Photochemistry and photophysics of guanines
by James Paul Morgan

A thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Chemistry
Montana State University
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Abstract:
Guanine and several derivatives undergo photochemistry in glycol-water glasses, below -100°C. The photochemistry can be sensitized with light of ?'s > 300 nm when acetone is added. The product is not photoreversible, and the molar extinction coefficients are 19.4xl03, 3.7xl03, and 8.3x103 at 225 nm, 265 nm, and 305 nm maxima. The product fluorescence maximum is at 418 nm. The fluorescence yield is 0.35 at -112°C in the 7:3 glycol-water glass. The photochemical quantum yield is 2% exciting at GMP's absorption maximum, and increases to more than 4% when exciting at ?'s > 285 nm. Wavelength dependent effects are seen for the luminescence yields of the derivatives studied. Cu2+, Co2+, and Ni2+ show evidence for com-plexation with GMP in the absorption and severely reduce the photochemical and luminescence yields.

The product reverts to the parent compound when warmed rapidly to room temperature. If the product is warmed slowly in the -85°C to -74°C region, a conversion to a stable product with a 360 nm fluorescence maximum results. At warmer temperatures, ~-70°C, only the stable product is produced. The 360 nm fluorescent product can be photosensitized at room temperature via acetone, even in t-RNA.

A photophysical, photochemical study of GMP containing dinucleotides was made. F, 3t, and rate studies indicated that: singlet electronic transfer in CpG and dpGpT is efficient; GpA, GpU, and GpC are partially in the form of exciplexes and the exciplexes intersystem cross as the nucleotide with the lower energy singlet. Photochemical and luminescence yields in ApG and GpA are nearly that of GMP, but are much less in CpG, GpC, UpG, and GpU.

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All computer assisted calculations cited in the thesis are the handiwork of Rob Wilson, my lab partner during graduate study. In addition to the numerous computer programs provided by Rob, he was a collaborator in defining the optical properties of molecules which were of mutual interest. An even disposition and a selfless attitude by Rob made my tasks easier.

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ABSTRACT

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A photophysical, photochemical study of GMP containing dinucleotides was made. Φ, $^3\tau$, and rate studies indicated that: singlet electronic transfer in CpG and dpGpT is efficient; GpA, GpU, and GpC are partially in the form of exciplexes and the exciplexes intersystem cross as the nucleotide with the lower energy singlet. Photochemical and luminescence yields in ApG and GpA are nearly that of GMP, but are much less in CpG, GpC, UpG, and GpU.

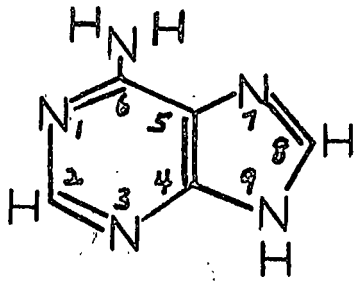
INTRODUCTION

The Molecules

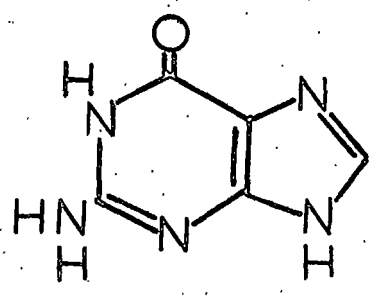
The chromophores responsible for photophysical and photochemical effects in GMP and GMP containing dinucleotides are the nucleic bases. What are thought to be the principle tautomeric forms are shown below, along with the numbering systems used for the bases, depending on whether they are purine or pyrimidine derivatives.

Purines

Adenine

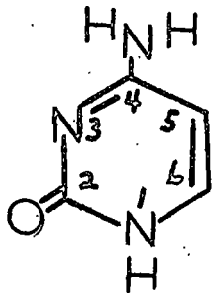


Guanine

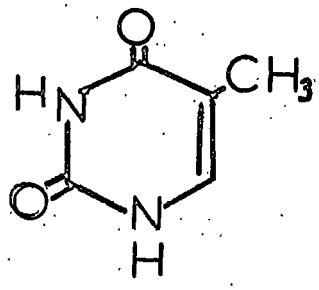


Pyrimidines

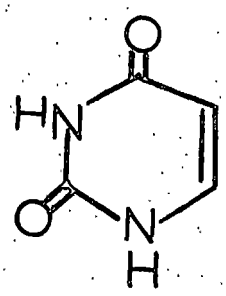
Cytosine



Thymine



Uracil



In the form of a nucleoside, there is a substitution at the 9 position in the purines, and at the 1 position in the pyrmidines. The substituting group is ribose in the case of a ribonucleoside; or it is deoxyribose in the case of a deoxyribonucleoside. The substitution is by a glycoside linkage at the 1′ position of the sugar. Adenosine, guanosine, cytidine, thymidine, and uridine are the terms applied to the ribonucleosides. Deoxyribonucleosides have the prefix deoxy added to the terms used for the ribonucleosides.

Nucleotides are the sugar-O-phosphate esters of the nucleosides, with substitution at the 2′, 3′, 5′ positions possible in ribosides, and substitution at the 3′ and 5′ positions in the deoxyribosides. Adenosine monophosphate is abbreviated AMP, and similar abbreviations apply to the other nucleotides: GMP, CMP, TMP, and UMP. Since TMP exists only as the derivative of a deoxyribonucleoside, it is understood to be such. dAMP, dGMP, and dCMP designate the deoxynucleotides of adenosine, guanosine, and cytidine. UMP does not exist in a deoxy form.

A short segment of a nucleic acid polymer may be made by the formation of two sugar-O-phosphate ester bonds with two nucleosides and one phosphate. A molecule of this

type, such as adenyl, 3′, 5′ guanylate, is abbreviated to ApG(3′, 5′). The conformation in a dinucleotide is believed to be similar to that of the Watson-Crick geometry for a nucleic acid polymer. Crystal structures have been determined for two dinucleotides, ApU (1) and GpC (2).

Principles of Photophysics

The basic elements of the principles, which are believed to govern photophysical processes, can be expressed in terms of a kinetic scheme portrayed on an energy level diagram. Such a diagram, commonly referred to as a Jablonski diagram, is shown in Figure 1.

In the diagram, the vertical transitions of increasing energy are representative of absorption events related to a change in vibrational-electronic energy states. The absorption intensity is related to two considerations: 1) the degree of interaction of the light wave with the molecule, and 2) the population differences between the initial and final states.

Experimentally, the transmitted intensity at a given energy of excitation is observed to be governed by; the concentration of the molecules in the medium, the distance of penetration of the light wave into the absorbing medium,

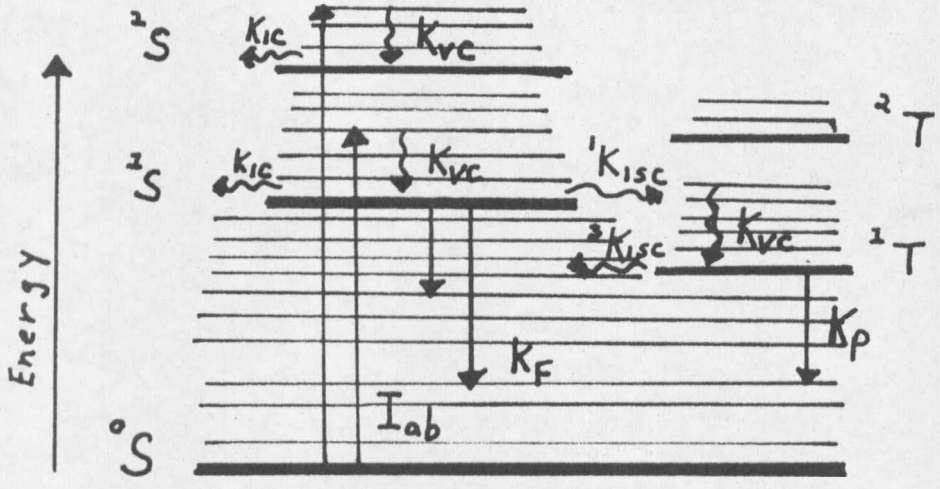


Figure 1. Jablonski diagram for widely separated chromaphores. Straight vertical lines depict radiative processes. Wavy lines represent nonradiative processes. Dark horizontal lines represent electronic state energy minima. Lighter horizontal lines represent combined vibrational-electronic states.

and a constant characteristic of the absorbing molecule. The expression relating these effects is

$$I(x) = I_0 10^{-\epsilon cx}$$

where $I(x)$ = the intensity of the light at distance x in the medium,

I_0 = the intensity of the light just as it enters the medium,

c = concentration of the absorbing molecules in moles per liter,

ϵ = a constant related to the degree of interaction of the molecule with the light wave being absorbed, and given the name molar extinction coefficient.

The intensity of the absorbed light, I_{ab}, is the difference between the intensities of, the incident light, I_0, and the light at distance x, $I(x)$, so that

$$I_{ab} = I_0 - I(x)$$

$$= I_0(1 - 10^{-\epsilon cx}).$$

I_{ab} is observed to vary as the energy of excitation is varied, when I_0, c, and x are held constant. Therefore, ϵ can only be considered a constant for a given excitation energy.

The variation of ϵ with energy of excitation in the visible and UV regions of the spectrum is approximately a series of gaussian shaped curves, with varying degrees of overlap, beginning at the minimum excitation energy for absorption, and continuing to point where ϵ becomes a constant, indicating photodissociation. Molecules in the vapor phase, and some molecules in condensed media, show additional fine structure in the electronic absorption bands, whose energy spacings are of the nature of vibrational transitions. However, many complex molecules in solution, and at room temperature, display smooth unstructured bands with band widths of from 3,000 cm^{-1} to 10,000 cm^{-1}. These individual bands are considered to be the result of transitions between the spin-paired ground energy state ^0S, and some higher spin-paired electronic state ^nS. The variation in intensity across a particular band is attributed to a variation in "overlap" between the lowest vibronic state of the ground electronic state and the vibronic states of some higher energy electronic state. The

value of ϵ at its maximum within an electronic band is found to vary by many orders of magnitude (10^0 to 10^5). This variation is thought to be the result of certain selection rules which apply to an electronic transition; for example, transitions between states of different spin are not "allowed", nor would the transition between two states with a zero value of the integral $\langle\psi_1|\sum_i q_i r_i|\psi_2\rangle$ be allowed.

The time required for an electronic transition must be near that of the oscillation period of the light wave inducing the transition, $\sim 10^{-15}$ seconds.

Once a molecule reaches a higher electronic state, it does not remain there indefinitely but relaxes to the ^{0}S state, after a time interval dependent on the individual molecule and the degree of external perturbation.

Most often, the vibronic state reached on excitation is some higher vibrational level of the ^{n}S. As, with only a few known exceptions, the observed relaxation processes of the spin-paired ^{n}S states take place only from the lowest vibrational level of ^{1}S, the dissipation of energy in excess of ^{1}S must be more rapid than a process such as the re-emission of the light. Rentzepis, by an elegant technique using laser excitation, was able to observe a

vibration relaxation time of 20 picoseconds (10^{-12} sec.) for an excited vibronic state of benzophenone (3). The rates of vibrational cascade, k_{vc}, and k_{IC} ($^{n}S \rightarrow {}^{n-1}S$) of other molecules may also be of the order of 10^{11} - 10^{13} sec^{-1}. The invariance of observable relaxation processes with excitation energy is commonly referred to as Kasha's rule.

The depopulation of lowest vibrational level ^{1}S state occurs via three pathways: 1) emission to some vibrational level of ^{0}S (fluorescence) at a rate k_f (10^6 - 10^9 sec^{-1}); 2) nonradiative isoenergetic transfer to a spin-unpaired triplet state, ^{1}T, (intersystem crossing) at a rate, $^{1}k_{ISC}$ ($10^{?}$ - 10^9 sec^{-1}); and 3) nonradiative isoenergetic transfer to a highly excited vibrational level of ^{0}S with retention of paired spin-state (internal conversion) at a rate k_{IC} ($10^{?}$ - $10^{?}$ sec^{-1}).

The idea of invariance of the fluorescing state with excitation energy has its basis in three experimental observations: 1) the highest energy fluorescence seen is very close to the lowest possible absorption energy, 2) the shape of the fluorescence band is not dependent on excitation energy, and 3) the intensity of fluorescence at any

one point in the fluorescence band is directly proportional to the ϵ values at varying excitation energies.

Additionally, from 1) above, the energy of the transition of minimum energy absorption and maximum energy fluorescence should be located at the intersection of the absorption and fluorescence bands (0-0 energy). From 2) above, since the shape of the fluorescence band for a particular species is not dependent on excitation energy, an observed variance is evidence of more than one species, thus providing a test for fluorescent impurities. From 3) above, by monitoring the fluorescence intensity at any given point in the fluorescence band, and then varying the excitation wavelength across the absorption band, what should be observed is a fluorescence intensity variation exactly parallel to the varying $\epsilon(\lambda_{ex})$, i.e., the absorption band shape should be reproduced.

While the basic features of absorption and fluorescence of molecules can be understood by an extension of Einstein's treatment of transitions in atoms (see Birks (4)), nonradiative deactivation processes such as internal conversion and intersystem crossing are the objects of intensive theoretical consideration. Nonradiative internal conversion from ^1S is often minor, and is thought to be so

because of the relatively large energy separation (1-8 eV) between ^0S and ^1S. However, in some cases (such as for the molecules being examined for this work), it is the dominant deactivation pathway even though the ^0S - ^1S energy separation is quite large (>7 eV for the nucleic acids). Intersystem crossing is formally forbidden by the selection rules, but is thought to be partially allowed by a quantum mechanical spin-orbit coupling of the ^1S and ^1T states.

The spin-unpaired triplet state, ^1T, once populated by nonradiative intersystem crossing (the radiative ISC is even more "forbidden") undergoes relaxation processes similar to those of the ^1S state, except due to the forbidden nature of decay to the ground ^0S state, the radiative process (phosphorescence) has a rate, k_p, (10^{-2} - 10 sec^{-1}) that is much less than k_F, so that is observed only when the nonradiative deactivation rate, $^3k_{ISC}$, is reduced substantially. Experimentally, this is achieved by immersing the molecules in a low temperature matrix. As indicated in the diagram, phosphorescence ensues at a lower energy than does fluorescence.

Two additional experimental parameters, related to the competition between radiative and nonradiative deactivation of the lowest lying excited states, are used to gain

information about excited state pathways. These are the relaxation times, and the efficiencies or quantum yields, of luminescence.

The relaxation time τ, or lifetime as it is normally called, is defined as the time that is required for an observed luminescence intensity to fall to 1/e of a given value on termination of the excitation beam.

$$I_{t=t} = I_{t=0}e^{-t/\tau}, \quad I_{t=\tau} = \frac{I_{t=0}}{e}$$

$^1\tau$ = lowest singlet state lifetime

$^3\tau$ = lowest triplet state lifetime

$^1\tau_0$, $^3\tau_0$ = natural radiative lifetimes (only radiative deactivation is possible)

$$\tau = \frac{1}{\sum_i k_i}, \text{ where } k_i = \text{any deactivation rate constant}$$

The quantum yield Φ is defined as the fraction of molecules undergoing a certain photophysical or photochemical process out of the total number of molecules excited.

$$\Phi_x = \frac{\text{Number of molecules undergoing process x}}{\text{Total number of molecules excited}}$$

In the steady state approximation, which usually applies in the absence of photochemistry, any sampling period

after the establishment of the steady state will give the same result for Φ_x.

The quantum yield can also be expressed in terms of rate constants

$$\Phi_x \quad \frac{k_x}{k_x + \sum_i k_i},$$

where k_x is the rate constant for the process whose quantum yield is being measured, and k_i is the rate constant for any other process proceeding from the same excited state.

The previous discussion alluded to the situation where the molecules were dissolved in an inert, transparent medium at low concentration ($<10^{-5}$ M), so that the interaction between solute chromophores was negligible. As the concentration is increased and the solute molecules are brought closer together, they interact more strongly, and a variety of effects may be evidenced as a result. Varying solvent conditions may also produce photophysical effects. Some of the more commonly observed effects are listed below.

1. Inner filter effect - A decrease in luminescence intensity due to reabsorption within the solution.

2. Nonlinear absorption dependence on concentration - Ground state dimer formation.

3. Excimer fluorescence - An unstructured fluorescence band 5,000 cm^{-1} to the red (lower energy) of the monomer fluorescence due to excited state dimer formation.

4. A variation in the O-O energy due interaction of the solute chromophores, in the excited state or ground state, with solvent molecules or other solutes.

5. A shortened lifetime of some solute chromophores and a related luminescence yield increase of other solute chromophores, due to nonradiative electronic energy transfer.

6. A variation in lifetimes and yields due to a change in the rates of internal conversion or collision deactivation.

Nucleic Acids and UV Light

Since the discovery of the nucleic acids as the genetic material, scientists have attempted to gain exacting information about these very important molecules by a variety of methods. The ideal method to apply would be one where the investigational probe would have a perturbing effect that was transitory in time and minor in degree, so that it would be possible to elicit information about the detailed functioning of the system, without serious

disruption of the system. Nonionizing electromagnetic radiation in the ultraviolet region would seem suited to these purposes as the time of the perturbation is quite small, ~10^{-8} seconds, and only a very small fraction of the molecules need be disturbed to quantitatively gauge the overall conditions.

Indeed, the absorption of UV light by the nucleic acids has served well as a detection method for the molecules, both quantitatively and qualitatively. Even such subtle effects as the degree of helical conformation in a nucleic acid polymer may be obtained by means of absorption spectroscopy, due to the variation of absorbance properties of the helical conformer as opposed to the non-helical form. As convenient as the UV absorption method is, it is not without limitations.

While only 10^{-15} seconds is required to absorb the UV light, as much as one second may be required for an excited molecule to relax back to the ground state under certain conditions. It is known that the nucleic acids undergo photoalteration to a small degree within this relaxation period, so that the perturbing effect is not entirely transitory. Additionally, the individual nucleotide components of the nucleic acids have very similar absorbance

properties, so that it is nearly (if not totally) impossible to excite any one nucleotide independently of the others in a polymer.

Luminescence measurements allow one to detect excited molecules as they relax, via a radiative mechanism, to the ground state. If the efficiencies, lifetimes, and/or energies of the radiative processes are sufficiently different for molecules which have similar absorbance properties, then these detection methods may be used in place of absorption techniques to monitor a particular chromophore. It has been found that the individual nucleotide components do have luminescence properties (considering both fluorescence and phosphorescence) which do differ enough that luminescence measurements are capable of distinguishing any one nucleotide.

What is unfortunate, as far as the technique is concerned, is that it is necessary to have the nucleotides dissolved in a low temperature glass, in order to observe the fluorescence (this is always the case for phosphorescence) by conventional techniques. This is necessary because the nucleotides exhibit a high degree of nonradiative decay (internal conversion) at room temperature, so that the efficiency for the fluorescence process is only about

10^{-4}. Even at liquid nitrogen temperature, the internal conversion processes account for up to 90% of the deactivation for the nucleotides. This is somewhat unusual in that most molecules which fluoresce, do so at room temperature, internal conversion not being an efficient deactivation pathway between the ground state and the first excited singlet level. The low quantum efficiencies of luminescence (on the order of a few percent), even at 77°K, has been the cause of a rather slow development in this area of research. Only within the last fifteen years or so has the necessary instrumentation been devised which could give good results for such low luminescence yields and which are so environmentally dependent. A group of investigators at Bell Telphone Laboratories, Murray Hill, New Jersey has been most responsible for defining the properties of the excited states of the nucleotides. The bulk of their published work appeared in the years 1966-1970. Eisinger and Lamola have published a review of the group's research results incorporated in a general review of the area (5). In Table 1 are listed some of their results for the nucleotides as given in the above mentioned review.

In 1971, Daniels and Hauswirth published luminescence and excitation spectra for the nucleic bases