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Peroxidase-Catalyzed Covalent Binding of the Antitumor Drug N^2-Methyl-9-hydroxyellipticinium to DNA in Vitro†

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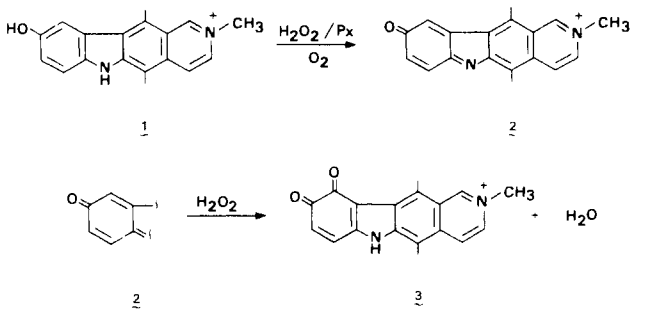
ABSTRACT: In the presence of DNA, the antitumor drug N^2-methyl-9-hydroxyellipticinium (elliptinium; NMHE) [Le Pecq, J. B., Gosse, C., Dat-Xuong, N., & Paoletti, C. (1975) *C. R. Seances Acad. Sci., Ser. D 281*, 1365–1367] is oxidized by the horseradish peroxidase–hydrogen peroxide (HRP–H_2O_2) system to the quinone imine derivative N^2-methyl-9-oxoellipticinium (NMOE) [Auclair, C., & Paoletti, C. (1981) *J. Med. Chem. 24*, 289–295], which interacts with DNA according to the intercalation mode. When excess H_2O_2 was used, the major part of the quinone imine was further oxidized to the *o*-quinone N^2-methyl-9,10-dioxoellipticinium [Bernadou, J., Meunier, G., Paoletti, C., & Meunier, B. (1983) *J. Med. Chem. 26*, 574–579]. In the presence of stoichiometric amounts of H_2O_2 (H_2O_2/NMHE = 1), NMOE reacts with DNA, yielding a fluorescent compound irreversibly linked to the nucleic acid, which is related to the covalent binding of the ellipticinium chromophore. Under optimal reaction conditions, NMHE binding occurs according to a first-order process ($k = 4.3 \times 10^{-3}$ min^{-1}) with a linear increase with respect to drug to nucleotide ratio up to a maximum binding of 1 NMHE per 20 base pairs ($r = 0.05$). The fluorescence spectra (ex, 330 nm; em, 548 nm) of NMHE bound to DNA, the occurrence of energy transfer from the DNA to the drug, and the DNA length increase of the DNA–NMHE adduct suggest that the binding occurs at the intercalating site with limited denaturation of the DNA helix. The fluorescence properties of the ellipticine chromophore covalently bound to DNA are consistent with linkage between the C10 of NMHE and a primary amine of DNA [Auclair, C., Meunier, B., & Paoletti, C. (1983) *Biochem. Pharmacol. 32*, 3883–3886].

The oxidative bioactivation of the antitumor drug N^2-methyl-9-hydroxyellipticinium (NMHE)[1] has been found to occur through peroxidase and oxidase reactions leading to the generation of the reactive quinone imine N^2-methyl-9-oxoellipticinium (NMOE) (Scheme I) (Auclair & Paoletti, 1981; Auclair et al., 1983a; Bernadou et al., 1983). In the presence of suitable nucleophiles such as O, N, or S donor containing compounds, including amino acids, proteins, glutathione, and ribonucleosides, the oxidation of NMHE to NMOE results in the formation of covalent adducts in vitro (Auclair et al., 1983b,c, 1984; Meunier et al., 1983; Bernadou et al., 1984). Evidence for the occurrence of the oxidative bioactivation of NMHE in vivo was provided by the detection of the glutathione–NMHE adduct in biological fluids such as bile and urine of patients and animals treated with the drug (Monsarrat et al., 1983; Maftouh et al., 1984). Consequently, hypotheses concerning the mechanism of the cytotoxicity of NMHE should take into account its possible covalent binding to biological nucleophiles. Among the various cellular nucleophiles, double-stranded nucleic acids can be considered as preferential targets for NMHE since the drug interacts with nucleic acids through an intercalating mode of a high apparent affinity (Le

Scheme I



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[1] Abbreviations: NMHE, N^2-methyl-9-hydroxyellipticinium; NMOE, N^2-methyl-9-oxoellipticinium; NMDOE, N^2-methyl-9,10-dioxoellipticinium; Gly-NMHE, 7,10,12-trimethyl-6*H*-[1,3]-oxazolo[5,4-*c*]pyrido-[3,4-*c*]carbazole; Px, peroxidase; H_2O_2, hydrogen peroxide; HRP, horseradish peroxidase; r_i, initial drug to nucleotide ratio; ex, excitation; em, emission.

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Pecq et al., 1974a). In this way we have recently observed that the treatment of cultured L1210 cells by NMHE resulted in the irreversible binding of the drug to the cellular DNA (Dugué et al., unpublished results). In order to known whether such a binding may arise from the oxidative bioactivation process, we have investigated the occurrence and the modality of the covalent binding of NMHE to DNA in vitro upon the oxidation of the drug by the peroxidase–H_2O_2 system.

Materials and Methods

Chemicals. N^2-Methyl-9-hydroxyellipticinium (NMHE) (**1** in Scheme I) was synthesized according to Dalton et al. (1967) and was provided by the Sanofi Co. (Sisternon, France). The intercalating fluorescent probe 7,10,12-trimethyl-6*H*-[1,3]-oxazolo[5,4-*c*]pyrido[3,4-*c*]carbazole (Gly-NMHE) was synthesized as previously described (Auclair et al., 1984; Gouyette et al., 1985). The *o*-quinone 9,10-dioxo-2-methylellipticinium (NMDOE) (**3** in Scheme I) was prepared according to Bernadou et al. (1983). Horseradish peroxidase (HRP) was obtained from Sigma Chemical Co., St. Louis, MO.

Identification of the o-Quinone NMDOE. NMDOE was purified from the assay mixtures by using a hydrophobic XAD2 column (Servachrom). The purified compound was identified by high-performance liquid chromatography (HPLC) and mass spectrometry. HPLC analysis was performed on a μBondapak C_{18} column with an elution mixture composed of methanol–water 60:40 containing 1 g/L heptanesulfonate and 2 mL/L acetic acid. Retention time of the product was compared with that of the authentic compound. Mass spectra were performed according to the desorption chemical ionization (DCI) (NH_3) method: NMDOE M cation MH^+ = 292.

Nucleic Acids. Calf thymus DNA was purchased from Boehringer Mannheim Germany and further purified by pancreatic ribonuclease treatment, followed by phenol extraction and dialysis against 0.5 M NaCl. Low molecular weight DNA was produced by 10 1-min cycles of sonication. DNA sonication resulted in a molecular weight drop from about 8×10^6 to 3×10^5 without a noticeable change in the T_m and absorption spectrum. Covalently closed circular DNA from bacteriophage PM2 was obtained from Boehringer Mannheim Germany and further purified according to Le Pecq (1971).

DNA Binding Parameters of the Quinone Imine NMOE. A stable solution of NMOE can be obtained in phosphate buffer (pH 5) through the oxidation of NMHE in the presence of excess HRP–H_2O_2. After the oxidation reaction was completed, H_2O_2 remaining in the solution was destroyed by the addition of catalase. Both enzymes were removed from the solution by filtration under nitrogen pressure using a Diaflo system equipped with an Amicon XM-50 membrane. In these conditions, more than 99% of the NMHE present in the solution is oxidized to NMOE.

The DNA binding constant of NMOE was determined at pH 5.0 in phosphate buffer by taking advantage of the hypochromic effect at 310 nm resulting from the binding of the drug to DNA according to Kohn et al. (1975). The amount of drug bound to DNA (C_b) was estimated according to the equation:

$$C_b = (\Delta OD/X - 1)(1/\epsilon_b)$$

where OD = absorbance of the drug without DNA minus absorbance of the drug in the presence of DNA, X = molar extinction coefficient of the free drug/molar extinction coefficient of the bound drug, and ϵ_b = molar extinction coefficient of the bound drug.

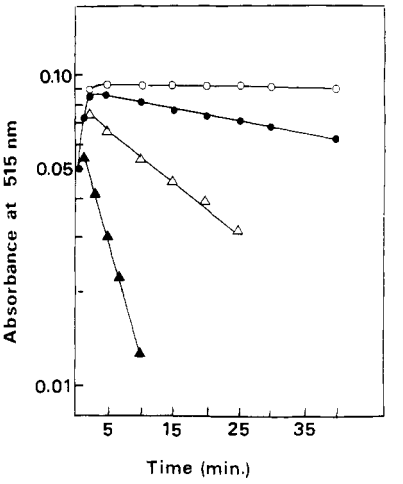

FIGURE 1: Absorbance decay behavior of NMOE in the presence of calf thymus DNA. Experiments were carried out at 25 °C in mixtures containing 0.025 M phosphate buffer, pH 7.40, 5×10^{-5} M HRP, 1.95×10^{-4} M DNA, and 1.25×10^{-5} M NMHE. Oxidation of NMHE was started by the addition of H_2O_2: 1.25×10^{-5} (●), 5×10^{-5} (△), or 5×10^{-4} M (▲) at pH 7.40 or 1.25×10^{-5} M at pH 5.0 (○).

The intercalation process was investigated by measuring the increase in length of sonicated DNA and the unwinding angle of the covalently closed circular DNA helix. Both DNA length increase and relaxation of the supercoiled DNA were estimated by viscosimetry as previously described (Revet et al., 1971; Saucier et al., 1971).

Covalent Binding of NMHE to DNA. In 0.025 M phosphate buffer (pH 7.40) (20 °C) was dissolved 10^{-7} M HRP, 1.95×10^{-4} M calf thymus DNA, 2.5×10^{-5} M NMHE-([^{3}H]NMHE at 0.6 mCi/mmol) (NMHE/nucleotide r_i = 0.13), and 2.5×10^{-5} M H_2O_2. After termination of the reaction, the DNA was precipitated at 0 °C by the addition of 4 M NaCl (1/0.5 v/v) and ethanol (1/2.5 v/v). After centrifugation, the pellet was redissolved in water and dialyzed against a 0.01 M phosphate buffer (pH 7.40) containing 0.1 M NaCl. Amounts of NMHE irreversibly bound to DNA were estimated by the radioactivity of each sample compared to a calibration curve obtained with the [^{3}H]NMHE solution. This overall procedure was referred to as standard operating conditions. In some experiments, dialyzed samples were subjected to cesium chloride centrifugation according to standard procedures. Each gradient was fractionated into aliquots on which were estimated absorbance at 260 nm, fluorescence, radioactivity, and DNA content.

Results

Oxidation of NMHE by Peroxidase–H_2O_2 in the Presence of DNA. It has previously been shown that, near neutral pH, the addition of HRP–H_2O_2 as oxidizing system in an NMHE-containing mixture resulted first of all in a rapid increase in the absorbance in the 500-nm region, followed by a rapid bleaching of the mixture (Auclair & Paoletti, 1981). This sequence of events corresponds to the oxidation of NMHE to the quinone NMOE, followed by the further degradation of NMOE either to the *o*-quinone NMDOE in the presence of excess H_2O_2 (Bernadou et al., 1983) (Scheme I) or to polymers of undefined structure. Figure 1 shows that in the presence of DNA and in the standard operating conditions (NMHE/nucleotide = 0.13, H_2O_2/NMHE = 1), the HRP–H_2O_2 system readily oxidizes NMHE to NMOE, which is measured by the increase in absorbance at 515 nm. The absorbance undergoes a slow exponential decay with a rate

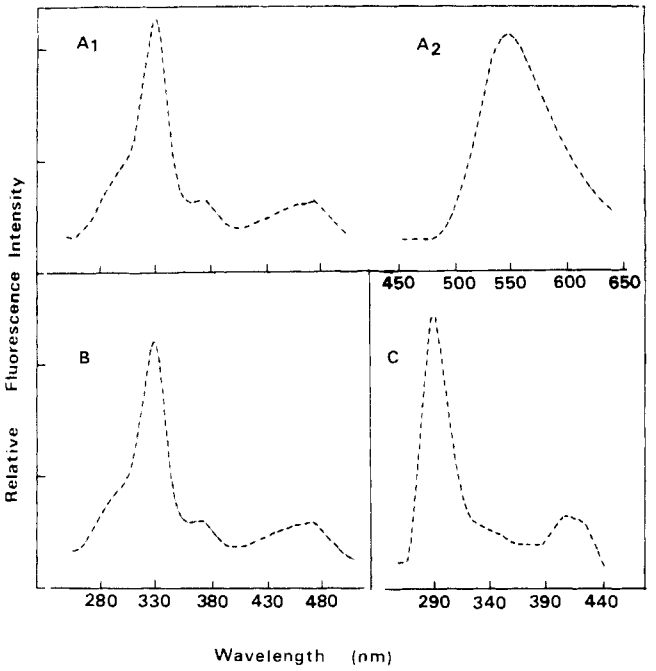

FIGURE 2: (A) Uncorrected fluorescence spectra [excitation (A_1) and emission (A_2)] of NMHE irreversibly bound to DNA. Spectra were recorded after the decay in absorbance at 515 nm was completed (12 h) in a medium composed of 0.025 M phosphate buffer, pH 7.40, 5×10^{-7} M HRP, 1.95×10^{-4} M DNA, 1.25×10^{-5} M NMHE, and 1.25×10^{-5} M H_2O_2. (B) Uncorrected excitation spectrum of Gly-NHME intercalated in the DNA carried out on a mixture composed of 0.025 M phosphate buffer, pH 7.40, 1.95×10^{-4} M DNA, and 5×10^{-7} M Gly-NMHE. (C) Uncorrected excitation spectrum of NMDOE dissolved in 0.025 M phosphate buffer, pH 7.40.

constant $k = 4.5 \times 10^{-3}$ min^{-1}. At pH 5.0, the absorbance remains quite stable during the time of the experiment. At pH 7.40 and after the decay time was completed, the mixture exhibits fluorescence spectra (ex, 330 nm; em, 545 nm) whose excitation and emission envelopes are indicated in Figure 2A. These spectra are similar to those obtained with the fluorescent ellipticinium derivative Gly-NMHE upon intercalation into DNA (Figure 2B). Ethanol precipitation followed by dialysis failed to remove fluorescence, indicating that the chromophore is irreversibly associated to DNA. NMHE oxidation using increasing concentrations of H_2O_2 (H_2O_2/NMHE ratios greater than 1) results in a strong increase in the decay rate of the absorbance at 515 nm (Figure 1) and is accompanied by the appearance of a fluorescence spectrum markedly shifted toward short wavelengths (ex, 290 nm; em, 490 nm). This spectrum is similar to the one exhibited by the authentic *o*-quinone NMDOE (Figure 2C). Ethanol treatment and dialysis completely remove the 290/490-nm fluorescence. HPLC experiments performed on the supernatant obtained after the ethanol precipitation step show the presence of a single fluorescent peak clearly identified as the *o*-quinone (see Materials and Methods). From these series of experiments, we conclude that in the presence of DNA (i) HRP–H_2O_2 catalyzes the oxidation of NMHE to NMOE, (ii) in the presence of stoichiometric amounts of H_2O_2 (H_2O_2/NMHE = 1) NMOE reacts with the DNA yielding a fluorescent compound irreversibly associated to the nucleic acid, and (iii) in the presence of excess H_2O_2 the major part of NMOE is readily oxidized to the *o*-quinone NMDOE, which does not react with DNA.

Peroxidase-Catalyzed Covalent Binding of NMHE to DNA. It has previously been demonstrated that the oxidation of NMHE by peroxidase in the presence of proteins resulted in

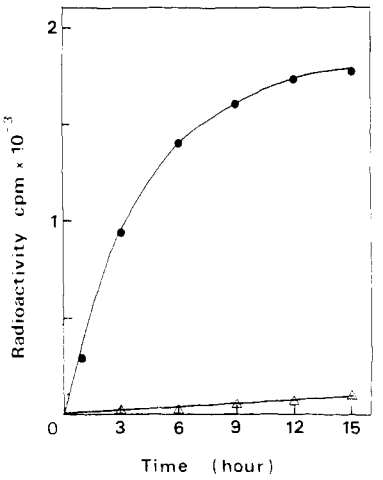

FIGURE 3: Time course of the incorporation of tritiated NMHE into DNA. Experiments were performed at 25 °C in medium composed of 0.025 M phosphate buffer, pH 7.40, 5×10^{-7} M HRP, 1.95×10^{-4} M DNA, 1.25×10^{-5} M [^{3}H]NMHE, and 1.25×10^{-5} M H_2O_2. For each indicated time, aliquots were removed from the solution and treated as indicated under Materials and Methods. The symbol △ indicates that peroxidase was omitted.

the covalent binding of the drug to the protein. In that case, covalent binding was accompanied by the appearance of fluorescence (ex, 315 nm; em, 525 nm) of the ellipticine chromophore (Auclair et al., 1983c). From these data, it can be expected that the remaining fluorescence irreversibly associated with DNA upon NMHE oxidation comes from the covalent binding of the drug to DNA. Evidence for such binding was provided by the time-dependent incorporation of radioactivity into the DNA remaining after ethanol precipitation and dialysis of a mixture containing initially radiolabeled [^{3}H]NMHE, DNA, and peroxidase–H_2O_2 (Figure 3). When banded on a cesium chloride gradient, all radioactivity and fluorescence (ex, 330 nm; em, 548 nm) were located within the band containing DNA (Figure 4). In the standard operating conditions, the amount of NMHE irreversibly bound to DNA (r) increased linearly as a function of the NMHE/nucleotide ratio (r_i) (Figure 5A). The experimental values show that about 25% of the NMHE irreversibly binds the DNA under these conditions. At NMHE concentrations that quite saturate the intercalating binding sites ($r_i > 0.2$), one molecule of NMHE is bound for ten base pairs, indicating a markedly high binding efficiency. The use of a H_2O_2/NMHE concentration ratio greater than 1 results in a marked decrease in the extent of NMHE binding likely due to the increase in the NMDOE production rate. As r bound increases, one observes a linear increase in the fluorescence intensity (ex, 330 nm; em, 548 nm) of the DNA–NMHE adduct (Figure 5B), indicating that the recurrence of the fluorescence property of the ellipticine chromophore is connected with the formation of the covalent binding. It should be noted that NMHE reversibly associated to DNA through intercalation exhibited no fluorescence at all.

Nature of the Covalent Binding Sites. Since it can be reasonably assumed that the covalent binding of NMHE to DNA results from the reaction between the electrophilic quinone imine NMOE and the nucleophilic centers in DNA, it is of interest to investigate first of all the DNA binding parameters of NMOE. Because of the relative stability of NMOE at acidic pH (Figure 1), it is possible to determine both the binding constant and the ability to intercalate between DNA base pairs. Figure 6A shows the binding curve plotted according to Scatchard and obtained from the hypochromic effect resulting from NMOE binding to DNA (see Materials

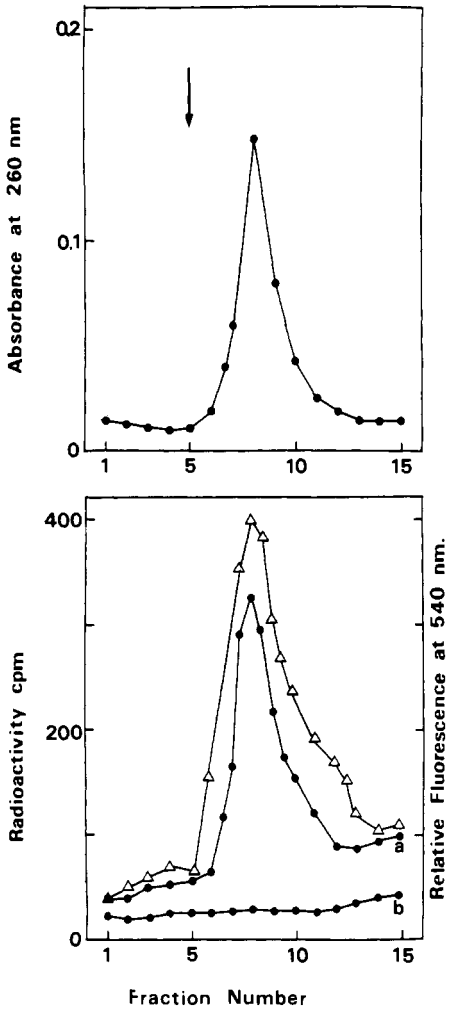

FIGURE 4: Cesium chloride gradient of calf thymus DNA labeled with radioactivity on incubation with HRP–H_2O_2 and [^{3}H]NMHE. The incubation was performed at 25 °C during 6 h with conditions as described in Figure 3. After ethanol treatment and dialysis overnight, DNA was banded on the gradient as described under Materials and Methods. In the upper figure, the arrow indicates the position of the peak of native DNA. In the lower figure, Δ indicates radioactivity, curve a (●) the fluorescence at 548 nm, and curve b (●) the fluorescence of samples containing DNA and noncovalently bound NMHE.

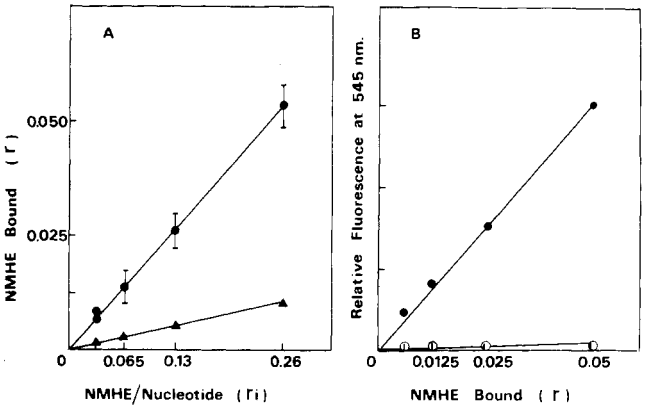

FIGURE 5: Quantitative estimation of the binding of NMHE to DNA. Experiments were performed at 25 °C in medium composed of 0.025 M phosphate buffer, pH 7.40, 5×10^{-7} M HRP, 1.95×10^{-4} M DNA, and various concentrations of NMHE. The H_2O_2/NMHE ratio was equal to either 1 (●) or 4 (▲). Six hours after the addition of HRP–H_2O_2, samples were subjected to ethanol precipitation and dialysis and checked for radioactivity and fluorescence. Amounts of NMHE bound to DNA were expressed as NMHE/nucleotide ratio (r) ± the standard deviation (SD). In (B), O indicates the fluorescence of mixtures containing NMHE reversibly intercalated into the DNA.

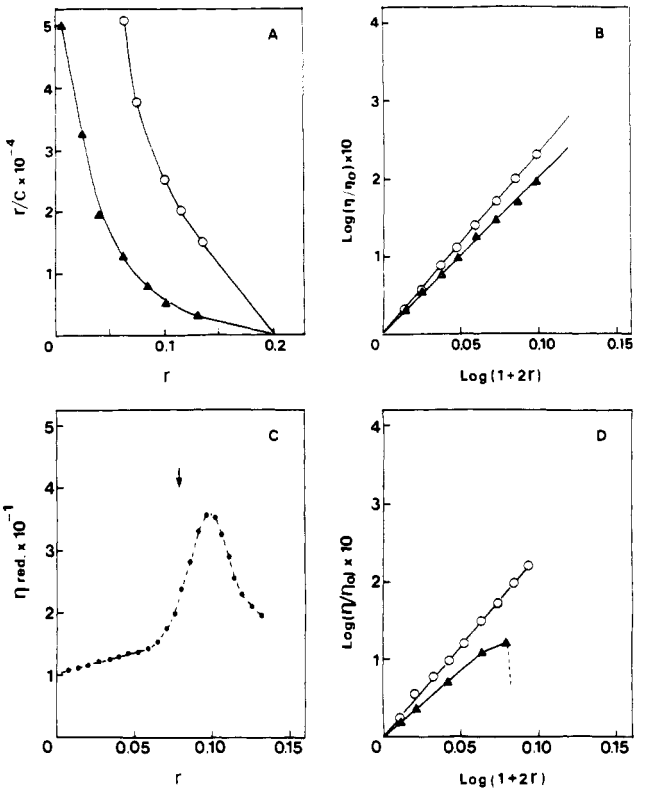

FIGURE 6: DNA binding parameters of the quinone imine NMOE (A–C) and of NMHE covalently bound to DNA (D). (A) Scatchard plots for binding of NMOE (▲) and NMHE (O) to native calf thymus DNA in 0.025 M phosphate buffer, pH 5 (final volume, 4 mL). The nucleotide concentration was 2×10^{-4} M. (B) Variation of the viscosity of sonicated DNA as a function of the number of bound NMOE (▲) and NMHE (O) per nucleotide. The assay medium was composed of 0.025 M phosphate buffer, pH 5.0, and the DNA concentration was 3.6×10^{-4} M. (C) Reduced viscosity of covalently closed circular DNA from phage PM2 as a function of the number of bound NMOE per nucleotide. The assay mixture was as in (B), and the DNA concentration was 2.5×10^{-4} M. The arrow indicates the maximum viscosity obtained in the presence of NMHE. (D) Variation of the viscosity of sonicated DNA as a function of the number of reversibly bound NMHE (O) and covalently bound NMHE (▲) per nucleotide. The assay medium was composed of 0.025 M phosphate buffer, pH 7.40, and the DNA concentration in both assay was 3.5×10^{-4} M.

and Methods). The apparent association constant estimated from the slope of the initial part of the curve was 1×10^6 M^{-1}. In order to determine whether NMOE is able to intercalate in DNA, we have measured the relative viscosity of sonicated calf thymus DNA in the presence of increasing amounts of drug (Figure 6B). The value of the slope (2.10) of the straight line obtained is consistent with an intercalation process. This is confirmed by the unwinding of the double helix caused by the binding of NMOE to covalently closed PM2 DNA (Figure 6C). The value of the unwinding angle (15°) is very close to those of some other ellipticine derivatives (Le Pecq et al., 1974a) including NMHE. Since NMOE may intercalate within the DNA, covalent binding may occur at the intercalating sites. In order to investigate this possibility, we have measured first of all the effect of the covalent binding of the drug on the relative viscosity of sonicated DNA. Figure 6D shows that the covalent binding of NMHE to sonicated DNA results in an increase of the viscosity. The slope value (1.75) of the straight line obtained is significantly lower that the one obtained with NMHE (2.40) but remains in agreement with a monofunctional intercalating process. Additional information can be obtained from the determination of energy transfer from the DNA to the bound drug. Figure 7 shows that the variation of the fluorescence quantum yield of the DNA–NMHE adduct

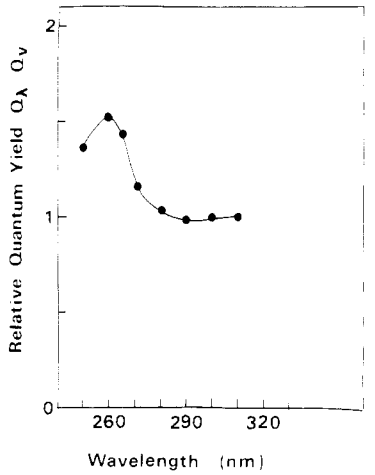

FIGURE 7: Energy transfer from DNA to covalently bound NMHE. The relative fluorescence quantum yield was estimated from the absorbance and corrected fluorescence excitation spectra of NMHE bound to DNA as previously described (Weil & Calvin, 1963). In this experiment, the amount of drug irreversibly bound to DNA r was equal to 0.05.

follows the variation of the DNA absorption spectrum. This is consistent with the occurrence of energy transfer and stongly suggests that the fluorescent chromophore is intercalated between DNA base pairs.

Discussion

The importance of oxidative bioactivation of various organic compounds including carcinogens and antitumor agents is gradually being recognized. The HRP–H_2O_2 system is a suitable promising model for studying these processes in vitro.

In this way, it has been shown previously that HRP–H_2O_2 may catalyzed in vitro the covalent binding of NMHE to various nucleophiles through the generation of the quinone imine, followed by a Michael addition reaction at the C10 position on the ellipticine chromophore (Meunier et al., 1983). The experimental data indicated in the present report clearly show that peroxidase–H_2O_2 catalyzes as well the covalent binding of NMHE to DNA. The primary step leading to the formation of the DNA–NMHE adduct appears to be the oxidation of NMHE to the quinone imine. The quinone likely reacts with the nucleophilic groups of DNA or is further oxidized to the *o*-quinone. In the standard operating conditions, the use of excess peroxidase, which readily oxidizes all of the NMHE initially present while consuming all H_2O_2 according to the stoichiometry of the reaction (1/1), minimizes the subsequent degradation of the quinone imine. In these conditions, the extent of NMOE further oxidized to NMDOE strikingly depends on the H_2O_2 concentration, accounting for the strong decrease in the NMHE binding when a H_2O_2 to NMHE ratio greater than 1 is used.

A tentative mechanism for the reaction of NMHE with DNA in the presence of HRP–H_2O_2 is

$$\text{NMHE} + \text{DNA} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{NMHE--DNA} \qquad (1)$$

$$\text{NMHE} + \text{HRP–}H_2O_2 \xrightarrow{k_2} \text{NMOE} \qquad (2)$$

$$\text{NMOE} + \text{DNA} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \text{NMOE--DNA} \qquad (3)$$

$$\text{NMOE--DNA} \xrightarrow{k_4} \text{NMHE–DNA} \qquad (4)$$

where NMHE--DNA and NMOE--DNA are the noncovalent complexes between drugs and DNA and NMHE–DNA refers to the covalent adduct between the drug and nucleic acid. The equilibrium binding of NMHE with DNA expressed by eq 1 involves the intercalation of the drug in the DNA. The association constant k_1/k_{-1} was estimated to be near 10^6 M^{-1} near neutral pH (Paoletti et al., 1979) with a saturation of the intercalating binding sites reached at $r = 0.2$ according to the site-exclusion model. In the standard operating conditions (r about 0.1) it can be assumed that most of the NMHE molecules are intercalated. However, it is reasonable to think that HRP–H_2O_2 reacts with the remaining free NMHE since the drug has to reach the catalytic site of HRP to be oxidized. It should be noted that the competition is not in favor of HRP since the apparent affinity of the enzyme for NMHE has been estimated to be $1/K_m = 2 \times 10^5$ M^{-1} (Auclair et al., 1983b). As free NMHE was oxidized, the equilibrum shown in eq 1 should be displaced toward the left until the reaction was completed. NMOE further reacts with DNA through an intercalation process according to the equilibrum shown in eq 3. From the K_{app} value obtained at pH 5, where NMOE exhibited two positive charges (the pK_a value of the indolic nitrogen can be estimated to be about 6.0–6.5), the association constant K_3/K_{-3} can be estimated in the range of 1×10^5 M^{-1} near neutral pH (Le Pecq et al., 1974b). In the standard operating conditions, ideally, a very small fraction of the NMOE is free in solution whereas the totality of H_2O_2 was readily reduced to water during the NMHE oxidation reaction. Under these conditions, a limited degradation of the NMOE occurs during the time course of the binding reaction, and the production of the DNA–NMHE adduct is a simple first-order process. However, it should be noticed that, at any NMHE per nucleotide ratio used, only 25% of the drug present in the assay medium prior to oxidation is recovered as covalent DNA–NMHE adduct, and the formation of unstable bonds that could be hydrolyzed during the dialysis step cannot be excluded. Since precipitation of the DNA--NMHE complex occurs when r_i is greater than 0.26, it was not possible to saturate the stable covalent binding sites.

Because NMOE interacts through intercalation, the covalent binding of NMHE to DNA may occur according to three different modes: (i) outside binding, (ii) binding at the intercalating sites, and (iii) insertion–denaturation binding (Fuchs et al., 1976). The DNA length increase resulting from the covalent binding of NMHE to the sonicated DNA strongly suggests that the binding occurs at the intercalating sites. Additional evidence is provided by the occurrence of energy transfer from DNA to the bound drug. However, it should noticed that the Q_{UV}/Q_{vis} value was only 1.55 for the DNA–NMHE adduct whereas it was found to be 4.3 for the fluorescent intercalating NMHE derivative Gly-NMHE (Banoun et al., 1984). Since the energy transfer efficiency is a function of both distance and relative orientation between the chromophores (Forster, 1959; Stryer et al., 1967), the low value obtained with DNA–NMHE may result from a local denaturation of the DNA helix produced by the insertion of the drug in agreement with the denaturation–insertion model. In fact, the extent of such a denaturation depends on the site of attachment of the drug to the DNA. The virtual identity of the fluorescence spectra of the DNA–NMHE adduct and amino acid–NMHE adduct such as Gly-NMHE suggests that NMHE is linked to DNA through a covalent bond between carbon 10 of the ellipticine chromophore and primary amines in DNA.

This assumption is supported by the fact that covalent adducts between NMHE and various nucleophiles involving a bond between the C10 of the ellipticine ring and secondary

amine, tertiary amine, oxygen, and thiol are not fluorescent in aqueous medium (unpublished results). Primary amines in DNA are at the N2 of guanine, N6 of adenine, and N4 of cytosine. The crystallogrphic data obtained with the iodoCpG–ellipticine complex (Jain et al., 1979) have indicated that when intercalated, the C10 of ellipticine can be very close to the primary amine of cytosine whereas the binding to the primary amine of either guanine or adenine should induce a strong modification in the arrangement of the DNA helix as postulated in the insertion–denaturation model. The binding process will be definitely determined by the identification of the chemical structure of nucleotide–NMHE adduct(s).

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Stereospecificity of the Fructose 2,6-Bisphosphate Site of Muscle 6-Phosphofructo-1-kinase†

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ABSTRACT: We explored the stereospecificity of the fructose 2,6-bisphosphate site of rabbit muscle 6-phosphofructo-1-kinase by determination of the activation constants (K_a) of several structurally locked analogues of this potent metabolic regulator. Under the assay conditions used, the K_a of fructose 2,6-bisphosphate was 0.12 μM. The most effective synthetic analogues and their K_a's were 2,5-anhydro-D-mannitol 1,6-bisphosphate (2.9 μM), 1,4-butanediol bisphosphate (6.6 μM), hexitol 1,6-bisphosphate (40 μM), and 2,5-anhydro-D-glucitol 1,6-bisphosphate (47 μM). Ten other bisphosphate compounds were much less effective as activators of the enzyme. These findings indicate that, unlike its active site, this allosteric site of 6-phosphofructo-1-kinase does not require the furanose ring. Its basic requirement seems to be a compound with two phosphate groups approximately 9 Å apart. Although the free hydroxy groups of the activator do not seem to be essential, their presence enhances appreciably the affinity of the ligand for this regulatory site.

The discovery of fructose 2,6-bisphosphate as an activator of 6-phosphofructo-1-kinase (Van Schaftingen et al., 1980a,b; Claus et al., 1981; Uyeda et al., 1981) opened new avenues of research on this enzyme. This modulator was discovered by workers investigating the effect of glucagon on hepatic 6-phosphofructo-1-kinase (Van Schaftingen et al., 1980a). The decrease in activity of the enzyme upon treatment of hepatocytes with glucagon was initially ascribed to be the result of its phosphorylation by cyclic AMP dependent protein kinase (Castano et al., 1979; Kagimoto & Uyeda, 1979, 1980; Claus et al., 1980). The potency of fructose 2,6-bisphosphate ac-

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