Accessibility to Bacterial Nucleic Acids of the Intercalating Drug Aliphatic Amino Acid Ellipticinium Derivatives in *Escherichia coli* and *Salmonella typhimurium*†

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Received April 13, 1984; Revised Manuscript Received August 28, 1984

ABSTRACT: The fluorescence of the aliphatic (amino acido)-N^2-methyl-9-hydroxyellipticinium (AA-NMHE) derivatives [Auclair, C., Voisin, E., Banoun, H., Bernardou, J., Meunier, B., & Paoletti, C. (1984) *J. Med. Chem. 27*, 1161–1166], namely, dehydroglycino-NMHE, dehydroalanino-NMHE, dehydrovalino-NMHE, and dehydroleucino-NMHE, has been characterized. The changes in the fluorescence properties of the drugs, including increase in quantum yields, increase in fluorescence lifetimes, and occurrence of energy transfer upon binding to DNA in vitro, have been further investigated. The measurement of the fluorescence increment of AA-NMHE when bound to fluorescent sites inside intact bacteria has been found to be suitable for the determination of the accessibility of the drugs to bacterial nucleic acids according to the method of Lambert and Le Pecq [Lambert, B., & Le Pecq, J. B. (1984) *Biochemistry 23*, 166–176]. With this methodology, the kinetics of drug uptake, the ability of the drug to reach the bacterial nucleic acids at equilibrium, and the nature of the ligand binding model have been determined in two AA-NMHE-sensitive strains, *Escherichia coli* BL 101 (Lambert & Le Pecq, 1984) and *Salmonella typhimurium* TA 98 [Ames, B. N., Lee, F. D., & Durston, W. E. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 782–786]. The main results obtained are the following: (i) At nonsaturating concentrations, each AA-NMHE exhibits a marked difference in its ability to reach the bacterial nucleic acids. This parameter seems to be correlated with the antibacterial efficiency of the drugs. (ii) The accessibility of AA-NMHE to the bacterial nucleic acids appears to be under the control of the hydrophobic character of the drugs on the one hand and the phenotype of the bacteria on the other hand. It is suggested that the increase in the accessibility to bacterial nucleic acids resulting either from a hydrophobic effect or from mutation is related to the ability of the drugs to reach a class of binding sites likely organized in a nucleosome-like structure.

N^2-Methyl-9-hydroxyellipticinium (NMHE)[1] is a monofunctional intercalating agent which exhibits a high cytotoxic activity against various experimental tumor cells (Le Pecq et al., 1975). Aliphatic (amino acido)-NMHE adducts, namely, dehydroglycino-NMHE, dehydroalanino-NMHE, dehydrovalino-NMHE, and dehydroleucino-NMHE (Table I), have been previously synthesized in our laboratory in order to obtain a homologous series of NMHE derivatives having various hydrophobic properties (Auclair et al., 1984). As compared to the parent compound NMHE, the most striking biological feature resulting from this structural modification is a strong increase in the antibacterial activity toward the *Salmonella typhimurium rfa* mutants (Ames et al., 1973) and the quarternary ammonium hypersensitive strain *Escherichia coli* BL 101 (Lambert & Le Pecq, 1982). The antibacterial efficiency of AA-NMHE has been found to be positively correlated with the hydrophobicity of the drugs. This relationship can be classically explained by an increase in the rate of passive diffusion across cell membranes (Cohn, 1971). However, it has been recently suggested that the uptake of psoralens in *E. coli acrA* mutants (Hansen, 1982) as well as the uptake of ethidium bromide in *E. coli* BL 101 strain (Lambert & Le Pecq, 1984) was controlled by the nucleic acid accessibility to these drugs inside the cells. Taking advantage of the fluorescence properties of AA-NMHE when bound to nucleic acids, we have investigated the quantitative aspects of the intracellular binding of AA-NMHE to nucleic acids in the sensitive strains *Salmonella typhimurium* TA 98 and *Escherichia coli* BL 101 and attempted to establish a relationship between the accessibility of AA-NMHE to nucleic acids, their hydrophobicity, and their antibacterial property.

Materials and Methods

Chemicals. N^2-Methylellipticinium (NME) was provided by Dr. N. Dat-Xuong (Institut de Chimie des Substances Naturelles, Gif sur Yvette, France). N^2-Methyl-9-hydroxyellipticinium (NMHE) was provided by SANOFI Co. (Sisteron, France). The aliphatic (amino acido)-NMHE adducts (dGly-NMHE, dAla-NMHE, dVal-NMHE, and dLeu-NMHE) were prepared as previously described (Auclair et al., 1984). Pancreatic deoxyribonuclease I, pancreatic ribonuclease I, micrococcal nuclease, calf thymus DNA, poly[d-(A-T)], and poly[d(G-C)] were from Boehringer (Germany).

Bacterial Strains and Media. Escherichia coli K12 strain AB 1157 was kindly provided by Dr. N. Otsuji (Fukuoka, Japan). *Escherichia coli* BL 101 strain was provided by Dr. B. Lambert (Villejuif, France). This strain derives from AB 1157 after a single mutation and was isolated and selected for its sensitivity toward ethidium bromide (Lambert & Le Pecq, 1982). *Salmonella typhimurium* strain TA 98 was provided by Dr. B. N. Ames (Berkeley, CA). This strain exhibits the *rfa* mutation resulting in increased permeability to bulk and hydrophilic compounds. Oxoid nutrient broth 2 was obtained from Oxoid Ltd. (England). M_9 medium contains the following per liter: Na_2HPO_4, 6 g; KH_2PO_4, 3 g; NaCl, 0.5 g;

† This work was financially supported by CNRS and INSERM.

[1] Abbreviations: ED_{50}, effective dose 50, the dose of drug reducing by 50% the bacteria growth rate as compared to controls; AA-NMHE, (amino acido)-N^2-methyl-9-hydroxyellipticinium; NMHE, N^2-methyl-9-hydroxyellipticinium; NME, N^2-methylellipticinium.

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NH_4Cl, 1 g; $MgSO_4$, 1 mM; $CaCl_2$, 0.1 mM; vitamin B_1, 1 mg; casamino acids (Difco, Detroit, MI), 3 g; glucose, 2 g. In anaerobiosis experiments, M_9 contains 16 mM succinate instead of glucose.

Fluorescence Measurements. Fluorescence spectra were recorded on a SLM 800 spectrofluorometer (Urbana, IL) equipped with a thermostated cell holder. This instrument was connected with an Ortec 9315 photon counter, interfaced through an Ortec 779 interface controller, to a Minc digital computer. Fluorescence spectra were corrected for the variation of the lamp excitation intensity as previously described (Le Pecq & Paoletti, 1967). In the presence of bacteria, corrections for inner filter effect were performed as described by Lambert & Le Pecq (1984). Kinetics of fluorescence increment in the presence of bacterial suspensions were performed by using an SFM 23/B Kontron spectrofluorometer (Zurich, Switzerland). Fluorescence lifetime was measured by the time-correlated single photon counting technique as previously described (Wahl et al., 1970; Yguerabide, 1972), using an instrument built in the laboratory.

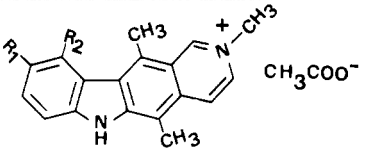
Fluorescence Titration of DNA, Poly[d(A-T)], and Poly[d(G-C)]. The fluorescence increment (ΔI_F) was the difference of fluorescence intensity between the solution containing drug and DNA and the solution containing drug alone. The values of the increasing factor of fluorescence intensity between free and bound drug (V) were calculated according to Eisinger et al. (1965). When the binding of the drug was not complete, the concentration of bound drug was computed according to Le Pecq & Paoletti (1967). The binding constants and the number of binding sites were obtained from Scatchard plots. In all cases, fluorescence titrations were performed in quartz fluorescence cells (1-cm path length) containing 3 mL of buffered solutions thermostated at 25 °C. The excitation wavelength was set at 330 nm and the emission wavelength at 550 nm.

Kinetics of Fluorescence Increment and Fluorescence Titration of Bacterial Suspensions. Bacteria were grown overnight in oxoid medium at 37° in a rotative shaker and then diluted (4%) in M_9 medium up to an absorbance of 0.6 at 650 nm. The bacterial suspension was adjusted to 10^8 cells/mL in prewarmed M_9 medium and allowed to grow 10 min in the rotative shaker at 37 °C (cell counts have been performed by plating efficiency and using a Malassez hematocytometer). Bacteria were used as they exist for fluorescence assays or were disrupted in a French press (Aminco). For kinetic measurements, the fluorescence intensity was continuously recorded after the addition of various concentrations of drug to the bacterial suspension. The rate constants were computed from the curves obtained. For the fluorescence titrations, ΔI_F was taken as the difference between the fluorescence intensity at the steady state of the mixture containing the drug and the fluorescence intensity of the free drug. The latter was measured either with drug alone in M_9 or with drug in the presence of the insensitive strain AB 1157 under similar experimental conditions. Experimental data obtained were treated as described for DNA.

Energy Transfer Measurement. The occurrence of energy transfer from nucleic acids to the bound drug was determined according to Weil & Calvin (1963). The relative quantum yield ratio of the bound drug upon excitation in the UV region vs. excitation in the visible region was determined by using the following equation:

$$\frac{Q_{UV}}{Q_{vis}} = \left(\frac{I_{UV}}{I_{vis}}\frac{E_{vis}}{E_{UV}}\right)_{bound}\left(\frac{I_{vis}}{I_{UV}}\frac{E_{UV}}{E_{vis}}\right)_{free}$$

Table I: Structure of Ellipticine Derivatives



compd	R_1	R_2	R_3
NME	-H	-H	
dGly-NMHE	-OH	-N=C(R_3)COOH	-H
dAla-NMHE	-OH	-N=C(R_3)COOH	$-CH_3$
dVal-NMHE	-OH	-N=C(R_3)COOH	$-CH(CH_3)_2$
dLeu-NMHE	-OH	-N=C(R_3)COOH	$-CH_2CH(CH_3)_2$

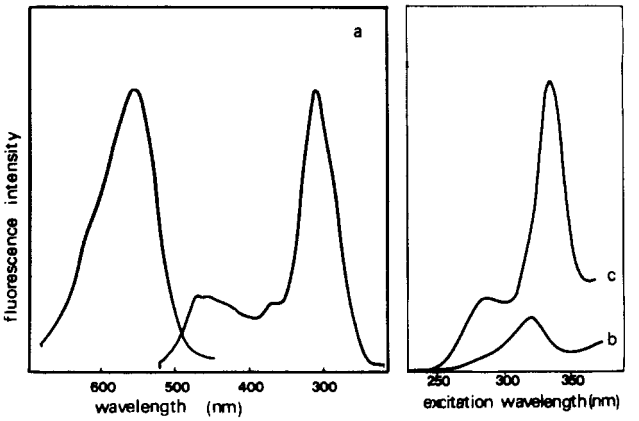


FIGURE 1: Fluorescence spectra of AA-NMHE free and bound to DNA: (a) uncorrected excitation and emission spectra of dVal-NMHE (2 μM); (b) part of uncorrected excitation spectra of dGly-NMHE (2 μM); (c) same spectra with an excess of calf thymus DNA (30 μM). Solutions were in 0.1 M cacodylate buffer, pH 7.

where I and E are respectively the fluorescence intensity and the molar extinction coefficient for $\lambda_{ex,vis}$ = 315 nm and $\lambda_{ex,UV}$ = 260 nm.

Enzymatic Procedures. When RNase A alone was allowed to work, DNase residual activity was removed by heating 10 min at 60 °C. Then bacterial broken cells (10^8 cells/mL) were treated with 5 units/mL RNase A for 45 min at 37 °C. For DNase treatment, DNase I was allowed to work 80 units/mL for 90 min and the micrococcal nuclease worked 2 units/mL for 90 min at 37 °C to yield mononucleotides from nucleic acids.

RESULTS

Fluorescence Properties of AA-NMHE. In the series of ellipticines, the derivatives hydroxylated at the C-9 position (Table I) exhibit no detectable fluorescence in aqueous media. The presence of the amino acid moiety linked at the C-10 position to N^2-methyl-9-hydroxyellipticinium results in the recovery of the fluorescence properties of the ellipticine chromophore. Figure 1a shows the uncorrected fluorescence spectra of dVal-NMHE dissolved in water. Maximum excitation and emission wavelengths and the various quantum yields of each AA-NMHE are indicated in Table II. The values corresponding to the nonhydroxylated compound N^2-methylellipticinium are shown as reference for the unsubstituted ellipticinium chromophore.

Fluorescence Properties of AA-NMHE Bound to DNA. As previously described for intercalating fluorescent dyes, it can be expected that free molecules in aqueous solution surrounding DNA and those intercalated into DNA might have different fluorescence properties (Le Pecq & Paoletti, 1967). Accordingly, the addition of DNA to a solution of AA-NMHE results in a marked increase in the fluorescence intensity of

Table II: Fluorescence Characteristics of AA-NMHE and NMHE Free in Solution

compd	emission max (nm)	noncorrected excitation max (nm)[a]	corrected excitation max (nm)[b]	Q_f[c]
NME	542	309, 358, 422	280_{sh},[d] 300, 360, 425	0.21
dGly-NMHE	546	310, 361, 464	282, 310_{sh}, 360, 425	0.21
dAla-NMHE	550	308, 361, 465	283, 315_{sh}, 370, 425	0.10
dVal-NMHE	548	306, 361, 452	278, 315_{sh}, 370, 420	0.14
dLeu-NMHE	554	306, 361, 466	279, 312_{sh}, 365, 425	0.14

[a] Underlined numbers are the highest maxima. [b] Correction is achieved for lamp intensity. [c] Q_f is the quantum yield of the drug calculated in comparison with that of ellipticine (Le Bret et al., 1977). [d] sh, shoulder.

Table III: Fluorescence Characteristics of AA-NMHE Bound to Calf Thymus DNA

compd	$Q_{b,UV}/Q_{b,vis}$[a]	τ (ns)[b]	V[c]
dGly-NMHE	4.3	24	25
dAla-NMHE	6.0	17	28
dVal-NMHE	5.1	16	23
dLeu-NMHE	3.8	17	25

[a] Ratio of the quantum efficiency of drug bound to DNA at 260 nm vs. the same quantity at 315 nm. [b] τ is the fluorescence singlet lifetime of the drug bound to DNA (r = 0.06). [c] V is the fluorescence increment between free and bound drug at 330-nm excitation and 25 °C in 0.1 M cacodylate buffer, pH 7.

Table IV: Calf Thymus DNA Binding Constants of AA-NMHE and NME[a]

compd	K_{app} (M^{-1}) at pH 5	K_{app} (M^{-1}) at pH 7
NME	9.9×10^5	4.4×10^5
dGly-NMHE	4.3×10^6	1.7×10^6
dAla-NMHE	1.0×10^7	7.8×10^5
dVal-NMHE	5.0×10^6	5.0×10^5
dLeu-NMHE	1.8×10^6	3.9×10^5

[a] pH 5 is obtained with 0.1 M acetate buffer and pH 7 with 0.1 M cacodylate buffer.

Table V: Poly[d(A-T)] and Poly[d(G-C)] Binding Parameters of AA-NMHE in 0.1 M Cacodylate Buffer, pH 7

compd	poly[d(A-T)] V[a]	poly[d(A-T)] K_{app}[b]	poly[d(G-C)] V[a]	poly[d(G-C)] K_{app}[b]
dGly-NMHE	28	7.8×10^5	22	8.0×10^5
dAla-NMHE	28	1.0×10^6	22	1.1×10^6
dVal-NMHE	26	3.3×10^5	20	7.0×10^5
dLeu-NMHE	28	6.0×10^5	22	1.0×10^6

[a] V is the fluorescence increment between free and bound drug at 330 nm. [b] K_{app} is the apparent affinity constant in M^{-1}.

the drug and in marked modifications of the fluorescence spectra. Figure 1b,c shows the excitation spectra obtained in the UV region for free and DNA-bound dGly-NMHE. The increase in the fluorescence intensity associated with a shift toward longer wavelengths can be related to a hydrophobic effect (data not shown) as previously reported in the case of ethidium bromide when intercalated (Olmsted & Kearns, 1977). The appearance of a shoulder in the 260-nm range is consistent with an energy transfer process from DNA to the intercalated drug as demonstrated for ethidium bromide (Le Pecq & Paoletti, 1967). No shift in the visible region of the fluorescence excitation spectrum, as well as in the emission spectrum, is observed. The occurrence of the energy transfer process can be revealed by calculation of the ratio $Q_{b,UV}/Q_{b,vis}$ (see Materials and Methods). If energy transfer occurs, this ratio must be greater than unity. The values indicated in column 2 of Table III show that energy transfer effectively occurs with all AA-NMHE. Energy transfer has been suggested to take place only with compounds which are intercalated in nucleic acids (Le Pecq & Paoletti, 1967; Reinhardt et al., 1982), and therefore, these results corroborate the viscosimetric data previously obtained concerning the DNA length increase in the presence of AA-NMHE (Auclair et al., 1984). In contrast, the quantum yield enhancement resulting from the hydrophobic effect may be due to the intercalation process and/or to outside binding. These possibilities can be investigated by measurement of the fluorescence lifetimes. The values of the fluorescence lifetimes of each AA-NMHE bound to DNA (pH 7, 0.1 M NaCl) are indicated in column 3 of Table III. These values are quite similar to those previously obtained in the series of ellipticine (Le Bret et al., 1977) and are consistent with intercalation.

At total dye to nucleotide ratios ranging from 0.01 to 0.20, each bound AA-NMHE exhibits a single fluorescence lifetime (single exponential decay); similar data were obtained at various ionic strengths (0.01–1 M Na^+), indicating that under these conditions external binding does not occur. AA-NMHE DNA binding parameters such as binding constant and number of sites can therefore be evaluated by fluorescence titration. The values of the fluorescence increment index (V) obtained for the different AA-NMHE adducts are indicated in column 4 of Table III (V is the ratio $Q_b\epsilon_b/Q_f\epsilon_f$, where Q_b and Q_f are the quantum yields of bound and free drug, respectively, and ϵ_b and ϵ_f are the extinction coefficients of the bound and free drug at 330 nm). The equilibrium between free and bound molecules is given by the Scatchard plot. The Scatchard plots of the fluorometric titration of calf thymus DNA by AA-NMHE yield slightly concave-upward curves, usually obtained with monofunctional intercalating compounds. For all the AA-NMHE, the maximum value of the drug bound per nucleotide ratio obtained was 0.2, which is consistent with the neighbor-exclusion model. The DNA binding constants obtained for the different drugs at pH 7.0 and 5.0 are indicated in Table IV. The fluorometric characteristics of drugs upon binding to DNA through intercalation could be dependent on the sequence of the DNA (Michelson et al., 1972; Le Pecq et al., 1975), and moreover, the drugs may exhibit a sequence-specific binding. Fluorometric titration of the homopolymers poly[d(A-T)] and poly[d(G-C)] shows that the fluorescence increment, the stoichiometry of binding, and the affinity for these polynucleotides are quite similar for each AA-NMHE (Table V).

Fluorometric Measurements of AA-NMHE Uptake in Bacteria. The experimental data described above indicate that binding of AA-NMHE adducts to DNA through intercalation resulted in a strong increase in the fluorescence of the drugs. With this occurrence, it can be assumed that in AA-NMHE-containing cell suspensions, an increase in fluorescence reflects the amount of drug which has reached fluorescent sites, including intercalation sites inside the cells. This technique has proved to be suitable for the study of ethidium bromide uptake in *E. coli* (Lambert & Le Pecq, 1984). Therefore, we have first of all investigated the kinetics of fluorescence increment of AA-NMHE in the presence of the sensitive strains *E. coli* BL 101 and *S. typhimurium* TA 98. Figure 2 shows that addition of different AA-NMHE derivatives to the bacterial suspensions resulted in a marked fluorescence enhancement as a function of time. The initial part of each curve

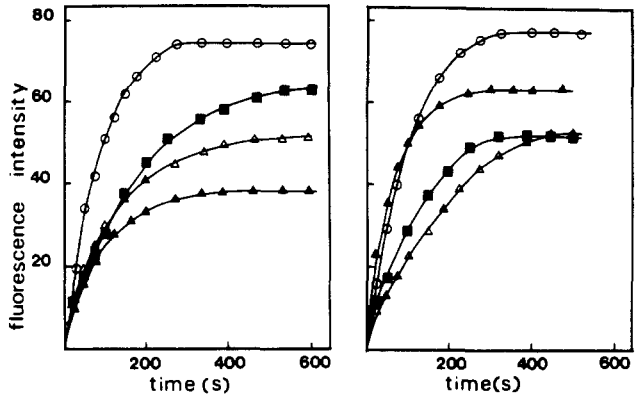


FIGURE 2: Time course of fluorescence increment of AA-NMHE in living cells: (Δ) dGly-NMHE; (■) dAla-NMHE; (○) dVal-NMHE; (▲) dLeu-NMHE. Intact cells were prepared as described under Materials and Methods. In the left panel, BL 101 was tested. In the right panel, curves refer to the TA 98 strain.

Table VI: First-Order Rate Constants of AA-NMHE Uptake in Bacteria

compd	k (min^{-1}) BL 101	TA 98
dGly-NMHE	0.396	0.314
dAla-NMHE	0.277	0.546
dVal-NMHE	0.572	0.693
dLeu-NMHE	0.438	0.924

can be fitted by a single exponential, yielding first-order rate constants ranging from 0.277 to 0.925 min^{-1} (Table VI). These values reflect the rate-limiting step of the accessibility of the drugs to the fluorescent sites inside the bacteria. This limiting step can be related to the rate of equilibration of the drug concentrations across the bacterial membrane and/or the rate of equilibration of the drugs with their fluorescent sites inside the bacteria. It must be noted that in the TA 98 strain the rate constant values were roughly correlated with the hydrophobicity of the drugs as expressed in terms of a capacity factor (Auclair et al., 1984). The addition of AA-NMHE to a suspension of wild-type *E. coli* AB 1157, which is insensitive to AA-NMHE, is not followed by a fluorescence increase. This observation suggests that the ability of the drugs to reach the fluorescent sites is related to their antibacterial activity.

Nature of the AA-NMHE Binding Sites in Bacteria. The fluorescence increment of AA-NMHE when associated with bacteria may come from the binding of the drugs to nucleic acids as well as to various hydrophobic components of the cells. Evidence for the involvement of double-stranded nucleic acids as fluorescent sites in bacteria is provided by determination of the fluorescence lifetimes and the occurrence of energy transfer. The values of the fluorescence lifetime of AA-NMHE in bacteria were found to be 24, 17, 18, and 16 ns for dGly-NMHE, dAla-NMHE, dVal-NMHE, and dLeu-NMHE, respectively. These values are quite similar to those obtained with calf thymus DNA in vitro (Table III). The occurrence of energy transfer from bacterial nucleic acids to the drugs is obvious from the data shown in Figure 3 which indicate that the variation of the quantum yield of AA-NMHE (dVal-NMHE in the figure) follows the variation of the nucleic acid absorption spectrum (see Materials and Methods). Quite similar spectra were obtained for all the AA-NMHE. As previously demonstrated for ethidium bromide (Lambert & Le Pecq, 1984), it appears from these results that the fluorescent binding sites for AA-NMHE in bacteria are the double-stranded nucleic acids. Additional information can be obtained from the titration curves of bacterial nucleic acids

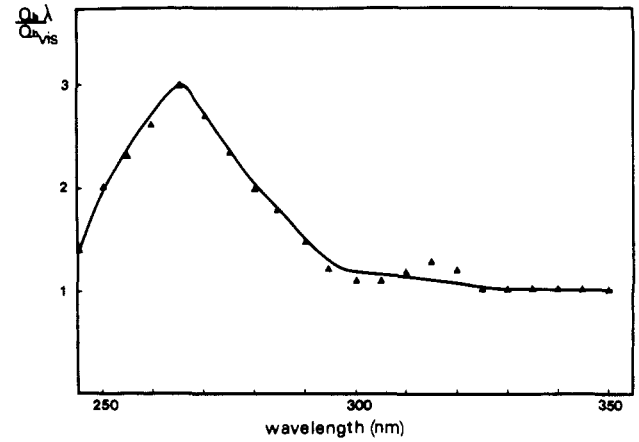


FIGURE 3: Energy transfer from bacterial nucleic acids to dVal-NMHE. The fluorescence excitation spectrum was corrected according to Lambert & Le Pecq (1984).

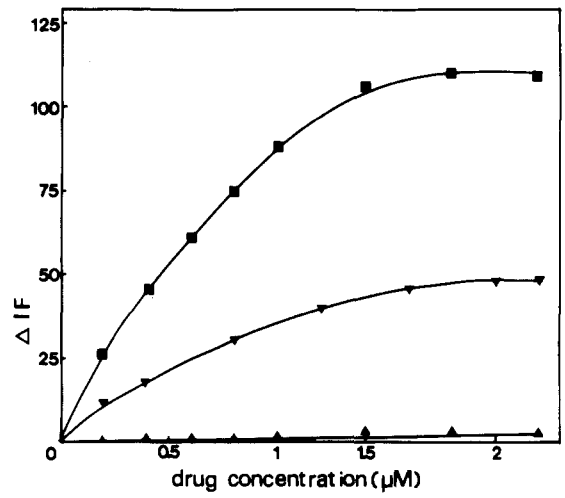


FIGURE 4: Fluorescence increment of dGly-NMHE upon binding to broken BL 101 cells. ΔI_F is the fluorescence increment between the drug in M_9 medium and the drug in disrupted cells, at the same concentration (■). (▼) Same titration after RNase- and DNase-free treatment; (▲) broken cells treated by RNase and subsequently by DNase.

performed in the presence of disrupted bacteria and subsequent treatments with nucleases. Figure 4 shows that the addition of RNase decreases by about 60% the fluorescence increment of AA-NMHE (dGly-NMHE in the figure) at saturation concentrations of drug. The further addition of DNases results in complete suppression of the fluorescence increment, indicating that in crude preparations of disrupted bacteria the fluorescent sites are located on nucleic acids only. Similar data were obtained with all the AA-NMHE.

Quantification of AA-NMHE Bound to Bacterial Nucleic Acids at the Steady State. The use of the extent of fluorescence increment to determine the amount of drug which has reached the nucleic acids inside the bacteria requires correction for the various fluorescence enhancement efficiencies occurring with each drug. This can be done by establishment of the titration curves of a nucleic acids containing suspension of disrupted bacteria. Moreover, in the range of concentrations used, each drug exhibits a single and constant fluorescence lifetime when bound to bacterial nucleic acids in both intact and disrupted cells. With this occurrence, the amount of drug bound to bacterial nucleic acids can be calculated according to Le Pecq & Paoletti (1967) as for purified DNA. For this purpose, it can be assumed that disruption of the bacteria results in the disappearance of the membrane barrier as well

Table VII: Differential Accessibility of AA-NMHE to Bacterial Nucleic Acids[a]

compd	BL 101		TA 98	
	b	*c*	*b*	*c*
dGly-NMHE	7	23	7	21
dAla-NMHE	12	43	7	25
dVal-NMHE	27	61	24	56
dLeu-NMHE	26	42	19	36
r[d]	−0.97	−0.92	−0.88	−0.81

[a] Values indicate the fraction (in percent) of the nucleic acid binding sites reached at the steady state for a concentration of 1 μM drug. [b] 100% is taken as the maximum binding to bacterial nucleic acids at saturating concentration of drug in the presence of disrupted bacteria. [c] 100% is taken as the binding to bacterial nucleic acids of 1 μM drug in the presence of disrupted bacteria. [d] The correlation coefficient, r, accounts for the correlation between percent of AA-NMHE accessibility to bacterial nucleic acids and the cytotoxicity of each drug.

as in the disorganization of the nucleosome-like structures of the cells (Pettijohn, 1982). These circumstances correspond to the maximum accessibility to the fluorescent sites. Therefore, at each concentration of the drug, the fluorescence increment corresponds to 100% of the potential accessibility to the fluorescent binding sites. Thus, as the fluorescence increment is proportional to the bound drug concentration (Le Pecq & Paoletti, 1967), in disrupted cells

$$\Delta I_F = kC_B$$

where C_B is the bound drug concentration. In intact cells, the fluorescence increment is

$$\Delta I_F' = kC_B'$$

Thus, the amount of drug bound to nucleic acids in intact bacteria expressed as the percent of maximum binding is

$$C_B' = C_B \frac{\Delta I_F'}{\Delta I_F}$$

with $C_B = 100\%$.

Therefore, at a given concentration of drug, the amount of drug bound to bacterial nucleic acids can be expressed either as the percent of the maximum accessibility at this concentration or as the percent of the maximum accessibility at saturating concentrations of drug. Table VII summarizes the values so obtained and shows that at equilibrium each AA-NMHE exhibited a marked difference in accessibility to bacterial nucleic acids in both strains. The highest values were obtained with the most antibacterial drug, dVal-NMHE, whereas the lowest values were obtained with the less active drug dGly-NMHE. The value of the linear correlation coefficient between the BL 101 antibacterial efficiency of AA-NMHE and their differential accessibility (columns *b*) is statistically significant for this relation as measured by the Student's test ($p < 0.05$). Apparent binding constants and maximum binding of AA-NMHE to bacterial nucleic acids can be investigated by Scatchard treatment of the fluorescence titration curves of intact bacteria by AA-NMHE according to the following equation:

$$r/c = K(n - r)$$

where r is the amount of AA-NMHE retained by 10^8 bacteria, c is the micromolar free concentration of AA-NMHE, K is the apparent binding constant, and n is the maximum number of AA-NMHE sites per 10^8 bacteria. Figure 5 shows Scatchard plots of these data obtained with dGly-NMHE and dVal-NMHE. In the BL 101 strain, dGly-NMHE and dVal-NMHE interact with bacterial nucleic acids according to two different models of binding. dVal-NMHE interacts

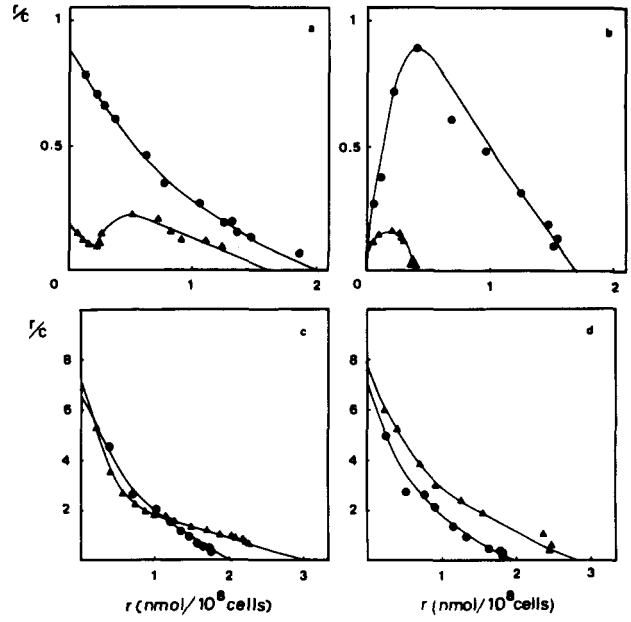


FIGURE 5: Scatchard representations of fluorometric measurements of dGly-NMHE (▲) and dVal-NMHE (●) retained by BL 101 (a) and TA 98 (b) cells at equilibrium. Bacterial suspensions (10^8 cells/mL) were separated in several samples and incubated till equilibrium with various drug concentrations. All incubations and measurements were done at 37 °C. In panels c (BL 101 strain) and d (TA 98 strain), dGly-NMHE (▲) and dVal-NMHE (●) binding to disrupted cells at 37 °C (equilibrium is instantaneous).

through the anticooperative binding model involving one class of binding sites as exhibited by the concave upward curved plot. In contrast, the binding of dGly-NMHE to bacterial nucleic acids occurs according to a sequential process involving two classes of binding sites (as described in the case of ethidium bromide interaction with lymphoblastic chromatin; Paoletti et al., 1977). At low concentrations, the drug reaches the first accessible sites whereas at higher concentrations, the second class of sites is filled according to a cooperative process. For both drugs, the maximum binding obtained at saturating concentration was about 2 nmol per 10^8 bacteria, which is in agreement with the value reported for ethidium bromide (Lambert & Le Pecq, 1984). In the TA 98 strain, dGly-NMHE and dVal-NMHE interact with bacterial nucleic acids in a similar way, suggesting a cooperative binding model involving a single class of binding sites. However, in this case, the values of maximum binding at saturating concentration of drug were found to be markedly different (0.35 nmol/10^8 bacteria for dGly-NMHE and 1.80 nmol/10^8 bacteria for dVal-NMHE). Strictly, the appearance of the two classes of binding sites in the BL 101 strain and the cooperative processes in both strains require integrity of the bacteria. As shown in Figure 5c,d, the Scatchard plots obtained from the fluorescence titration curves of disrupted bacteria by dGly-NMHE and dVal-NMHE exhibited in all cases concave upward curved plots, yielding quite similar binding constants in the range of 10^6 M^{-1} and a similar maximum number of binding sites at saturating concentrations of drug.

Discussion

Among the different possibilities which may account for the relation between the antibacterial properties of the AA-NMHE adducts and their hydrophobic properties, two hypotheses have been retained: (i) The increase in the hydrophobic character of AA-NMHE could be associated with an increase in the transport of the drugs across cell membranes (Hansch, 1971). (ii) The increase in the hydrophobic character

of AA-NMHE may result in the facilitation of the accessibility to bacterial nucleic acids. In this field of investigation, we have first of all characterized the fluorescence properties of AA-NMHE free in aqueous solution and bound to double-stranded nucleic acids. These fluorescence properties have been used to determine various DNA binding parameters, and the following results have been obtained: (1) All AA-NMHE interact with DNA through an intercalating process as revealed by DNA to drug energy transfer. (2) The DNA binding constants and the number of DNA binding sites are quite similar for all AA-NMHE. (3) AA-NMHE exhibit no sequence specificity toward the synthetic homopolymers poly[d(A-T)] and poly[d(G-C)].

These results clearly indicate that in the series of AA-NMHE neither the hydrophobicity nor the steric bulkiness of the linked amino acid significantly affects the in vitro DNA binding parameters of the drugs as previously suggested (Auclair et al., 1984).

The fluorescence properties of the AA-NMHE have been further used to investigate their uptake and their quantitative binding to bacterial nucleic acids according to the procedure previously described for ethidium bromide (Lambert & Le Pecq, 1984). In the case of AA-NMHE, the suitability of this technique was supported by the following observations: (1) In intact bacteria, the excitation spectra of the drugs in the UV region are consistent with the occurrence of energy transfer from the bacterial nucleic acids to the drugs. (2) The fluorescence lifetimes of AA-NMHE bound to DNA in vitro and bound to bacterial nucleic acids in intact cells are quite similar. (3) In the presence of disrupted bacteria, the fluorescence increment was completely suppressed following the addition of RNase plus DNase, indicating that the possible interaction of the drugs with various hydrophobic components of the cells, including membrane fragments, does not result in a detectable fluorescence increment.

Therefore, it can be stated that the fluorescence increment of AA-NMHE occurring in the presence of bacteria results from the uptake of the drugs followed by their binding to bacterial nucleic acids. From this event, the following three parameters can be determined: (i) the kinetics of access of the drugs to bacterial nucleic acids; (ii) the amount of drugs bound to bacterial nucleic acids at the steady state; (iii) the amounts of drugs bound to bacterial nucleic acids at various concentrations which allow estimation of the qualitative way of AA-NMHE binding to nucleic acids in vivo.

The kinetics of the access of the drugs to nucleic acids investigate the rate of equilibration of the drugs across cell membranes and their rate of equilibration with their fluorescent sites inside the cells. Data obtained show that in all cases these kinetics are first order. From a pharmacological point of view, the different values of the rate constants can be considered in the same order of magnitude. This indicates that neither the rate of diffusion of the drugs across cell membranes nor the rate of their binding to the accessible sites was a limiting step controlled by the hydrophobicity of the drugs. At the steady state, the amount of drug bound to the fluorescent sites (i.e., to the intercalating sites) reflects the accessibility of the drug to the bacterial nucleic acids. At a concentration of 1 μM, which is a dose yielding a strong difference in the antibacterial efficiency of the AA-NMHE (Auclair et al., 1984), a marked difference was observed in the accessibility of the drugs to bacterial nucleic acids in both BL 101 and TA 98 strains. The relative accessibility of the drugs to bacterial nucleic acids is related to their antibacterial activity as expressed in terms of ED_{50}. It must be pointed out that, in contrast with ethidium bromide (Lambert & Le Pecq, 1984), the accessibility of AA-NMHE to bacterial nucleic acids was not dependent on the energization of the bacterial membrane since neither the deprivation of oxygen nor the addition of cyanide (data not shown) affects the amount of drug bound to nucleic acids at the steady state. It can therefore be hypothesized that regulation of the accessibility occurs at nucleic acid levels. Scatchard plots of the binding of dGly-NMHE to BL 101 suggest the presence of two accessibility classes of binding sites which where sequentially filled as the concentration of drug increased. The sharp transition observed in the Scatchard plots reflects a cooperative effect in the binding of dGly-NMHE to bacterial DNA and can be due to the appearance of new binding sites for intercalation. This type of sequential filling has been previously described for the binding of ethidium bromide to chromatin (Paoletti et al., 1977). In this way, it has been suggested that the DNA in the bacterial chromosome is coiled in a nucleosome-like structure [see Pettijohn (1982) for a review]. In contrast, Scatchard plots of the binding of dVal-NMHE to BL 101 show a homogeneous filling of the binding sites as observed in the presence of purified DNA or disrupted bacteria. One of the possible explanations is that the lipophilic character of dVal-NMHE allows one to overcome a hydrophobic barrier which may exist at the bacterial nucleoid level. This phenomenon may account for the differential accessibility of these two drugs to *E. coli* nucleic acids at nonsaturating concentrations. At saturating concentrations of drug, the number of binding sites appears to be quite similar for both drugs. In the TA 98 strain the binding of dGly-NMHE and dVal-NMHE can be interpreted as a cooperative process involving in each case the interaction with a single class of binding sites. However, in that case, at saturating concentrations of drug, the number of binding sites is markedly lower for dGly-NMHE. The occurrence of two different models of ligand binding corresponding to each strain, BL 101 and TA 98, is in agreement with the hypothesis of Lambert & Le Pecq (1984) that the mutation in BL 101 is associated with modifications of the bacterial chromosome resulting in an increase in DNA accessibility. This property may explain the differential accessibility of AA-NMHE in the two strains and the marked higher antibacterial activity of the drugs toward BL 101 especially for the less hydrophobic compounds dGly-NMHE and dAla-NMHE.

From all these observations, we have the following three conclusions: (i) The different antibacterial activity of AA-NMHE is related to the different accessibility of these drugs to bacterial nucleic acids. (ii) The accessibility to bacterial nucleic acids is under the control of the hydrophobicity of the drugs. (iii) The increase in the hydrophobic character of the drugs increase their ability to reach a protected class of binding sites likely located in a nucleosome-like structure of the bacterial chromosome.

ACKNOWLEDGMENTS

We are very grateful to Dr. B. Lambert for providing the bacterial strain BL 101. We are indebted to Professor C. Paoletti for helpful discussions and critical evaluation of the manuscript.

Registry No. NMHE, 58337-34-1; dGly-NMHE, 89683-28-3; dAla-NMHE, 89683-32-9; dVal-NMHE, 89683-27-2; dLeu-NMHE, 89702-39-6.

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Adducts of the Antitumor Drug *cis*-Diamminedichloroplatinum(II) with DNA: Formation, Identification, and Quantitation†

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Received July 2, 1984

ABSTRACT: Salmon sperm DNA, treated with the antitumor agent *cis*-diamminedichloroplatinum(II) (*cis*-DDP), was enzymatically degraded to (oligo)nucleotides. Four Pt-containing products were identified by ^{1}H NMR after preparative chromatography on a diethylaminoethyl-Sephacel column at pH 8.8. In all identified adducts, comprising approximately 90% of the total Pt in the DNA, Pt was linked to the N7 atoms of the nucleobases guanine and adenine. The two major adducts were *cis*-Pt$(NH_3)_2$d(pGpG) and *cis*-Pt$(NH_3)_2$d(pApG), both derived from intrastrand cross-links of *cis*-DDP on neighboring nucleobases. Only the d(pApG) but not the d(pGpA) adduct could be detected. Two minor adducts were Pt$(NH_3)_3$dGMP, resulting from monofunctionally bound *cis*-DDP to guanine, and *cis*-Pt$(NH_3)_2$d$(GMP)_2$, originating from interstrand cross-links on two guanines as well as from intrastrand cross-links on two guanines separated by one or more bases. For analytical purposes we developed an improved method to determine *cis*-DDP adducts. Routinely, 40-μg samples of enzymatically degraded *cis*-DDP-treated DNA are now analyzed by separation of the mononucleotides and Pt-containing (oligo)nucleotides on the anion-exchange column Mono Q (FPLC) at pH 8.8 (completed within 14 min) and subsequent determination of the Pt content in the collected fractions by atomic absorption spectroscopy. The method was used to optimize the digestion conditions for *cis*-DDP-treated DNA. In kinetic studies on the formation of the various adducts, a clear preference of the Pt compound to react with guanines occurring in the base sequence d(pGpG) was established.

The antitumor drug *cis*-diamminedichloroplatinum(II) (*cis*-DDP)[1] is a frequently used chemotherapeutic agent; its working mechanism, however, is not fully clarified yet [for reviews, see Marcelis & Reedijk (1983) and Lippard (1982)]. It is generally accepted now that the antineoplastic activity of the drug is based on its interaction with cellular DNA leading to the formation of various types of adducts. In Figure 1 the hitherto known *cis*-DDP-DNA adducts are schematically depicted. Apart from these adducts, DNA–protein cross-links are formed by *cis*-DDP (Plooy et al., 1984), but these adducts are outside the scope of this study. Recently, we (Fichtinger-Schepman et al., 1982) and other investigators (Johnson, 1982; Eastman, 1983) reported methods to inventory the adducts in DNA by degrading *cis*-DDP-treated DNA, followed by chromatography of the resulting products.

† This work was supported by Grants MBL 79-1 and MBL 83-1 from the Koningin Wilhelmina Fonds (Dutch organization for fight against cancer), Amsterdam, The Netherlands, and by the Netherlands Foundation for Chemical Research (SON) with financial aid of the Netherlands Organization for the Advancement of Pure Research (ZWO), Grant 11-28-17.
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[1] Abbreviations: *cis*-DDP, *cis*-diamminedichloroplatinum(II); *cis*-DDP-DNA, salmon sperm DNA modified with *cis*-DDP in vitro; r_b, number of Pt atoms bound per nucleotide; r_t, total input drug–nucleotide ratio in solution; AAS, atomic absorption spectroscopy; ^{1}H NMR, proton nuclear magnetic resonance; FPLC, fast protein liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl.

0006-2960/85/0424-0707$01.50/0