MASTERFluorescence Properties of Visual Pigments^{1a}Anthony V. Guzzo, Gary L. Pool^{1b}, and Calvin B. LemanChemistry Department
University of Wyoming
Laramie, Wyoming 82070

The act of seeing, i.e. vision, requires a chromophore capable of absorbing light. In a continuing series of studies Wald, Hubbard, and co-workers have shown that this visual pigment is formed from the combination of a protein with a particular polyene aldehyde. By far the greatest attention has been centered on the night vision pigment, rhodopsin. Its chromophore is derived from 11-cis retinal by reaction of the aldehyde group with a specific amino₄ group on the protein (although there is some controversy over this point⁴). Further, the spectral properties of rhodopsin are best reconciled with the protonated (or acidified) form of this Schiff base⁵.

In discussing the spectroscopic and photochemical properties of rhodopsin, a number of investigators have suggested these are essentially derived from those of the polyene chromophore and that the protein has little effect. Such an extreme view is useful and, since the retinal derived Schiff bases are relatively easy to prepare, provides a good working hypothesis. It is of interest, then, to see which of the spectroscopic or photochemical properties of the visual pigment are derived from those of the polyene and then how they are perturbed by the presence of the protein. For instance, it has been assumed in studies concerning the potential barrier to photoisomerization of the polyene^{6,7} that the barrier is essentially that barrier to be found in the isolated Schiff base (the acidified form) and that the protein contributes little.

In the visual cell electron microscope studies have shown that the pigment molecules are arranged in layers, probably only one molecule thick. Such an arrangement is analogous to the arrangement of chlorophyll molecules in the chloroplast and the possibility of energy migration between rhodopsin molecules immediately suggests itself. We may argue then that such energy transfer may also be exhibited by the Schiff base at appropriate concentrations.

The present study was carried out then with this in mind. Our work on the fluorescence characteristics of rhodopsin and of its intermediates formed upon bleaching naturally led to a study of the Schiff bases derived from retinal with particular reference to the possibility of radiationless energy migration.

LEGAL NOTICE

This report was prepared as an account of Government sponsored work. Neither the United States, nor the Commission, nor any person acting on behalf of the Commission:

A. Makes any warranty or representation, expressed or implied, with respect to the accuracy, completeness, or usefulness of the information contained in this report, or that the use of any information, apparatus, method, or process disclosed in this report may not infringe privately owned rights; or

B. Assumes any liabilities with respect to the use of, or for damages resulting from the use of any information, apparatus, method, or process disclosed in this report.

As used in the above, "person acting on behalf of the Commission" includes any employee or contractor of the Commission, or employee of such contractor, to the extent that such employee or contractor of the Commission, or employee of such contractor prepares, disseminates, or provides access to, any information pursuant to his employment or contract with the Commission, or his employment with such contractor.

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED

fig

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

EXPERIMENTAL DETAILS

All-trans-retinal and retinol (Distillation Products, Inc.) were stored in a freezer under a nitrogen atmosphere and were checked for spectral purity before use. The Schiff bases were synthesized by mixing an excess of the appropriate amine with a solution of all-trans-retinal in the presence of K_2CO_3 to remove the water produced and allow the reaction to proceed to completion. On formation of the Schiff base the retinal absorption (385 m μ) is replaced by the imine absorption at 365 m μ . Completion of the reaction was checked by the absorption spectrum or treatment of the reaction mixture with Carr-Price reagent which will react with any unreacted retinal to produce a bright blue color. Protonation of the Schiff bases to effect bathochromic shifts in the absorption maxima was achieved by additions of trichloroacetic acid to a solution of the base in methylcyclohexane. Several different amines were used and all were similar in their fluorescence characteristics. The data presented here refers to the Schiff base formed from 1-amino-2-propanol and all-trans-retinal - all-trans-N-retinylidene-1-amino-2-propanol - which will be abbreviated as NRAP, the protonated form will be referred to as NRAPH⁺.

Because of the low fluorescence yields of the compounds studied, except for retinol, all spectra were obtained at liquid nitrogen temperature. Methylcyclohexane was most frequently used as the solvent since it allowed the larger bathochromic shifts to be observed upon protonation and formed a reasonably good glass at low temperatures. Methyltetrahydrofuran was used as the solvent in the polarization studies since it formed an excellent glass. The fluorescence spectra were obtained using an Aminco-Bowman spectrophotofluorometer and were corrected for wavelength response of the detector (the R136 or 1P28 photomultiplier detectors).

In order to measure the polarization of the fluorescence emission, the quantity $P = \frac{I_{VV} - I_{VH}}{I_{VV} + I_{VH}}$ was measured;¹¹ here the term I_{VV} represents the intensity of fluorescence excited by vertically polarized light and measured through a vertical polarizer; I_{VH} is the intensity of fluorescence excited by vertically polarized light but measured through a horizontal filter. Polaroid films HN22 were used to obtain the desired polarizations. No correction terms for instrumental response were applied to this equation since we were interested only in comparisons of polarizations between the various systems investigated, i.e. retinol, the Schiff base (NRAP), and the protonated Schiff base (NRAPH⁺).

The polarization measurements were obtained at 77° K from cylindrical films formed between the surfaces of a solid glass rod and a sealed off glass tube of slightly larger inside diameter than the solid rod. All polarization values were reproducible to within 10%.

RESULTS AND DISCUSSION

I. Absorption and Fluorescence Spectra

The fluorescence and excitation maxima obtained for retinol (Table I) correspond with the fluorescence maximum observed by Kahan¹² and the absorption maximum at liquid nitrogen temperature¹³ respectively. For retinal the observed fluorescence maximum corresponds to that observed previously¹⁴, allowing for detector response corrections.

All-trans-N-retinylidene-1-amino-2-propanol (NRAP) has an absorption maximum at 365 mμ at room temperature and a fluorescence excitation maximum at ~375 mμ at -196°C. The bathochromic shift is presumably a result of the temperature difference since it is of the order of magnitude expected¹³. The fluorescence maximum was at 500 mμ at -196°C. The addition of an equimolar quantity of trichloroacetic acid to the Schiff base produces a bathochromic shift in the absorption maximum to 450 mμ (25°C). The excitation and fluorescence maxima at -196°C were 460 mμ and 550 mμ respectively. Additions of a one hundred fold excess of trichloroacetic acid produces a further bathochromic shift in the absorption maximum to 470 mμ with corresponding excitation and fluorescence maxima at -196°C of 480 mμ and ~590 mμ respectively. The excitation and fluorescence maxima for this series of compounds are also tabulated in Table I.

TABLE I
FLUORESCENCE AND EXCITATION MAXIMA OF RETINOL,
RETINAL, AND MODEL SCHIFF BASES^a

Compound	Excitation Maximum (mμ)	Fluorescence Maximum (mμ)	$\Delta\nu^b$ (cm ⁻¹)
Retinol	335	465	8300
Retinal	(385) ^c	520	6800
NRAP	375	500	6700
NRAPH ⁺ (equimolar TCA) ^d	460	550	3500
NRAPH ⁺ (excess TCA)	480	590	3900

^aAll-trans isomers at -196°C in methylcyclohexane solvent.

^bThe difference in the excitation and fluorescence maxima expressed in cm⁻¹.

^cBecause of the distorted excitation spectrum the absorption maximum at -196°C was used.

^dTCA = trichloroacetic acid.

Table I shows that the energy separation between the absorption and fluorescence maxima of the unprotonated Schiff base is large and comparable to the same separation in retinal and retinol, whereas the protonated Schiff bases have a considerably decreased separation. This correlates well with the observations on rhodopsin and its bleaching intermediates⁹ given in Table II. In this series rhodopsin, lumirhodopsin, and metarhodopsin I have small separations whereas metarhodopsin II reverts to a large separation comparable to retinal, retinol, and the unprotonated Schiff base. According to present understanding of the bleaching cycle, metarhodopsin II is the first species in the cycle which contains the deprotonated Schiff base linkage. Thus the decrease in the absorption-fluorescence separation in both the rhodopsin system and in the model Schiff bases apparently arises from the protonation.

TABLE II

FLUORESCENCE AND ABSORPTION MAXIMA OF BOVINE RHODOPSIN AND
SOME OF THE INTERMEDIATES FORMED ON BLEACHING^a

Species	Absorption Maximum (mμ)	Fluorescence Maximum ^b (mμ)	$\Delta\nu^c$ (cm ⁻¹)
Rhodopsin	500	600	3300
Lumirhodopsin	497	600	3300
Metarhodopsin I	478	580	3500
Metarhodopsin II	380	535	7600

^aReferences 8, 9.

^bObserved at -196°C from sample of rod outer segments.

^cThe separation between the absorption maximum and the fluorescence maximum expressed in wavenumbers.

TABLE III

POLARIZATION VALUES OBTAINED FROM THIN FILMS OF RETINOL,
NRAP, AND NRAPH⁺^a

Concentration	Retinol	NRAPH ⁺	NRAP
less than 10 ⁻⁵ M	60		
2.7 x 10 ⁻¹ M		5	20
2.7 x 10 ⁻² M		23.6	34.8
2.7 x 10 ⁻³ M		40	43.5
2.7 x 10 ⁻⁴ M		37.4	41.5

^aAll values are in percentages and were obtained from the expression

$$P = 100 \times \frac{I_{VV} - I_{VH}}{I_{VV} + I_{VH}}$$

II. Polarization Studies

There are several ways of estimating the extent of energy transfer between a possible donor and acceptor¹⁵ which, in the systems studied here, are the same molecule. In our system we are limited by the intense absorption of the polyene chromophore and thus decreases in fluorescence intensity can not be reliably used as a measure of transfer. Further, lifetime studies are experimentally quite difficult. Polarizations techniques appeared to provide the best way of estimating transfer.

If a fluorescence is excited with polarized light we expect a retention of polarization in the emitted light if 1) no molecular tumbling has occurred in the lifetime of the excited state, 2) scattering is negligible, and 3) energy transfer has not occurred.¹⁶ Since our work was done at 77°K, the first point is of little consequence. Scattering is impossible to eliminate but can be accounted for by using some standard that does not exhibit energy transfer. In our case retinol served as the standard since we obtained no evidence of transfer with it except at very high concentrations. The third point--the existence of energy transfer--could then be determined from the retention of polarization measured in our systems at various concentrations.

In discussing transfer the "critical" transfer concentration C_o and "critical" transfer distance R_o are quantities often used. These terms refer to that concentration and that average interchromophore distance at which energy transfer is equally as probable as all other means of deactivation of the excited state. The two quantities are related by the expression¹⁵

$$C_o = \frac{397}{R_o^3} \dots\dots\dots (1)$$

Thus assuming no molecular tumbling, and zero scattering we expect a fifty percent retention of polarization if energy migration occurs in a system at the critical concentration.

The polarization results obtained from thin films of retinol, NRAP, and NRAPH⁺ in methyltetrahydrofuran are given in Table III. The result for retinol was obtained for the lowest concentration of this compound that still allowed a reasonable spectrum to be recorded. As discussed in the previous section, the energy separation between the absorption and fluorescence maxima of retinol is quite large and energy transfer is not expected to occur. Thus a polarization value of 60% indicates instrumental scattering of at least 40%. For the most dilute samples of the Schiff base and its protonated form, the maximum polarization values obtained are near 45%. This value would suggest instrumental scattering of at least 55%. There is thus uncertainty in this point; nevertheless, in the following we will use both of these values and hopefully obtain a range of "critical" transfer distances.

If energy transfer occurs, then at the critical concentration the polarization should fall to one half its value in the absence of transfer. For the scattering limit determined by the retinol case this is $P = 30\%$; while for the scattering limit determined from the Schiff base study this is near 23%.

The polarization values obtained for NRAPH⁺, which are plotted in Figure 1, give for $P = 30\%$ and then 23% "critical" concentrations of 0.012M and 0.027M respectively. Using equation 1 these "critical" concentrations give R_o values between 25Å and 32 Å, values typical for singlet-singlet transfer¹⁵ (a similar

analysis for the unprotonated Schiff base gives values near 15 Å for R_0 indicating very inefficient transfer as expected).

Figure 1. Polarizations of Thin Films of NRAP and NRAPH⁺ Versus Concentration.

We may now return to rhodopsin and, considering its protonated Schiff base nature, suggest that R_0 values in the 25-32 Å range should also hold for its transfer characteristics. The absence of photodichroism in rod outer segments has been observed by Hagins and Jennings¹⁸ and more recently by Pak and Helmrich.¹⁹ Results in this laboratory using the fluorescence depolarization technique strongly suggest that this is a true energy migration phenomenon.

III. Spectral Overlap Predictions of Transfer

In our study of the depolarization of the rhodopsin fluorescence¹⁹ we were unable to obtain accurate polarization values and could not then calculate R_0 . We may calculate this quantity following Förster's results for dipole-dipole or resonant transfer.²⁰ Förster's equation in the form used by Tweet, Bellamy, and Gaines²¹ is

$$R_0^6 = \frac{9000 \ln 10 \langle k^2 \rangle n_0}{128 \pi^6 n^4 N \bar{\nu}^4} \int_0^\infty e(\tilde{\nu}) f(\tilde{\nu}) d\tilde{\nu} \dots \dots \dots (2)$$

where η_0 is the quantum yield of fluorescence in the absence of transfer, n is the index of refraction of the medium, N is Avagadro's number, $\langle k^2 \rangle$ is an average over the angular dependence of the dipolar interaction, $\bar{\nu}$ is an appropriate average wavenumber usually found as the average position of the absorption and fluorescence maxima; and the integral is over the product of the absorption spectrum of the acceptor (given as the molar decadic extinction coefficient versus wavenumber) and the fluorescence spectrum of the donor normalized to unity on a wavenumber scale. Clearly the more widely separated are the absorption and fluorescence spectra the less probable will be transfer.

Using data obtained previously⁸ we have calculated R_0 for rhodopsin. With $\eta_0 = 0.005$, $\langle k^2 \rangle = 0.66$, $n = 1.2$, $\bar{\nu} = 18,400 \text{ cm}^{-1}$, and a calculated overlap integral of 4,300 liters/mole, we obtain $R_0 = 11 \text{ \AA}$; a value which would predict transfer to be negligible.

For the protonated Schiff base we may calculate an R_0 in the same way.¹ Using the measured value of $\eta_0 = 0.008$, $\langle k^2 \rangle = .66$, $n = 1.2$, $\bar{\nu} = 19,400 \text{ cm}^{-1}$, and a measured value of 8600 liters/mole for the overlap integral, R_0 is again found to be 11 $\text{\AA}$. Again we obtain a value of the transfer distance which is too small for transfer to be important.

Of the factors that influence these results, the quantum efficiency of fluorescence η_0 is the only one that can vary enough to make a large difference in calculated result. It is clear that the very low quantum yield measured for the NRAPH⁺ and rhodopsin fluorescences have produced these low "critical" transfer distances.

We are then faced with a contradiction. It is difficult to accept that our measured depolarization for both the protonated Schiff base and rhodopsin are greatly in error, especially in the face of the lack of transfer we do¹⁹ seem to observe for the un-protonated Schiff base and for metarhodopsin II. Förster's equation has been verified for a number of systems and appears valid. However, his analysis was developed for excited states that had reached thermal equilibrium much before the process of emission.²² There is¹⁴ some evidence that this may not be the case, at least for retinal. Becker¹⁴ has shown that the fluorescence yield of retinal is wavelength dependent thus indicating the presence of processes competing with thermal relaxation in the excited state. If similar phenomena existed within the protonated Schiff bases and in rhodopsin we would expect that Förster's resonant transfer approach would not apply.

Summary

Our experiments indicate that the fluorescence changes observed as rhodopsin bleaches to its various intermediates are entirely consistent with a change from a protonated to an unprotonated polyene Schiff base. In the protonated form both rhodopsin and the Schiff base have extensively overlapping absorption and fluorescence spectra indicating the possibility of radiationless migration of energy between chromophores. Polarization studies on rhodopsin and the protonated Schiff base do give evidence of such transfer. However, if we attempt to calculate a "critical" transfer distance using the theoretical expression obtained by Förster, we obtain values which are much lower than the measured quantity. It is our belief that Förster's equation does not apply to these polyene systems since photochemistry from the excited vibronic levels may take place. As discussed by Förster, transfer from such non-equilibrated excited states should not conform to the theory developed. It is a matter of future research to see if photochemical processes do exist to a measureable extent, and if they are important in the process of vision.

Bibliography

1. a) We gratefully acknowledge support for this work by the Atomic Energy Commission through contract No. AT(11-1) - 1627; b) NIH Predoctoral Fellow (1966) supported through grant No. 1 - F1 - GM - 36, 155-01: Now at Chemistry Department, University of Minnesota.
2. G. Wald, P. K. Brown, and I. R. Gibbon, J. Opt. Soc. Amer., 53, 20 (1963); also R. Hubbard, J. Biol. Chem., 241, 1814 (1966).
3. R. A. Morton and G. A. J. Pitt, Biochem. J., 59, 128 (1955).
4. E. W. Abrahamson, R. P. Poincelot, P. G. Millar, and R. L. Kimbel, Jr., Nature, 221, 256 (1969).
5. H. J. A. Dartnall, The Visual Pigments, 1957, John Wiley, New York.
6. K. Inuzuka and R. S. Becker, Nature, 219, 383 (1968).
7. J. R. Wiesenfeld and E. W. Abrahamson, Photochem. Photobiol., 8, 487 (1968).
8. A. V. Guzzo and G. L. Pool, Science, 159, 312 (1968).
9. A. V. Guzzo and G. L. Pool, Photochem. Photobiol., 9, 565 (1969).
10. J. O. Erickson and P. E. Blatz, Vision Res., 8, 1367 (1968).
11. A. C. Albrecht, J. Mol. Spect., 6, 84 (1961).
12. J. Kahan, Acta Chem. Scand., 21, 2515 (1967).
13. L. Jurkowitz, J. Loeb, P. Brown, and G. Wald, Nature, 184, 614 (1959).
14. D. E. Balke and R. S. Becker, J. Amer. Chem. Soc., 90, 6710 (1968).
15. N. Turro in Molecular Photochemistry, W. A. Benjamin, Inc., New York, 1967, Ch. 5.
16. E. Gaviola and P. Pringsheim, Z. Physik, 24, 24 (1924).
17. W. A. Hagins and W. H. Jennings, Disc. Fara. Soc., 27, 180 (1959).
18. W. L. Pak and H. G. Helmrich, Vision Res., 8, 585 (1968).
19. C. B. Leman, Ph.D. Thesis, University of Wyoming 1969.
20. Th. Förster, Fluoreszenz Organischer Verbindungen, Vandenhoeck and Ruprecht, Göttingen, 1951.
21. A. G. Tweet, W. D. Bellamy, and G. L. Gaines, Jr., J. Chem. Phys., 41, 2068 (1964).
22. Th. Förster, Disc. Fara. Soc., 27, 7 (1959).