 2.8 to 625 M^{-1}. In agreement with the model, a correlation was observed between the detergent affinity of these molecules and the level of enzyme stimulation under the standard assay conditions. However, at high Triton X-100 concentration (5%), this correlation was abolished. Under these conditions, the enzyme activation, in the presence of the drugs which display the highest affinities for the detergent, was drastically decreased (compound III), or even completely lost (compound IV). There again, the formation of a detergent–drug complex, instead of a ternary complex, would explain this effect.

As discussed above, our model accounts for the results obtained with most of the phenanthridinium derivatives. However, the behavior of some molecules is still difficult to understand. For example, compound XVI, which carries a 6-β-naphthyl, has DNA and Triton X-100 affinities very close to that of EtdBr. Nevertheless, its activation capacity under the standard assay conditions is significantly lower and completely abolished in 5% Triton X-100. Presently, this discrepancy remains unexplained.

Another puzzling problem is the apparent inhibition by excess substrate which was observed when the enzyme activity was measured at different substrate concentrations (DNA–EtdBr, r = 0.16) and in the presence of a fixed concentration of detergent. At low DNA–EtdBr concentrations, the enzyme activity first increases until a maximum is reached. Beyond this point, further increase of the substrate concentration led to a rapid decrease of the enzyme activity. The interpretation of these results is very difficult. According to our model the maximum enzyme activity should be reached when the concentration of the ternary DNA–drug–detergent complex is maximum. However, the formation of this complex, which depends on a number of parameters, such as the DNA–EtdBr and Triton X-100 concentrations and the respective affinities of the drug for the DNA and the detergent, cannot be analyzed experimentally. The structure of this complex, i.e., the number of detergent molecules per DNA molecules, is also undetermined. However, knowledge of this structure might be an important parameter in order to understand the decrease of the enzyme activity which is observed at high DNA concentrations. We may assume that the optimal enzyme activity requires an optimal ratio of Triton X-100/DNA, corresponding to a discrete number of DNA molecules per detergent micelle. When the DNA concentration increases, two possibilities should be considered: either the detergent molecules will be evenly distributed in the population of DNA molecules or the number of DNA molecules per micelle will be increased, as described by Yedgar & Gatt (1976) in the sphingomyelin Triton X-100 system. In both cases, variation of the Triton X-100/DNA ratio would result in a decreased affini of the enzyme for the substrate and would account for the served apparent inhibition by excess of substrate. Better understanding of this phenomenon will require further experimental analysis.

The in vivo formation of this complex, and its possible involvement in the induction of the yeast "petite" mutation,

is now under investigations. It should be pointed out that Triton X-100 micelles are highly hydrated structures which contain about 40 molecules of water per molecule of detergent (Yedgar et al., 1974). Although the EtdBr DNase activation is not specific for Triton X-100 and was observed with other nonionic detergents, it remains uncertain whether an identical effect would be obtained in the lipidic environment of the biological membranes. However, Mahler (1973) has shown that the structural requirements of the phenanthridinium derivatives for the mitochondrial mutagenesis in yeast are identical with those that we observed for the EtdBr DNase activation. Also, Hall et al. (1977) observed that the ability of a series of phenanthridinium derivatives to induce mitochondrial DNA degradation is related to the hydrophobicity of the substituent at position 6. In both cases, the results are interpreted in terms of a model that requires interaction of the mutagen with the mitochondrial inner membrane as well as with the DNA. From a pharmacological point of view, this mechanism, which relies on drug-mediated interactions between hydrophilic and hydrophobic structures, might be of general interest.

Acknowledgments

The authors are indebted to Dr. J. M. Saucier for help in preparing the manuscript and for critical comments and to Dr. J. B. Le Pecq for critical reading of the manuscript. We are greatly indebted to Drs. Barbet, Dat-Xuong, Roques, and Waring for generous supplies of numerous compounds used in this work. The excellent technical assistance of Miss E. Franque is gratefully acknowledged.

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