Pure & Appl. Chem., Vol. 49, pp. 333-339. Pergamon Press, 1977. Printed in Great Britain.

PHOTOCHEMICAL STUDIES OF VISUAL PIGMENTS

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Abstract - The photochemistry of retinal, synthetic model retinals and the rhodopsins formed from these retinals has been studied with the purpose of securing information which allows a clearer picture to be obtained of the intermediates and steric requirements of the opsin receptor site. The retinals were separated by hplc and their structures elucidated by pmr spectroscopy. Single photon two bond isomerizations have been observed in some cases. The 11,12-dihydroretinal is capable of binding to opsin to give a rhodopsin which is not bleached by light under normal conditions.

All of our studies described below were carried out with bovine opsin. Since the pioneering studies of Wald and Hubbard (1952), it is now understood that the visual pigment rhodopsin, molecular weight ca. 40,000, contains 11-cis retinal as the chromophore, which is bound via a protonated Schiff base to the lysine terminal amino group in opsin. Rhodopsin absorbs at 500 nm and hence is orange-red colored. Upon irradiation with light, a fast photochemical reaction takes place, and this is followed by a rapid series of thermal reactions going through various intermediary stages to yield all-trans retinal and the apoprotein opsin as the final products (Figs. 1 and 2). Since the absorption maxima of the products are at 380 nm and 270 nm, this decolorization process is called bleaching.

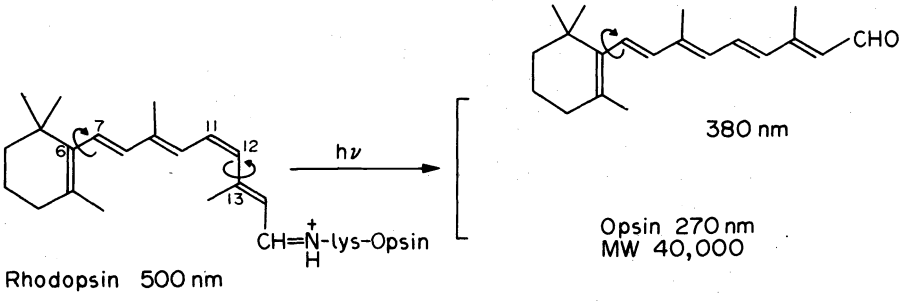

Fig. 1 Bleaching of rhodopsin (1)

The main purpose of our studies is to secure a clearer picture of the intermediates and the steric requirements of the opsin receptor site by studying the isomers by hplc. A hexane/ether solvent system and 5-10 micron glass beads coated with silica affords satisfactory separation. However, the drawbacks of this system are the severe limitation imposed on the quantity of retinals which can be separated by one run (1-2 mg), and the damage caused by contaminant detergents when the retinals originate from detachment of rhodopsins. We have recently been able to achieve gram scale separation of retinals and intermediates by usage of pressurized prep columns (9) (Waters Assoc.). The usage of "micro-CN" columns has also made it possible to pass detergents through the expensive hplc columns without damaging them (10). The advantage of these columns is that after separation of retinals by conventional solvents, the absorbed polar detergents can be washed out with aqueous methanol by using the column packing in the reverse phase mode.

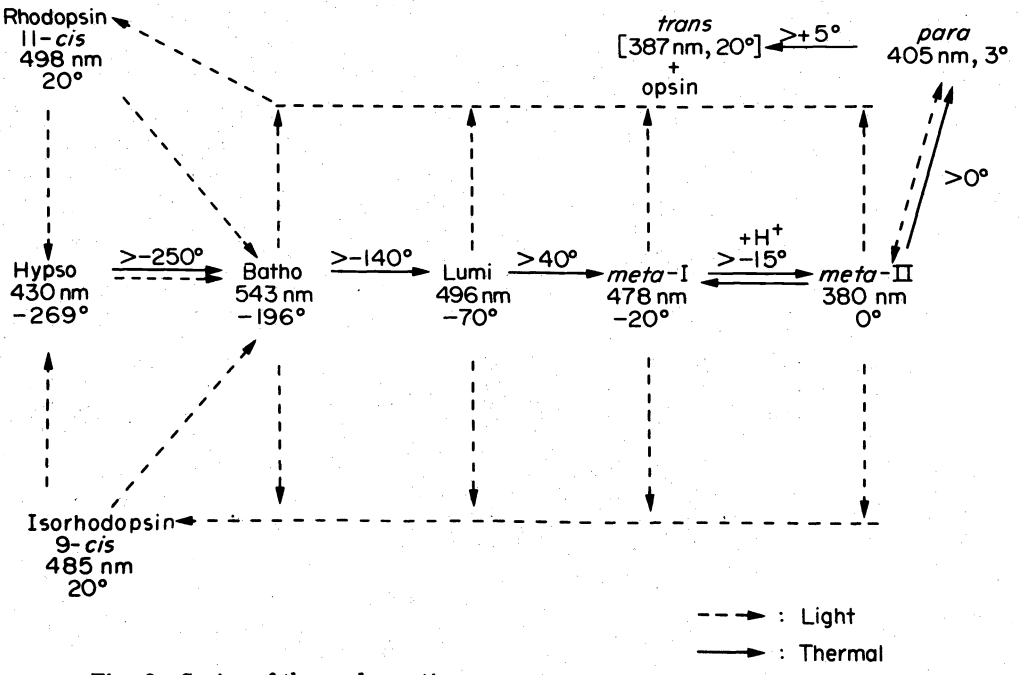

Fig. 2 Series of thermal reactions

The retinals thus separated are then submitted to extensive pmr studies at 220 MHz, 270 MHz (5) to clarify the cis-trans nature of various double bonds. The particular 14-methylretinal series were used to show that (Fig. 3) the solution conformation of 11-cis retinal is predominantly 12-s-cis (by uv), (2, 4) as theoretically predicted by Honig and Karplus (1971) (11) and shown spectroscopically by nmr (15) and by resonance laser Raman (7). In rhodopsin however, the 11-cis retinal chromophore appears to adopt a shape closer to the 12-s-trans form (2, 4, 7).

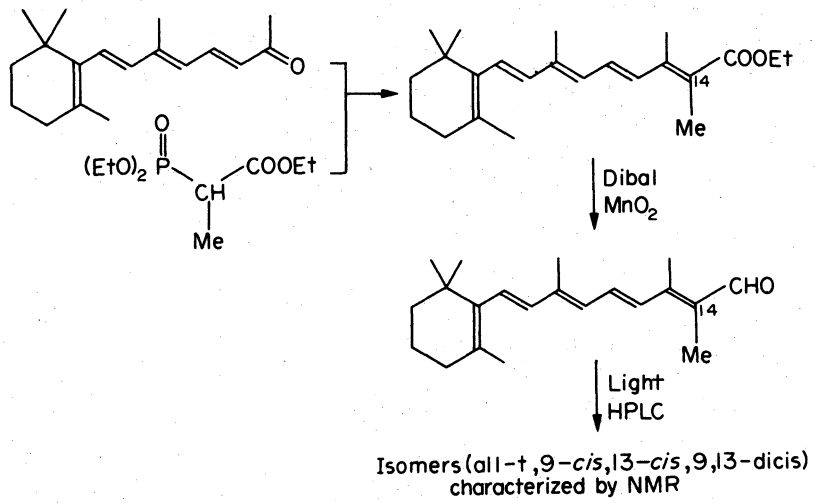

Fig. 3 14-Methylretinal

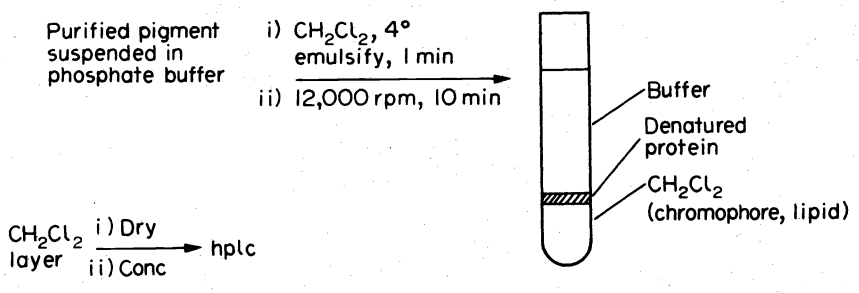

Fig. 4 A simple method for detaching the chromophore from rhodopsin without using heat or light (10).

The method consists of shaking the pigment, i.e., rhodopsin, with aqueous CH_2Cl_2 and submitting the CH_2Cl_2 extract to hplc analysis. Presumably, the organic solvent denatures the protein and exposes the Schiff linkage, which is spontaneously hydrolysed. Since neither heat nor light is employed, there is no danger of isomerization, and hence it is possible to determine the double-bond geometry of the chromophore in the form it was attached to the opsin. The micro-CN column is used for hplc since common silica hplc columns are rapidly deteriorated by the detergent or rod outer segment membrane.

This simple method clearly affords great advantage for characterization of chromophores and has recently been applied to clarify some complex aspects of bacteriorhodopsin species (12).

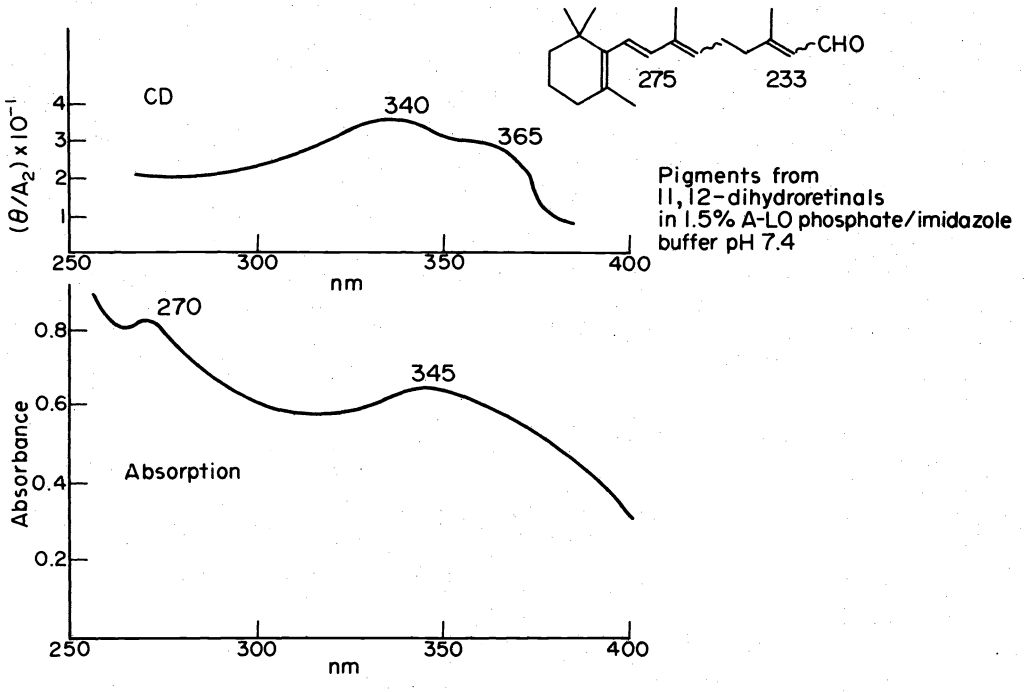

Fig. 5 A nonbleachable rhodopsin absorbing at 350 nm instead of 500 nm (13).

Although the synthetic 11-dihydroretinal isomers (four have been separated by hplc) have as yet not been tested individually for their binding properties with opsin, the mixture of four isomers has been shown to bind with opsin to afford a pigment absorbing ca. 345 nm, cd ca. 350 nm. Since 11-cis, 9-cis, and 9, 13-dicis retinals (3) all bind with opsin, it is quite possible that all four of the dihydroretinals, presumably all-trans, 9-cis, 13-cis, and 9, 13-dicis, will form pigments, too. The chromophore is apparently flexible enough to adopt a shape close to 11-cis when it binds to the protein.

The pigment is interesting in that it is the first non-bleachable rhodopsin, i. e. photostable, and hence will allow basic pigment studies to be carried out in the light and at room temperature instead of dark or liquid nitrogen conditions. Also a correlation between the 233 nm enone uv absorption and the 345 nm band of the pigment will undoubtedly contribute to the clarification of the origin of the 500 nm rhodopsin band (it is not understood why the protonated Schiff base band at 440 nm in retinylidenebutyl amine is shifted to 500 nm in rhodopsin).

DIRECT EXCITATION OF RETINALS

	quantum yield	products
all-trans	0. 04	13-cis
9-cis	0. 18	2:1 trans: 9, 13-dicis
11-cis	0. 24	5:1 trans: 11, 13-dicis
13-cis	0. 21	trans
9, 13-dicis	0. 20	1:1 9-cis: 13-cis

in 3-methylpentane, rt

ca. 15% conversions, $\phi_{PI} \pm 15\%$

Xe 50 μsec flash, hplc

(in MeOH: ϕ_{PI} = 0.-006 for trans, 0. 04 for cis)

Fig. 6 Direct excitation of retinals (8).

The hplc method described in Fig. 4 is directly applicable to product analysis of retinal and rhodopsin photochemistry. Direct photoisomerization of retinal isomers were studied in 3-MP and methanol. It was found that within experimental error, all cis isomers have similar quantum yields which are 4-5 times lower in the polar solvent methanol. In spite of the solvent effects, bonds which undergo isomerizations are the cis double bonds and the terminal 13-ene. The one photon/one double isomerization assumption applies to this class of polyenals.

BIACETYL ^{3}SENSITIZATION OF RETINALS

	quantum yield	products
all-trans	0. 003	13-cis
9-cis	0. 20	5:1 trans: 9, 13-dicis
11-cis	0. 17	trans
13-cis	0. 15	trans
9, 13-dicis	0. 20	2:3:5 trans: 9-cis: 13-cis

in acetonitrile, rt, deaerated

ca. 20% conversions, $^3\phi_{PI} \pm 20\%$

ϕ_{ET} = ca. 0. 75

Fig. 7 Triplet sensitized isomerization of retinals (8).

i) All-trans retinal underwent no detectable triplet isomerization.

ii) The quantum yields of all-trans and 11-cis are essentially identical to values reported by Rosenfeld, et al. (14) in hexane when biacetyl or biphenyl are used as sensitizers.

iii) Addition of oxygen has no measurable effect upon the quantum yield of 11-cis, as reported previously (14).

iv) Formation of the all-trans isomer from the 9, 13-dicis isomer is presumably due to a secondary photoreaction.

v) Product ratios and quantum yield considerations of the direct and biacetyl sensitized isomerizations lead to the conclusion that photoisomerization of cis retinals occur via the singlet as well as the triplet state.

First, direct irradiation of 9, 13-dicis retinal in methanol yields ca. 5% of all-trans (trans: 9-cis: 13-cis ratio is 1:8:10), whereas upon biacetyl sensitization a 20% yield is obtained. If the direct excitation involved only the triplet state, a higher yield of trans retinal would be expected in methanol.

Second, the intersystem crossing efficiency of Φ_{ISC} of retinals is ca. 0.5 and higher (14). Since the total isomerization Φ_{PI} observed upon direct excitation is equal to the isomerization in the singlet and triplet states:

$$\Phi_{PI} = {}^{1}\Phi_{PI} + {}^{3}\Phi_{PI} \cdot \Phi_{ISC}$$

and since for 11-cis retinal, $\Phi_{PI} = 0.24$ and ${}^{3}\Phi_{PI} = 0.16$ in nonpolar solvents (14), it follows that ${}^{1}\Phi_{PI} = 0.16$, namely, approximately one-third is accounted for by the triplet state. If Φ_{ISC} and ${}^{3}\Phi_{PI}$ (nonpolar solvents) were of comparable magnitude as for the 11-cis isomer, then a similar argument can be proposed for the other cis retinals.

TRIPLET SENSITIZED ISOMERIZATION
OF RETINALS

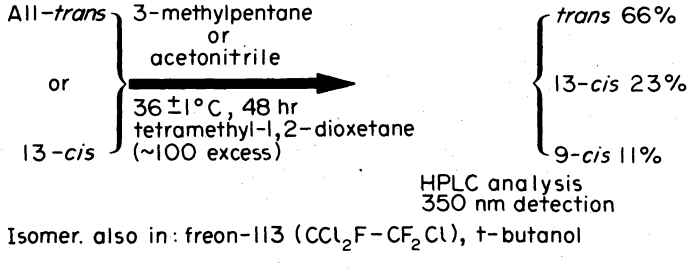

Fig. 8 Thermally generated triplet sensitization of retinals (8).

A steady state mixture of trans:13-cis:9-cis - 66:23:11 was generated with tetramethyl-1, 2-dioxetane.

PULSED LASER EXCITATION OF PIGMENTS

	quantum yield	products
rhodopsin	0.67[a]	50:1 trans : 9-cis
isorhodopsin	0.33	20:1 trans : 13-cis
9, 13-isorhodopsin	0.33	15:1 trans : 11-cis

[a] Hubbard, Bownds, Yoshizawa (1965)

in Ammonyx-LO phosphate buffer, room temperature

ca. 50% conversion, error of cis ± 33%

460 nm, 300 nsec pulse

Fig. 9. Pulsed laser excitation of rhodopsins.

Under the conditions shown, i.e., a single 300 nsec laser pulse (460 nm in Ammonyx-L), trans retinal is the predominant isomer from all three rhodopsins. The formation of all-trans from 9, 13-isorhodopsin is probably a one-photon/two-bond isomerization in spite of the shorter life-time of excited rhodopsin as compared to that of the laser pulse. The reasons for this assumption are: (1) only 50% of the pigment was bleached ; (2) the number of photons and molecules was approximately equal ; and (3) kinetic studies (3) conclude that it is a one photon process.

PULSED LASER EXCITATION OF RHODOPSIN

Detergent	quantum yield	products (retinals)
Triton X-100	0.64	trans
Ammonyx-LO	0.62	50:1 trans: 9-cis
Cetyltrimethyl-ammonium bromide	0.69	30:3:1 trans: 9-cis:13-cis
Sodium desoxycholate	0.55	20:3:1 trans:13-cis: 9-cis

50 mM in phosphate buffer, pH 8.0, rt

50-75% conversion, $\phi \pm 5\%$

error of cis ca. 20%

460 nm, 300 nsec pulse

Fig. 10. Pulsed laser excitation of rhodopsin in various detergents (6).

Cd studies of rhodopsin in various detergents have shown that the intensity ratios of the 500 nm α band and 335 nm β band differ according to the detergent, digitonin giving the highest ratio of 0.87 while the others range from 0.36 to 0.46. Since cd curves reflect the conformation of the rhodopsin molecule, caution should be exercised in comparing data of rhodopsin studies carried out in different micelle environments.

This applies to the photochemical results as well. Of the four detergents tested, the nonionic micelles Triton X-1-0 and A-LO, in contrast to the cationic micelles CTAB and NaDOC, give none or only a small amount of cis retinal. It is only Triton X-100 which gives, similar to natural ungenerated rhodopsin, the trans retinal as the sole product. Here again the question of multiple photon events becomes relevant ; however, in addition to the reasons given in Fig. 4, the fact that in Triton X-100 no cis retinals were formed favors a one-photon/two-bond isomerization.

It is clear that the environment alters the rhodopsin shape and consequently the protein-chromophore intereaction, as reflected in the differences in photoproducts.

TRIPLET SENSITIZED ISOMERIZATION OF RHODOPSIN IN TRITON X-100 at 0° C.

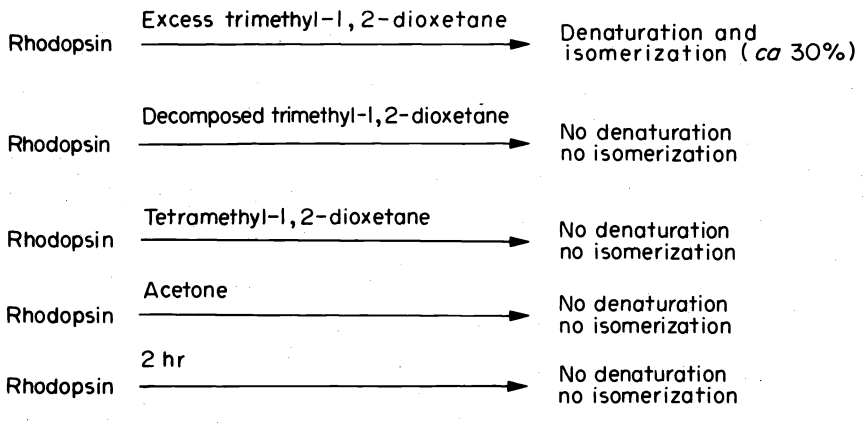

Fig. 11. Thermally generated triplet sensitization of rhodopsin (8).

Rhodopsin is thermally decomposed at 36° C, i.e., the temperature which induces tetramethyldioxetane to yield triplet acetone. Hence, trimethyl-1,2-dioxetane, which thermally decomposed to triplet excited states at temperatures where rhodopsin is thermally stable (ca. 0° C) was used. The pigment was completely bleached in the dark by this trimethyldioxetane, the products being a mixture of 9-cis and all-trans (product ratio is unimportant since multiple isomerizations could have occurred). As shown in Fig. 11, all control experiments led neither to denaturation nor bleaching of rhodopsin. Since trimethyl-1,2-dioxetane forms excited state acetone with an efficiency of ca. 10% and has a triplet/singlet ratio greater than 300, it is clear that, besides permeation of dioxetane into the protein, rhodopsin undergoes a triplet sensitized decomposition to retinal and opsin, similar to that observed for direct excitation. However, it is not clear whether the triplet state is involved in the photoisomerization process upon direct excitation of visual pigments.

Acknowledgement - I have only given a partial account of the visual pigment studies carried out at Columbia which were started around 1973, and have deleted many non-photochemical results. I am most indebted to the following members of my group who have contributed to the studies on a full-time or part-time basis:

1. Former colleagues: Dr. W.-K. Chan, Dr. R. Crouch, M. Handler, Dr. N. Harada, V. Purvin, Dr. M. Hashimoto, Dr. W. Waddell, and Dr. A. P. Yudd.

2. Current colleagues: M. Arnaboldi, Dr. V. Balogh-Nair, Dr. R. Brown, M. Gawinowicz, L. Ghei, M. Pettei, Dr. F. Pilkiewicz, J. Sabol, and S. Tanis. I am also grateful for the fruitful collaborations and discussions with Prof. R. Callender, Prof. T. Ebrey, Prof. B. Honig, Dr. G. Olson, and Prof. N. J. Turro. The studies were supported by grants from the National Institutes of Health and the Hoffmann-La Roche Company.

REFERENCES

1. For an excellent collection of reviews of vision chemistry, see Acc. Chem. Res., 8, March issue (1975).
2. W. K. Chan, K. Nakanishi, T. G. Ebrey, and B. Honig, J. Amer. Chem. Soc., 96, 3642 (1974).
3. R. Crouch, V. Purvin, K. Nakanishi, and T. Ebrey, Proc. Nat. Acad. Sci., USA, 72, 1538 (1975).
4. T. Ebrey, R. Govindjee, B. Honig, E. Pollock, W. Chan, R. Crouch, A. Yudd, and K. Nakanishi, Biochemistry, 14, 3933 (1975).
5. K. Nakanishi, A. P. Yudd, R. K. Crouch, G. L. Olson, H.-C. Cheung, R. Govindjee, T. G. Ebrey, and D. J. Patel, J. Amer. Chem. Soc., 98, 236 (1976).
6. W. H. Waddell, A. P. Yudd, and K. Nakanishi, J. Amer. Chem. Soc., 98, 238 (1976).
7. R. H. Callender, A. Doukas, R. Crouch, and K. Nakanishi, Biochemistry, 15, 1621 (1976).
8. W. H. Waddell, R. Crouch, K. Nakanishi, and N. J. Turro, J. Amer. Chem. Soc., in press.
9. F. Pilkiewicz and M. Pettei, unpublished.
10. F. Pilkiewicz, M. Pettei, A. P. Yudd and K. Nakanishi, submitted for publication.
11. B. Honig and M. Karplus, Nature, 229, 558 (1971).
12. M. Pettei, A. P. Yudd, K. Nakanishi, and W. Stoeckinius, submitted for publication.
13. J. Sabol, V. Balogh-Nair, unpublished.
14. T. Rosenfeld, A. Alchalal, and M. Ottolenghi, J. Phys. Chem., 78, 336 (1974).
15. R. Rowan, A. Warshel, B. Sykes, and M. Karplus, Biochemistry, 13, 970 (1974).