STUDIES OF THE FLUOROPHORE SEMPERVIRENE AND ITS COMPLEXES WITH DNA

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ABSTRACT Evidence that the fluorophore sempervirene binds to nucleic acids is presented. The complexes were studied by fluorescence intensity, spectra, decay lifetime, and polarization methods. Both fluorescent and nonfluorescent complexes are formed. The sempervirene is rigidly fixed to DNA. If ethidium bromide and sempervirene are bound to DNA, energy can be transferred from sempervirene to ethidium. Sempervirene is taken up by mammalian cells and appears in the cytoplasm. This unusual new probe should be useful in molecular and cellular investigations.

INTRODUCTION

A number of fluorescent compounds which complex specifically with nucleic acids have been studied in recent years. These molecules are especially suited for use as probes or "reporter" molecules because their fluorescent characteristics alter in response to complexing and these changes allow the investigator to look into some of the otherwise inaccessible properties of nucleic acid biopolymers. Extensive investigations have been carried out with ethidium bromide in vitro (1–3) and in vivo (4), and with acriflavine, proflavine (5–7), and a few others.

The present paper describes studies with a new probe, sempervirene. This probe is chemically dissimilar to those which have been extensively investigated. It has been reported that sempervirene is an efficient inhibitor of cholinesterase (8) but other biological effects of this compound have been little studied. Among the compounds which have been reported to complex DNA the only one somewhat similar in structure to sempervirene is berberine (9). We have found that sempervirene complexes with DNA, RNA, and enters mammalian cells freely. The characteristics of DNA-sempervirene complexes are described and interpreted, and the interaction of ethidium and sempervirene in multiprobe complexes with DNA is reported.

METHODS

DNA was obtained from Miles Laborties, Inc. (Kankakee, Ill.) and was highly polymerized calf thymus DNA. Sempervirene nitrate was obtained from Mann Research Labs, New York. Ethidium bromide was from Boots Pure Drug Company, England.

Fluorescence spectra and polarization were determined with a Perkin-Elmer 203 spectrofluorometer (Perkin-Elmer Corp., Norwalk, Conn.), having excitation and emission monochromators and with modified filter holders to permit polarization measurements. For measurements at other than room temperature, a jacketed quartz cuvette was used in conjunction with a circulating precision water bath. Polarization filters were Polacoat PL40 (excitation) and MB105 (emission) (Polacoat, Inc., Cincinnati, Ohio). Polarization was obtained from the formula

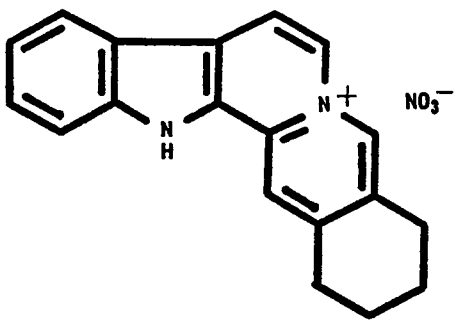
$$P = (I_{vv} - GI_{vH})/(I_{vv} + GI_{vH}),$$

where I_{VV} and I_{VH} are the measured fluorescence intensities with the analyzers vertically and horizontally oriented, and the correction factor $G = I_{HV}/I_{HH}$ is obtained with the incident beam horizontally polarized (10). Decay time measurements were made with the TRW nanosecond decay time fluorometer (TRW Instruments, El Segundo, Calif.) (11, 12), and Corning filters (Corning Glass Works, Corning, N.Y.) were used to select excitation and transmission bands.

RESULTS

Sempervirene Analysis

The structure and absorption spectrum of sempervirene nitrate have been determined by Ban and Seo (13). Ban and Seo also refer to this compound as 1,2,3,4-tetrahydro-13H-benz(g)indolo[2,3a]pyrido-colinium nitrate and give the structure as:



The absorption spectrum of the sample of sempervirene nitrate used in our studies was determined. Absorption maxima at wavelengths of 243, 248, 296, 348, and 387 nm were found in ethanol. These compare with maxima at 242, 248, 297, 346, 387 nm reported by Ban and Seo.

Excitation Spectra of Free and Bound Sempervirene

The excitation spectrum of free sempervirene in an aqueous solution containing 0.01 M NaCl and 10^{-3} M EDTA is shown in Fig. 1. Sempervirene concentration was 0.17 μg/ml and the emission was measured at the wavelength of maximum intensity,

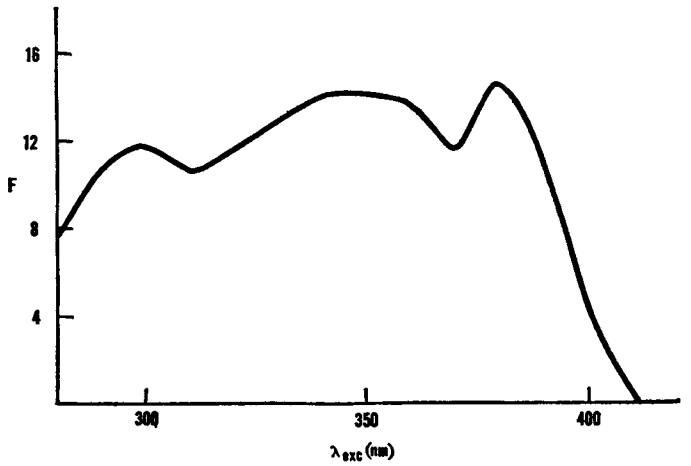


FIGURE 1 Excitation spectrum (corrected) of free sempervirene in 0.01 M NaCl. Sempervirene, 0.17 μg/ml. Emission wavelength, 437 nm. F is fluorescence intensity and λ_{exc} is excitation wavelength.

437 nm. This spectrum, and those of Fig. 2, were corrected for dependence of excitation intensity on wavelength by reference to a quinine sulfate standard (14). The excitation spectrum for $\lambda > 280$ nm shows maxima at 300, 345, and 380 nm. The corresponding absorption maxima measured for sempervirene in aqueous salt solution are 292, 340, and 380 nm.

Fig. 2 shows corrected excitation spectra for a DNA-sempervirene mixture in two different concentrations of NaCl. DNA concentration is 25 μg/ml, sempervirene is 0.17 μg/ml, and the nucleotide to dye ratio (N/D) is 168. The upper curve is for DNA-sempervirene in 10^{-3} M NaCl and the lower is for 10^{-2} M NaCl. In both cases 10^{-3} M EDTA is present to chelate divalent ion impurities. The fluorescent intensities F of Figs. 1 and 2 can be compared directly. The emission is measured at 435 nm, the emission maximum of DNA-sempervirene.

Comparing Fig. 2 and Fig. 1 it is seen that the fluorescence intensity of sempervirene complexed to DNA is much reduced compared with free sempervirene. At 380 nm, the intensity of free sempervirene is 4.5 times that of bound sempervirene. Another effect of DNA is to increase the intensity observed with excitation at 300 nm or shorter wavelengths relative to the intensity with long wavelength excitation. This effect is particularly pronounced in 10^{-3} M NaCl solution. Although DNA absorbs strongly at the shorter wavelengths the inner filter effect is small at the stated concentration of DNA and would, if significant, tend to reduce fluorescence intensity rather than increase it. Fluorescence enhancement at short wavelengths is more pronounced in 10^{-3} M NaCl than in 10^{-2} M NaCl. On the other hand this salt effect plays no part in the reduction in sempervirene fluorescence on binding when excitation is at 380 nm. At higher concentrations of salt than 10^{-2} M there is

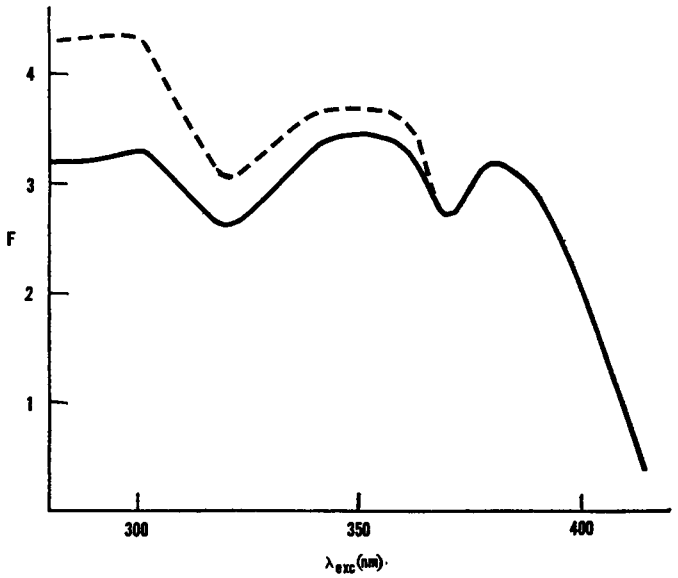


FIGURE 2 Excitation spectra (corrected) of sempervirene-DNA in 10^{-2} M NaCl (—) or 10^{-3} M NaCl (- - -). Sempervirene 0.17 μg/ml, DNA 25 μg/ml, N/D = 168. Emission wavelength 435 nm.

a progressive decrease in binding, as indicated both by intensity and polarization measurements, until in 0.5 M NaCl the presence of DNA has no effect on the fluorescence characteristics of sempervirene. High salt concentration then appears to prevent complexing of any type between sempervirene and DNA.

It is noteworthy that the excitation maximum at 380 nm is not shifted measurably when sempervirene binds to DNA. The absorption maximum in aqueous salt solution is, however, shifted to 395 nm. The extinction coefficient at 380 nm is 1.6 times greater for free than for bound sempervirene.

An investigation of possible interaction between sempervirene and RNA or bovine serum albumin (BSA) was also made. Sempervirene in 0.01 M NaCl appears to complex to rRNA because a decrease in fluorescence intensity and an increase in polarization similar to those found with DNA are observed. Sempervirene in the presence of high concentrations of BSA (1%) shows no fluorescence changes and therefore apparently does not complex to this protein.

Emission Spectra of Free and Bound Sempervirene

Fig. 3 shows uncorrected emission spectra for free sempervirene at 0.17 μg/ml and for sempervirene plus DNA at 25 μg/ml. Both are in aqueous solution with 10^{-3} M NaCl, 10^{-3} M EDTA, and excitation at 380 nm. Fluorescence intensity is presented as percent of intensity at 435 nm in order to show up the small spectral shift, and

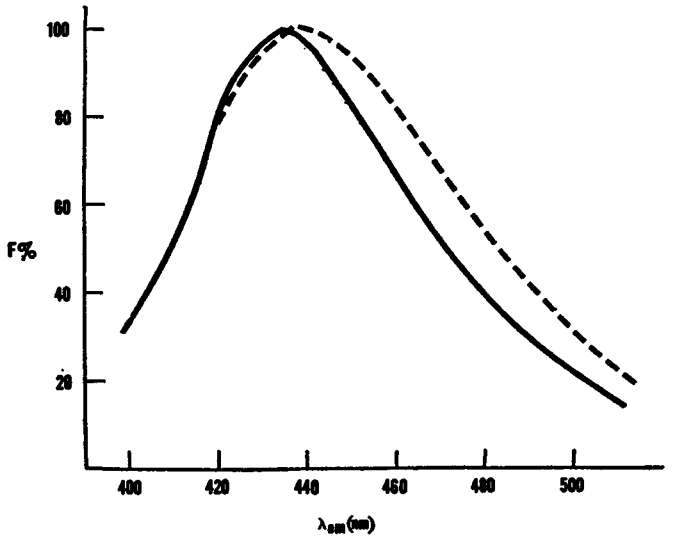


FIGURE 3 Emission spectra of free sempervirene and DNA-sempervirene in 10^{-3} M NaCl. Concentrations as in Figs. 1 and 2, excitation wavelength, 380 nm. Curves are normalized to 100 at λ_{em} = 435 nm. Free sempervirene (- - -), DNA-sempervirene (—).

$F\%$ is not comparable to F in Figs. 1 and 2. Binding of sempervirene to DNA shifts the emission maximum 2 nm lower. With excitation at wavelengths less than 380 nm and down to 280 nm the same 2 nm shift was found.

Fluorescence Decay Lifetimes of Free and Bound Sempervirene

Decay times were determined with the TRW nanosecond flash apparatus with D_2 lamp for excitation and Corning 7–54 filter in the excitation beam. The emission filter was a Corning 3–75. Decay times were measured for free sempervirene and for DNA-sempervirene with N/D = 7, 14, 28, 56, 112, and 168, and in 10^{-3} M NaCl or 10^{-2} M NaCl. All values of decay time obtained fell within the range 6.5±0.5 ns. Within the accuracy of the measurements, there is no significant effect of binding on sempervirene decay time.

Polarization of DNA-Sempervirene Fluorescence

Fig. 4 shows the polarization of fluorescence from sempervirene in the presence of DNA as a function of the nucleotide to dye ratio, N/D. The concentration of DNA was fixed at 25 μg/ml (except for N/D = 0, where DNA is absent) and the sempervirene concentration was varied to obtain different N/D. Excitation was at 380 nm and emission at 435 nm. The upper curve is for 10^{-3} M NaCl 10^{-3} M EDTA solution and the lower is for 10^{-2} M NaCl 10^{-3} M EDTA solution.

The polarization of free sempervirene (N/D = 0) is close to zero, and P in-

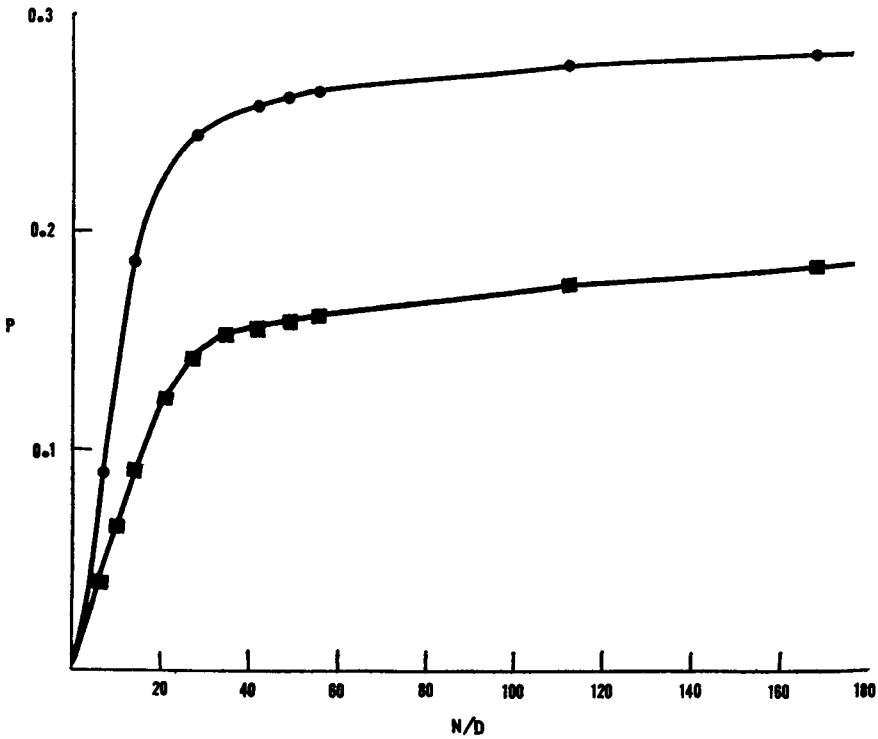


FIGURE 4 Polarization P vs. nucleotide to dye ratio N/D for sempervirene-DNA in 10^{-2} M NaCl (■) and in 10^{-3} M NaCl (●). λ_{exc} = 380 nm, λ_{em} = 435 nm.

creases rapidly between N/D = 0 and 20. In this region there is apparently both bound and free sempervirene so that the observed value of P is an average. Above N/D = 40 the P values increase only slightly as N/D is further increased, indicating that essentially all the sempervirene is bound. The maximum values of P obtained at N/D = 168 were 0.281 and 0.185 for 10^{-3} M NaCl and 10^{-2} M NaCl, respectively. The lower value of P in the higher salt concentration suggests that the sempervirene is bound in a more flexible way. As will be shown in a subsequent section, the maximum value of polarization for sempervirene, or P_o, has been measured as 0.27 for free sempervirene in viscous sucrose solution. Hence, the binding of sempervirene to DNA in 10^{-3} M NaCl appears to be rigid—that is, the molecule does not rotate appreciably during the period of fluorescence emission. At higher salt concentrations than 10^{-2} M, P declines rapidly until in 0.1 M NaCl P approaches zero, indicating free sempervirene is responsible for the fluorescence and virtually none of it is bound.

Perrin Plots for Free Sempervirene

The Perrin relation states that the reciprocal of polarization is linearly dependent on T/η (where T is absolute temperature and η is viscosity). Fig. 5 is a plot of $1/P$

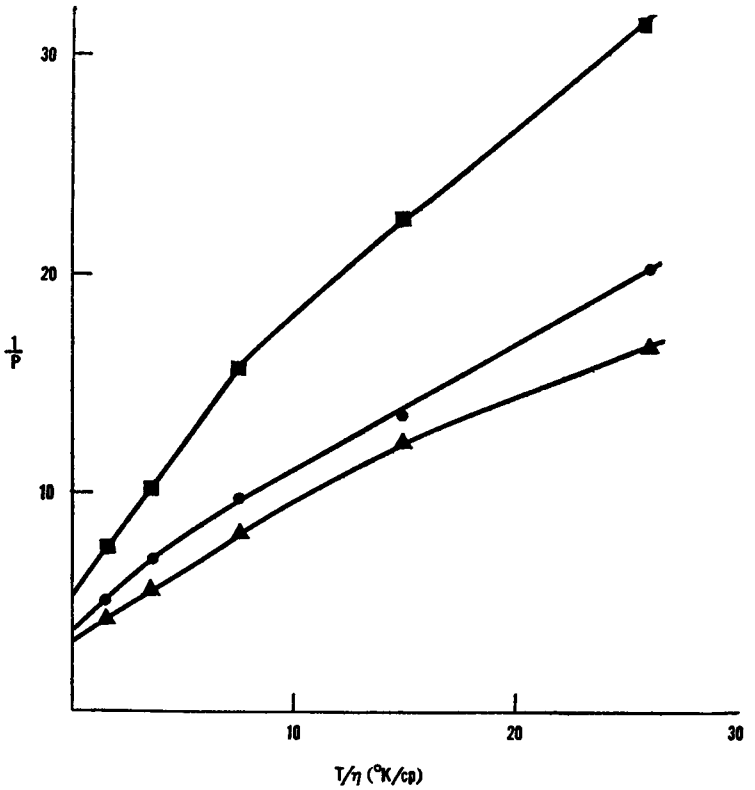


FIGURE 5 Perrin plots for free sempervirene. λ_{exc} = 380 nm (▲), λ_{exc} = 280 nm (●), λ_{exc} = 260 nm (■). λ_{em} = 437 nm for all. cp, centipoise. See text for details.

vs. T/η for free sempervirene in 10^{-3} M NaCl 10^{-3} M EDTA. The three curves correspond to excitation at 260, 280, and 380 nm; emission was measured at 437 nm in each case. Temperature was fixed at 300° K and η was varied by means of sucrose addition. The relationship is clearly not linear in the case of excitation at 260 nm. There is less indication of nonlinearity for excitation at the longer wavelengths. Polarization is much lower with excitation at 260 nm than it is for excitation at 280 or 380 nm. The P_o values (for T/η = 0) are 0.16 for 260 nm and 0.27 for 380 nm.

Simultaneous Binding of Sempervirene and Ethidium Bromide to DNA

The fluorophore ethidium bromide (EB) complexes with DNA and has been extensively studied (2, 3). EB is known to intercalate in DNA, energy transfer from DNA to EB occurs, binding characteristics have been determined, and the fluorescent properties of the complex studied. It was of interest therefore, to see if sempervirene competed with EB for binding sites and to see if interactions such as dye-to-dye energy transfer would occur. Bound EB has an excitation maximum at 500 nm, as well as at 300 nm, and an emission maximum at 585 nm. These maxima differ

from those of sempervirene sufficiently so that EB or sempervirene can be selectively excited in a mixture and the emission from one or the other selectively recorded.

The experimental design involved use of high N/D (=168) or low N/D (=28) sempervirene-DNA in combination with high N/D (=52) or low N/D (=13) EB-DNA. Measurements of fluorescence intensity, polarization, and decay times were made. The results are tabulated in Table I. Intensity and polarization were determined for three combinations of excitation and emission wavelengths: Column A is for excitation at 380 nm and emission at 437 nm, column B for excitation at 500 nm and emission at 585 nm, and column C for excitation at 380 nm and emission at 585 nm. The fluorescence intensity values have not been corrected for variation with excitation wavelength. Therefore intensities should not be compared horizontally in the table but may be compared vertically. The polarization values can be compared horizontally as well as vertically; blanks appear where intensities were too low to permit calculation of polarization. Decay time was measured with excitation filter CS 7-54 and emission filter CS 5-59 in place. This combination results in detection of emission from sempervirene only.

Analysis of the results is difficult and must take account of the facts that sempervirene fluorescence increases when it is freed from complexing while EB fluorescence decreases when it is freed, and that fluorescence intensity could also be decreased by dye-to-dye quenching between the complexes. Free sempervirene and EB in solution do not quench each other. Polarization could be changed by quenching or energy transfer or by shift of the dye from complexed to free state.

The results of intensity and decay time measurements indicate that sempervirene

TABLE I

FLUORESCENT CHARACTERISTICS OF COMPLEXES OF DNA WITH ETHIDIUM BROMIDE AND SEMPERVIRENE

N/D		Polarization			Fluorescence intensity			Decay time
Semper-virene	EB	A	B	C	A	B	C	
								ns
168	52	0.244	0.287	0.070	31	46	19	4.1
168	13	0.016	0.202	0.053	38	172	56	4.1
28	52	0.160	0.280	0.093	139	56	61	6.0
28	13	0.001	0.193	0.062	430	165	115	6.5
168	—	0.278	—	—	51	0	0	6.3
28	—	0.221	—	—	253	0	0	6.6
—	52	—	0.283	0.043	2	56	15	—
—	13	—	0.203	0.043	2	173	44	—

A, excitation 380 nm, emission 436 nm; B, excitation 500 nm, emission 585 nm; C, excitation 380 nm, emission 585 nm. Fluorescence intensity is not corrected for variation with excitation wavelength. Decay time of sempervirene complex is tabulated.

fluorescence at sempervirene N/D = 168 is partly quenched by the presence of EB. Sempervirene at N/D = 28 however, seems to be extensively displaced from its sites on DNA by EB. Looking now at EB fluorescence it is seen that when EB is excited directly (column B) sempervirene has little or no effect. This fits in with the deduction that EB has greater affinity for DNA than sempervirene and that it binds to nearly the same extent whether or not it must displace sempervirene to bind to DNA. Column C, however, shows that EB emission at 585 nm increases in intensity several fold when sempervirene is present at N/D = 28 and excitation is at 380 nm. This strongly suggests energy transfer from sempervirene to EB (which fits in with the quenching of sempervirene fluorescence) and reemission of the energy as EB fluorescence. Also, it was found that the reemitted light from EB (column C) is depolarized relative to polarization of sempervirene fluorescence (column A), and this would be expected to result from energy transfer. Direct excitation of EB at 380 nm (column C) in the absence of sempervirene gives a lower value of polarization as well as a much lower intensity than is obtained with sempervirene present at N/D = 28.

Effects of Sempervirene on Living Cells

Mammalian cells in tissue culture were used in these studies, both bovine bone marrow cells and baby hamster kidney (BHK) cells. Both types of cell took up sempervirene readily. Much of the sempervirene in the cell leaked out quickly when the cells were placed in medium lacking sempervirene. Part was complexed in the cell and the measured polarization of this fraction was 0.10. Exposure to 5 μg/ml sempervirene for 2 h did not inhibit subsequent growth in sempervirene free medium. Cells continuously in 1 μg/ml sempervirene showed light inhibition of growth and this effect increased with concentration until inhibition was complete in 5 μg/ml.

Bovine bone marrow cells were observed in a fluorescence microscope. The sempervirene fluorescence appeared throughout the cell, except for the nucleus.

DISCUSSION

The fluorescent properties of the complex between sempervirene and DNA are complicated in some respects but it is possible to draw some conclusions from the available data. Firstly, there is a 4.5-fold decrease in fluorescence intensity when sempervirene complexes to DNA but only a 1.6-fold decrease in extinction coefficient. The major part of the decrease then would appear to result from a decreased quantum yield. However, quantum yield in theory is proportional to decay lifetime and this is essentially the same for free and bound fluorescent sempervirene. Furthermore, although the excitation maximum does not shift on binding, the absorption maximum does shift. Since the absorption and excitation spectra of the complex differ, this implies that there are at least two absorbing species. The excitation spectrum is unshifted on binding, indicating that the fluorescent species' absorption

spectrum is not shifted on binding. The constancy of decay lifetime shows that the rate constant for deactivation of the excited state is unaffected by binding, and this in turn implies that the rate constants for fluorescence and for radiationless decay are unchanged for the fluorescent species. From this it follows that there is a fluorescent species with the same rate constants, quantum yield, and absorption spectrum in the free and bound forms, and any number of nonfluorescent bound forms with maximum absorption at longer wavelengths. Assuming then that the extinction coefficient of the fluorescent complex is the same as for free sempervirene, it is estimated that there are 3.5 nonfluorescent complexes for each fluorescent complex (at high N/D). This behavior is similar to that of proflavine. Proflavine is thought to form three nonfluorescent complexes per each fluorescent complex (5–7). Proflavine and sempervirene show very little structural similarity and the basis for an apparent similarity in complexing behavior is not clear.

The fluorescence polarization data provides further information on the fluorescent complexes. Polarization approaches maximal values at N/D = 50 or so, and further increase in N/D causes little change. If the complex is in 10^{-3} M NaCl the maximum value of polarization is the same as that for free sempervirene in very high viscosity solution (0.16 for 260 nm excitation and 0.27 for 380 nm excitation). This indicates rigid binding of sempervirene to the DNA polymer.

Polarization is maximum (P_o) for excitation of DNA-sempervirene at 260 nm and at 380 nm, although DNA absorbs strongly at 260 nm but not at 380 nm. If a significant fraction of the fluorescent light emitted was transferred from DNA to the complexed sempervirene, depolarization would be expected for 260 nm excitation. There is however the possibility that the fluorescence transition dipole moment could be oriented substantially parallel to the DNA absorption dipole moment, and this could lead to some degree of polarization of radiation resulting from energy transfer. This would be consistent with the observation that bound sempervirene has a higher ratio of fluorescence intensity with 260 nm excitation to intensity with 380 nm excitation than does free sempervirene.

A salt concentration of 10^{-2} M reduces the polarization of bound sempervirene without dissociating the complex. Still higher salt concentrations begin to dissociate the complex. This suggests that the fluorescent complex binding is electrostatic in nature. Dyes that are known to intercalate, such as proflavine and ethidium bromide, are relatively unaffected by salt concentrations of 0.5 M or less, although electrostatic interaction can also occur for ethidium bromide in very low salt concentrations. A recent paper (15) has suggested that only the nonfluorescent sites of proflavine-DNA are stabilized by intercalation. This may be the case also for sempervirene.

The experiments with sempervirene and ethidium bromide together show that these fluorophores can bind to DNA simultaneously. At least some of the sites on DNA can be occupied by either fluorophore since EB can displace sempervirene from DNA. Complexed EB can quench fluorescence from complexed sempervirene,

but the reverse does not occur. Energy transfer from fluorescent sempervirene complexes to EB complexes seems to occur.

Our studies so far show that sempervirene is a fluorophore with properties that should prove useful in nucleic acid and cellular research. Further work is planned to determine additional characteristics of the compound and its complexes.

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