PYRIMIDINE ADDUCT FLUORESCENCE IN UV IRRADIATED NUCLEIC ACIDS

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ABSTRACT: The fluorescence properties of the pyrimidine photoadducts were studied. The observation of similar spectra in UV irradiated DNA's and polynucleotides implies that the formation of adducts may be monitored in biological systems by their emission signals. The fixed ratio of photochemical yields between adducts and other lethal products also suggests the use of adduct fluorescence to assay directly the degree of photodamage in DNA.

Much evidence suggests that pyrimidine dimers and hydrates as photoproducts in nucleic acids are responsible for lethal and mutagenic effects of uv irradiation on living organisms.[1] Recently, a third class of photoproducts, the pyrimidine adducts, from DNA,[2] RNA[3], and their pyrimidine bases[4] and nucleotides[5] have been characterized. Although the formation of adducts *in vivo* has been detected at $<10,000$ ergs/mm,[2] assay depends on acid hydrolytic procedures which may alter unstable primary products.[2] This general difficulty also applies to dimers and hydrates. Thus, it is highly desirable to develop an assay for *direct* detection and identification of photodamage in irradiated but otherwise intact DNA at low uv doses. Taking advantage of the fluorescence observed for all these adducts[4,5] and for irradiated dinucleotides and nucleic acids,[2] a potentially fast and sensitive method for estimating directly the adduct and indirectly the dimer production in DNA is suggested.

Material and Methods

Derivatives of pyrimidinone were prepared.[8] Adducts 1 to 6 and 8 (see Table) were previously prepared,[4,5] while 7 was isolated in a similar manner. Ammonium salts of dC-dT, and dC-dC were gifts of Prof. R. L. Letsinger.[9] dT-dC, dT-dT (P-L Biochemicals) and calf thymus DNA (Sigma) were used without

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further purification. H. Influenzae DNA[10] was a gift of Prof. R. M. Herriott.

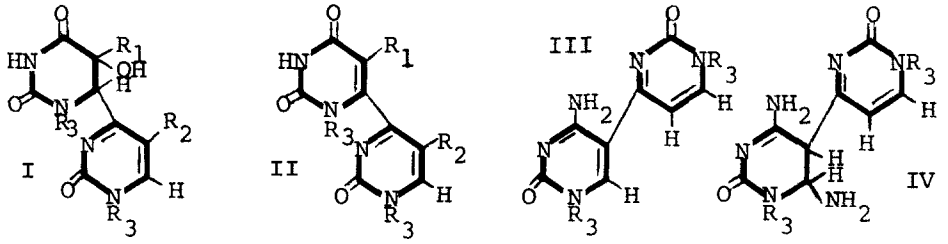
Absorption spectra were taken on a Varian model 635 and fluorescence spectra were recorded on an Aminco-Bowman SPF equipped with a 1P28 Photomultiplier tube. Two Perkin-Elmer Interpolating Vernistats and an Adjustable Function Generator were appended to correct the SPF output for its spectra sensitivity and variation in lamp output.[11] Fluorescence quantum yields were calculated relative to the corrected emission area vs. wavenumber of 1 μM diphenyloxazole (ϕ_f=0.83)[12] in nitrogen-flushed cyclohexane including a correction for the index of refraction. Water having no detectable fluorescence impurities at the highest sensitivity settings of the SPF was obtained by passing distilled water through a Continental Water Conditioning Corporation deionizing system. UV irradiations were carried out in a large monochromator[13] (a generous gift of Prof. Max Delbrück) with water dispersing elements and a PEK 1,000 watt air cooled mercury lamp. Dose rates (15-70 ergs/mm^2/sec) were measured by ferrioxalate actinometry.[14]

Fluorescence Properties of Pyrimidine Adducts

Fluorescence emission spectra and maxima (λ_f^{max}, corrected values in Table) for each adduct are independent of excitation energy over the low energy absorption manifold. These maxima appear either in the 375-390-nm region or around 450 nm. Maxima in the former region for compounds 1, 3, 6 to 8 although slightly red-shifted, agree in general shape and width with emission from the three pyrimidinones. This confirms previous molecular structural assignments[2,15] and the suggestion that the adduct chromophore resides in Pyo.[2-5] The spectral irregularity of compounds 4 and 5 are not critical to our conclusions but require additional consideration which will be presented elsewhere.

Excitation spectra are independent of emission wavelength within at least ±30 nm of λ_f^{max}, implying a single fluorescence excited state in each case. Moreover, the close agreement between absorption maxima (Table) and excitation maxima (λ_{ex}^{max}) confirms the authenticity of the fluorescence and makes it un-

likely that an impurity emission is involved. Fluorescence quantum yields (ϕ_f) exhibit one useful trend: adducts of structural type II or III that have undergone dehydration or deamination subsequent to adduct formation have $\phi_f < 3x10^{-3}$, while those of type I or IV have $\phi_f > 3x10^{-3}$.



Fluorescence Properties of Irradiated Polynucleotides

With the above information, a study of the appearance of similar emission signals upon irradiation of four dinucleotides and two DNAs (Table and Fig.1)

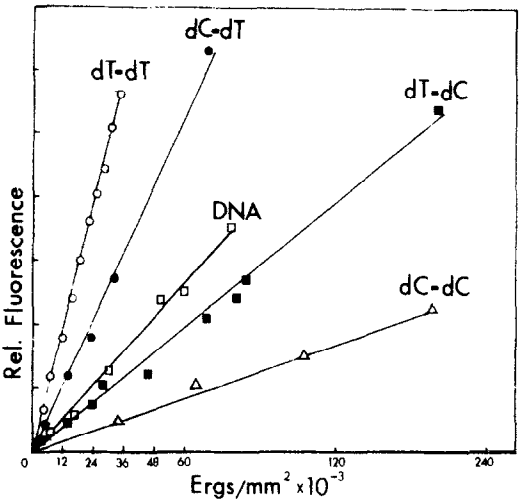


Figure 1. Production of photoadducts in nucleotides and DNA as a function of absorbed dose at 265 nm (2.4 nm bandwidth) in a stirred 1 cm cuvette at 27°C. Fluorescence was monitored at 400 nm with excitation at 310 nm in each case.

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was made. All exhibit new fluorescences that have λ_{ex}^{max} and λ_f^{max} in relatively good agreement with those for the isolated adducts. The action spectrum for fluorescence produced in calf thymus DNA (Fig. 1) is in reasonable agreement

TABLE: Fluorescence Properties of Pyrimidinones, Pyrimidine Adducts and Irradiated Polynucleotides.

Compound	λ_{ex}^{max} (nm)	λ_{f}^{max} (nm)	$\phi_f \times 10^3$
	Pyrimidinones#		
Pyo	295 (295)*	371	6
m^5Pyo	313 (310)	371	100
m^5Pdo	317 (315)	372	52
	Adducts#		
1 (I; R_1=R_2=CH_3; R_3=H)	317 (315)	388	72
2 (II; R_1=R_2=CH_3; R_3=H)	315 (319)	385	1
3 (I; R_1=CH_3; R_2=R_3=H)	307 (306)	375	99
4 (II; R_1=CH_3; R_2=R_3=H)	318 (313)	457	<1
5 (II; R_1=R_2=R_3=H)	308 (300) 338 sh (335)	445	3
6 (III; R_3=H)	297 (297) 321 (321)	385	1 (0.8)[5,+]
7 (I; R_1=R_2=CH_3; R_3=dR)	309 (309)	378	26
8 (III; R_3=dR)	328 (329)	391	1
	Irradiated Polynucleotides		
dT-dT	323 (325)	400	12(1.5) (1.2)[6]
dT-dC	310 (306)	405	5(0.8)
dC-dT	319 (317)	390	12(1.4)
dC-dC	297 (300) 325 sh (327)	405	3(1.1)
H. Influenzae DNA	318 (316)	410	6(0.9)
Calf Thymus DNA	317 (315)	411	5(0.8)

*The values given in ( ) are uv absorption maxima in nm.

#The following symbols are derived from the Recommendation of the IUPAC-IUB Commission on Biochemical Nomenclature [J. Mol. Biol., 55, 299 (1971)]. This usage has been discussed with Dr. Waldo E. Cohn, Director of the Office of Biochemical Nomenclature of the National Research Council and with Dr. Nelson J. Leonard, University of Illinois [as in J. Am. Chem. Soc., 94, 6178 (1972)]. Pyo is the symbol for 2-pyrimidinone, m^5Pyo for 5-methyl-Pyo and m^5Pdo for its deoxyribosyl derivative. Compound (1) is m^5Pyo(4-6)o^5hThy where 4-6 indicates the position of covalent linkage between the two moieties, o^5 stands for 5-hydroxy and h for dihydro. Similarly, (2) is m^5Pyo(4-6)Thy, (3) is Pyo(4-6)o^5hThy, (4) is Pyo(4-6)Thy, (5) is Pyo(4-6)Ura, (6) is Pyo(4-6)Cyt, (7) is m^5Pdo(4-6)o^5hdThd and (8) is Pdo(4-5)dCyd.

+The values given in ( ) are $\phi_A \times 10^3$.

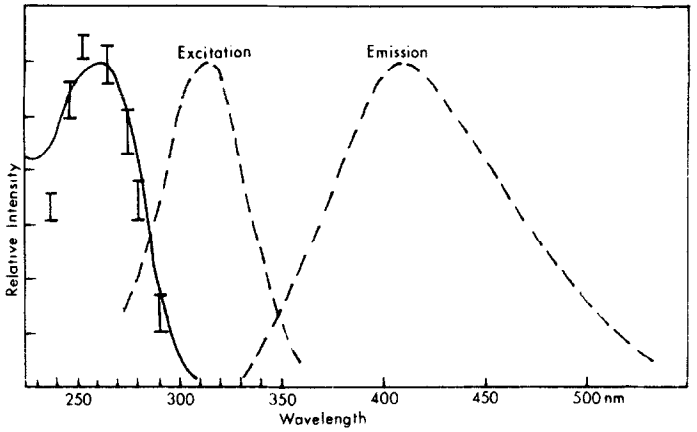

Figure 2. Spectral properties of calf thymus DNA (A_{260}=1.0 in 0.1 M NaCl) irradiated at 265 ± 5 nm to a total dose of 1.5×10^5 ergs/mm^2. Dashed curves are the corrected relative fluorescence excitation spectrum (emission monitored at 400 nm) and emission spectrum (excitation at 310 nm) produced during the irradiation. Solid curve is a relative % absorption spectrum at 2 nm resolution before photolysis and the bracketed lines are relative rates of 400 nm fluorescence increase as a function of incident wavelength (2.4 nm bandwidth) and constitute an action spectrum for its production.

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with its relative absorption spectrum thus eliminating the possibility of fluorescence due to photochemical reaction of an impurity and confirming that the initial step is light absorption by DNA. These properties strongly suggest the formation of photoadducts in these nucleotides, especially since fluorescence is not known for pyrimidine dimers or hydrates. Resultant ϕ_f's are all $>3 \times 10^{-3}$ and, in comparison with compounds containing a saturated pyrimidine moiety, (I) and (IV), are consistent with the notion that adducts do not spontaneously dehydrate or deaminate. Thus, the new emission is probably due to the production of photoadducts (I) and (IV). However, the possibility of micro-environmental factors resulting from polymeric DNA structure which may increase the ϕ_f of unsaturated adducts cannot be eliminated.

The close spectral similarity of adducts from dT-dT, dT-dC, dC-dT and dC-dC (2 positional isomers of each due to 3'→5' and 5'→3' phosphodiester linkages are possible) precludes resolution of DNA emission into its components. However, the similarity of ϕ_f's for irradiated dT-dT and dC-dT, and for dT-dC

and dC-dC suggests that the fluorescent moiety in the adducts for the former pair is m^5Pyo and for the latter is Pyo. All these pyrimidinones are 5'-phosphoric esters. Coupling this deduction with the similar ϕ_f's for irradiated DNAs, dT-dC and dC-dC all being close to Pyo itself, implies that adducts are formed in DNA from predominately thymidylyl or cytidylyl(3'-5')cytidine sequences. This is consistent with the only adduct (compound 4) so far isolated from DNA[2] and may also explain the lower quantum yields of cytidine dimerization relative to adduct formation observed in poly (rC).[5]

One important question that can be answered from this investigation is whether adducts are produced in DNA as primary stable species. A preliminary kinetic study (Figure 1) shows that the initial rates of fluorescence appearance in every case are unaltered over a significant dose range and extrapolate to the origin at the start of irradiation. This behavior would not be observed if the adducts were not formed from a single photochemical event; it is consistent with the suggestion that unstable oxetanes, azetidines or thietanes formed initially, will readily rearrange to the stable adduct.[2-6]

Proposed Assay for Nucleic Acid Damage

Photoadduct fluorescence is unique among known DNA photoproducts. Since DNA itself gives no interfering fluorescence this emission appears ideally suited as a convenient assay of uv damage in DNA. A qualitative measure can be gained simply by monitoring the fluorescence intensity after or concurrently with uv exposure since DNA absorption will not mask this effect and the ϕ_f's and adduct quantum yields[16] are sufficient to afford detection at biological doses. At such doses, adduct formation (or any photoproduct formation) should constitute a fixed fraction of all absorption events. Several lines of evidence provide experimental confirmation. Analysis of the acid hydrolysates of 253.7 nm irradiated E. coli DNA indicates about a 9:1 dimer:adduct ratio.[2] Photoreactivation, which is known to repair only dimers, gives a maximal recovery of 90%[17], also implying about 10% non-dimeric lethal photoproducts. From our data, the quantum yields of adduct formation can be estimated ($\sim 10^{-3}$)

and are an order of magnitude lower than reported photodimer yields[1b], in agreement with the two other independent estimates. Therefore fluorescence intensity also reflects total photoproduct content, the molecular source of DNA inactivation. Since the ratio between adducts and all products is expected to vary with the source of DNA and since base composition effects the relative abundances of photoproducts[18] including the adducts, a quantitative measure of inactivation would then require a preliminary determination of the relation between uv dose or inactivation and adduct fluorescence for each DNA of interest

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16. ϕ_A's were determined by irradiating dT-dT at 265 nm to a given quantal absorbed dose (D_{265}). Dimers were reversed by a short irradiation at 237 nm (D_{237}). The net loss in dT-dT (corrected for the small steady state dimer concentration at 237 nm, ref. 6) determined by absorbancy loss, was equated

with adduct production and an adduct extinction coefficient (ε_{325}) was determined from this concentration and the absorbancy at 325 nm (A_{325}) using Beer's Law. The resultant $\varepsilon_{325}=4.9\times10^{3}$ agrees with an $\varepsilon_{325}=4.83\times10^{3}$ calculated previously for this product although a different structure was assigned (ref. 6). Thus, Φ_A for dT-dT was determined as

$$\Phi_A(\text{dT-dT}) = \frac{A_{325} \cdot N}{\varepsilon_{325} \cdot (D_{265} + D_{237})}$$

where N = Avagadro's number. Other Φ_A's were determined relative to Φ_A(dT-dT) utilizing the adduct emission excited at 310 nm. Since the spectra are not identical for each adduct, corrected emission areas (B) were used. Thus

$$\Phi_A = \Phi_A\,(\text{dT-dT}) \cdot \frac{\Phi_f(\text{dT-dT})}{\Phi_f} \cdot \frac{B}{B\ (\text{dT-dT})} \cdot \frac{D(\text{dT-dT})}{D} \cdot F$$

where F is a factor determined from adduct absorption spectra and corrects for the relative absorption at 310 nm assuming that all adducts have approximately equal extinction coefficients at their absorption maxima.

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