MASTER

THREE YEAR REPORT

for

PHOTO INITIATED PROCESSES IN VISION

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Photo Initiated Processes in Vision

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Photo-Initiated Processes in Vision

Significance of the Area

The process of information transduction as carried out by the visual cell is one aspect of the broad problem of the effect of electromagnetic radiation on biological systems. Clearly the visual cells are constructed to respond to a select collection of wavelengths with a specific response. Nevertheless there seems to be a high probability that an understanding of sensory response in general will come about first for the visual system. This is partly because of the relative ease of isolation of the visual material and its ease of identification. The problem is only now becoming clarified to the extent that we can see that an understanding of the transduction process must also involve an understanding of excitable membrane phenomena in general.

Main Research Accomplishments

During the three year tenure of this research contract, November 14, 1969 to November 15, 1972, we recorded successes in the following areas:

- 1) The production of radical anions, with well resolved ESR hyperfine structure, of vitamin A aldehyde and the N-retinylidene Schiff base of all-trans retinal. The optical spectra of these species were also recorded.
- 2) The determination of several features of the radical anions of the α and β -ionones, short chain analogs of the retinal polyenes.
- 3) The determination of the isomerization pathway for several isomers of the retinals and measurement of the quantum yields of isomerization of the protonated Schiff bases of the retinals.
- 4) The determination of the kinetics of the rapid fluorescence change observed in the frog retina and from cattle rod-outer segments subsequent to a flash of light.

Area I.

From our studies concerning radical formation in the retinals and the N-retinylidene Schiff bases, we showed that well resolved ESR signals could be elicited from anions of these materials. Thus the failure to observe an ESR signal in the biological sample either in the dark or in light can not be due to a simple broadening phenomenon. Rather we are limited to assuming

1) that if electron exchange occurs in the formation of rhodopsin from opsin and 11-cis retinal the electrons must be tightly coupled and exist as a singlet state.

2) If electron transfer occurs upon photon absorption, then upon isomerization the reverse transfer must occur. For instance, if R is the polyene pigment and D is some electron donor, then we may

Upon isomerization of $\underset{11\text{-cis}}{R^{\cdot*}}$ to the all-trans form the electron reverts back to $D^{\cdot+}$. Thus the lifetime of the radical would be extremely short and no measureable population could be built up to be seen by ESR techniques.

3) If electron transfer occurs as in 2) the electrons could remain coupled to form a singlet or a triplet. Only in the latter case would any ESR signal be observed, however, our efforts at scanning the half field region (where a triplet signal would be expected) have been negative.

The restrictions we must place on radical participation in the primary process seem more and more to suggest that perhaps it should not be considered at all in the initial events of vision. The reason for postulating radical participation was to have some rationale for the high quantum efficiency of bleaching in visual materials¹ ($\phi = 0.6$). Retinal itself has only a low efficiency of isomerization² ($\phi = .2$) however we believe that our efforts in Area 3. provide us with a satisfactory answer to this point.

Area 3.

Our work with the protonated 11-cis retinylidene Schiff base shows that at room temperature the quantum efficiency of isomerization is between .3 and .4. However, it is well known that the extinction coefficient of the 11-cis isomer is temperature dependent. At room temperature $\epsilon = 27,000$ and increases at lower temperatures.³ This does not happen with the native visual pigment, since its extinction is high at all temperatures and we know it has the 11-cis isomeric form. Presumably the protein constrains the polyene chain much the same way that a lower temperature removes some degrees of rotational and vibrational freedom. Thus if the protein were to interact with the 11-cis Schiff base in the act of forming rhodopsin, its extinction would rise from the value $\epsilon = 27,000$ to $\epsilon = 43,000$ and the quantum efficiency would very likely increase by the same amount to $\phi = .48$. This is in the range of quantum yields that have been measured for rhodopsin bleaching.

Nothing more need be postulated and it becomes clear that the protonated Schiff base is indeed a very good model for the visual pigment chromophore at least from the spectroscopic viewpoint.

Area 4.

We are not yet sure of the significance of the fluorescence changes we have measured from frog retinas and from rod-outer segments after a flash of light. If as we suggest in our report C00-1627-26 the changes reflect the meta-I-to meta-II-rhodopsin conversion then it is merely another tool for recording this change. It may upon further investigation

only partly reflect this change. If this is the case then the phenomenon may shed some light on other events close to the excitation process.

The activation energy we measure for the process is 65 kcal./mole and is almost outside of the range of activation energies measured for the meta I to meta II rhodopsin conversion⁶ (34 to 55 kcal./mole.) This suggests that measuring the growth of meta II rhodopsin, if indeed this is what we are doing, may give different kinetics than measuring the decay of meta I rhodopsin. If this is true then perhaps this may be interpreted as evidence in support of intermediate steps in the meta I to meta II transformation (i.e. perhaps the meta I to meta I' equilibrium postulated by Williams⁴).

Present State of Knowledge

The visual response like other sensory responses must be investigated at different levels within the receptor cell. This hierarchy of levels is not differentiated so much in terms of difficulty but rather, as Kamen⁵ has done within the domain of photosynthesis, in terms of time elapsed after the absorption of a photon.

Time Period I

Within the lifetime of the excited state formed by the chromophore, within times less than 10^{-8} sec., we have the events which take place in the immediate environment of the chromophore. These changes are primarily conformational and configurational in nature and probably do not involve any mass transfer reactions such as ion flows.

Time Period II

The second time domain lasts to within one microsecond after the initial photon absorption. In this phase we again have no evidence of any ion flows, but rather see protein configurational changes⁶. It is during this time period that the conversion from pre-lumi rhodopsin takes place as follows:

These reactions occur in the dark and are reversible only in light.

Time Period III

The stage at which excitation takes place is considered by many to be the conversion of the meta I rhodopsin to meta II rhodopsin. This is the

time domain between 1 μ sec. and 1 msec. Since membrane permeability changes appear to occur in this interval some means of communication between the bleached visual protein and other structures within the cell must exist. For instance Hagins⁷ has given evidence of a very fast inward flow of Ca^{++} subsequent to a flash of light which precedes the sodium influx change. After the Ca^{++} has entered the cell, at a rate proportional to the number of photons absorbed, a permeability change occurs and sodium ions then are blocked from entering the receptor. Further Murikami and Pak⁸ have shown that the ERP recorded at quite early times is a plasma membrane phenomenon, yet earlier work has shown it is definitely related to rhodopsin bleaching.

Cone has argued that rhodopsin itself may act as a Ca^{++} transport agent⁹. According to the membrane model of Blaise and Worthington¹⁰, rhodopsin as a lipo protein is partially imbedded in the disc membrane. Upon entering the meta I - rhodopsin phase the chromophore finds itself less restricted in its movement and can sink into the lipid-hydrocarbon matrix of the membrane. Cone argues that perhaps a calcium ion could be transported through the membrane by this passive diffusional process. That is if conditions could be set up to have a Ca^{++} gradient, then the rather random motion of the visual pigment molecule could transport many hundreds of calcium ions during the lifetime of the meta-I intermediate.

This model has some difficulties associated with it as discussed by Dratz et al.¹¹. These workers found, on the basis of fluorescent labeling experiments, that very few hydrophilic groups on rhodopsin in the intact disk membrane were exposed to the aqueous environment. Only until the receptor disc was disrupted did labeling take place.

Further lipid depletion experiments showed that the reactive sites on the rhodopsin molecule (for the purpose of labeling) considered to be hydrophilic, are covered by lipid hydrocarbon. Some preliminary work mentioned by these same authors states that the labeling properties are quite different after light exposure. Thus structural features of the visual pigment do change with light and as suggested by other work probably change ⁱⁿ the meta I to meta II conversion.

Another study of great interest which may relate to the excitation process is the discovery that rhodopsin may act as a kinase and phosphorylate itself upon exposure to light. Bownd's et al.¹² showed that when 1% of the rhodopsin is bleached, the ratio of phosphate bound in the light to that bound in the dark has its maximum value (a value near 10). Further their data showed that very likely one phosphate was bound per rhodopsin. In connection with these findings Korenbrot and Cone¹³ find that the sodium conduction decrease becomes a maximum after 1% bleaching. Thus phosphorylation may be related to the fast Ca^{++} flow mentioned above and thus to the subsequent Na^{+} conductance change.

These findings suggest that the basic transduction mechanism may then be a combination of a chemical and physical event. That is, the protein unfolding produced by the absorption of a photon may release some sites which can catalyze the transfer of the terminal phosphate of ATP. Once the phosphate is bound, binding sites for Ca^{++} may develop.

Through our own studies and those of Inuzuka and Becker¹⁴ and Kropf and Hubbard², the basic isomerization process in ^{the} first time interval is

now better understood. It is clear that the retinals and the protonated retinylidene Schiff bases provide distinctly different models for visual pigment isomerization. The retinals being aldehydes have a non-bonding pair of electrons and thus a possible $n-\pi^*$ excited state. This is not the case with the protonated Schiff bases and we conclude that an efficient means of populating the triplet state is not present in this latter case. Thus the triplet state should be ruled out for the visual pigments if indeed the protonated Schiff base is a better model.

We summarize many of the features of the isomerization process via a state diagram such as given below. Not all features of this diagram have been experimentally established and some uncertainties still exist. Nevertheless the ordering of the states explains a number of points.

Let us first consider the absorption of a photon by all-trans retinal. The λ max is quite far removed from the 0-0 vibrational level (the 0-0 level is determined from the fluorescence emission curve) and it is not clear that relaxation down to this latter level occurs before other processes occur. Rather it appears that reactions can take place from higher vibrational levels. Kropf and Hubbard show that the quantum efficiency of isomerization does indeed vary as a function of wavelength for the reaction all-trans $\rightarrow$ 13-cis and 11-cis $\rightarrow$ trans. In the former case, the most efficient process is from the lowest vibrational level indicating that other reactions in all-trans retinal proceed out of the higher states. This is not the case for the 11-cis isomer.

Since isomerization is most efficient from the lowest level we consider the process to take place exclusively from that level. This is process

State Diagram of the Retinals Showing the Pathways of Isomerization.

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A in the figure. The activation energy for this step is ~ 5 kcal. In contrast to the assumption of Kropf and Hubbard² we have assumed that the activated state is the 13-cis activated or twisted state. This is because our work has shown that only the 13-cis isomers is formed with moderate irradiation times in non-polar solvents (TLC evidence). We postulate that similar activated or twisted states exist for the 9-cis and 11-cis isomers and that these must be higher in energy than ^{the} 13-cis activated state. This is reasonable since these isomers are not seen to be readily formed in the photo irradiation. If we assume the 9-cis A state is 1 kcal. higher than the 13-cis A state, and the 11-cis A state 2 kcal. higher then we have, on the basis of Boltzmann factors, rates of formation of these isomers in the ratio 1.0:0.18 : 0.03 . Thus if we irradiated an all-trans solution and obtained a 25% isomerization we would have

75% all- <u>trans</u> remaining
23% 13- <u>cis</u>
1.8% 9- <u>cis</u>
.2% 11- <u>cis</u>

Under ordinary TLC conditions it would be difficult to detect either the 9-cis or 11-cis isomers.

Now Kropf and Hubbard² find an activation E of ~ 2 kcal. for the 9-cis to all-trans reaction. Therefore we have placed the 9-cis (0-0) level 2 kcal beneath the 9-cis A state.

There was a negative E_A for isomerization of the 11-cis isomer. We shall explain this below but now indicate that the 11-cis (0-0) level must be $\gg$ the 11-cis A height. We have assumed the equal sign.

Now using the known fluorescence maxima of the 13-cis and 9-cis compounds we have located the ground state of these compounds ~ 2 and 6 kcal higher than the ground state of the all-trans isomer. We do not observe a fluorescence from the 11-cis isomer, but by analogy with the 9 and 13-cis isomer have assumed a similar transition energy and therefore have located the 11-cis ground state ~ 8 kcal./mole over the all-trans ground state. This last value compares quite well with the calculated value of Karplus (15) of 6.5 to 7 kcal./mole for this distortion energy in 11-cis retinal.

As we indicated above, the negative E_A for the isomerization of 11-cis retinal must be rationalized. In our fluorescence work, at least with all-trans retinal, we have found that there is an activation energy associated with the emission intensity and as expected, at lower T we have a higher intensity. This means that some process which depopulates the excited singlet is decreasing as the temperature is lowered. This, in effect, we see an increase in the singlet population at lower temperatures and since the isomerization process is directly proportional to the 11-cis singlet concentration, the yield increases at lower T. We have measured an E_A of ~ 1.0 kcal./mole for the effect; Kropf and Hubbard measure ~ 1.5 kcal. We believe that within the limits of the experiments there energies are the same and probably reflect a solvent relaxation process.

Another feature of the diagram is the location of the $n-\pi^*$ states. These positions are completely unknown except that they very likely are between the $(\pi-\pi^*)$ singlet and triplet. Again in our study of the fluorescence intensity of the all-trans retinal species, we found that

increasing solvent polarity increased the emission yield. This is a test for the presence of an $n-\pi^*$ state. Further Dawson and Abrahamson¹⁶ have shown that conversion to the triplet state in all-trans retinal is reduced in polar solvents. A similar argument involving the $n-\pi^*$ state was invoked to explain this effect.

Finally our value for the distortion energy of the 11-cis isomer, 8 kcal./mole, seems to be in disagreement with Hubbards¹⁷ value of 1.1 kcal./mole. However, in Hubbards study, she assumed that when 11-cis retinal was isomerized, using heat and an I_2 catalyst, the equilibrium mixture contained 11-cis retinal. We have shown rather that 13-cis and all-trans retinal are to be found in the equilibrium mixture. Very likely the process that occurred was

Thus the 1.1 kcal./mole value of the distortion energy could apply to the 13-cis case. Our diagram has used a value of 2 kcal; certainly our assumptions are not exact enough to say that this is a real difference. Further, the assumption involved in measuring these quantities, as in Hubbard's study, must be restudied in light of our TLC findings.

Photo-Initiated Processes in Vision

Plans for Continued Research

Part I.

There are several experiments which should be carried out on the basic spectroscopic properties of the polyene pigments in support of the figure on page 10. These should be considered not as new projects but rather as continuations of present studies.

If we are correct in our assertion that 11-cis retinal is not formed to any large extent in the thermal isomerization of all-trans retinal (at least in non-polar solvent) then Hubbards' work bears further analysis. It may be possible using activated TLC plates to detect smaller quantities of 11-cis formed. If this is the case then it would be possible to carry out a thermal isomerization study and determine the energy separation in the ground state of the all-trans and 11-cis isomers. We know that this study can be done with the 9-cis isomer since it is formed in 3-5% yield at 23°C. Perhaps the removal of the TCL spot and its spectral assay could tell the level of the isomer present to within the required accuracy. Our preliminary study of the emission yield of the retinals and the protonated Schiff bases should be put on a more quantitative basis. Quantum yields should be measured as a function of temperature, polarity (dielectric constant) of the solvent, and acidity. This information would provide us with activation energies that may help in identifying the relaxation processes involved in each excited state. For instance we observed that there was a large difference between the emission intensities of retinal and retinal in highly polar solvents. We did not observe this

great difference using the Schiff base. In the former case the evidence is consistent with the removal of an intermediate $n-\pi^*$ state. The same phenomenon should occur with the Schiff bases, but it did not.

It is possible that our observation can be understood once the experimental variables are worked out. Possibly we had used an excess acid in the Schiff base case and actually were observing an emission from some species other than a simple protonated base.

Part II.

In order to obtain more information about the changes occurring in time periods 2 and 3 we propose to continue our fluorescence studies such as given in COO-1627-26. We also wish to extend this research using various labeling techniques. Both fluorescence and spin labels will be considered.

Fluorescence Labeling

Numerous fluorescent labels have been catalogued and their specificities analyzed. The dye ANS(1-anilino-8-naphthalene sulfonate) seems to bind to specific protein sites¹⁸ and report on environment polarity. Other dyes have been incorporated into model membranes and report on rotational movement (by polarization studies).¹⁹ Still other classes of dyes become fluorescent only after hydrolysis.²⁰

We propose to examine retinal materials and possible membranes²¹ constructed from rhodopsin that have been incubated with various dyes that have specific labeling properties. For instance, if we could label only the rod plasma membrane much as was done with the liver cell membrane²² and the blood cell membrane²³ we could perhaps identify changes in the structure

subsequent to the light flash. Our work would aim towards a kinetic analysis of the effect at various temperatures.

Another interesting area which we propose entering again will utilize labeling techniques or possibly protein auto fluorescences will be upon a small protozoa Spirostomum Ambiguum. Some work carried out this past year in Professor Borsellino's laboratory in Genoa led me to consider this contractile protozoa as a species which might provide information on the receptor process in general.

Spirostomum Ambiguum (Sp. Am.) has received some attention in the past. Thus its photodynamic response has been analyzed through observation of its spontaneous contraction.²⁴ In the course of some further work at the same laboratory Carlini²⁵ discovered that a biphasic receptor potential could be elicited from individual Sp. Am. during the course of their stimulated contraction. I observed that this signal was much like that of the RI and RII response of the retina upon stimulation. These early receptor potentials (RI and RII) are directly related to the rhodopsin chromophore²⁶. It is thus my hypothesis that a similar process in the two system produces the biphasic response.

Much is known of the general features of the Sp. Am. contraction process through the work of Ettienne²⁷. In particular it was found that Ca^{++} is released in response to the contraction stimulus (an electrical potential). The Ca^{++} output was measured using the chemiluminescent protein aequorin which emits light upon interaction with a calcium ion. Contraction began when the free calcium concentration reached a given level suggesting that the mechanism initiating the contraction may have used or bound the ion.

A similar experiment might possibly be attempted using a rod-cell. Aequorin could be introduced via a micropipette (since the outersegments of the Gecko are quite large these would be used). A flash of light at a wavelength sufficient for bleaching, but removed from the region of chemiluminescent emission, would stimulate the system. The time course of the emission if it was observed could possibly identify the intermediate which either controlled the Ca^{++} release or reacted with released Ca^{++} .

In addition to the above kind of experiment, it would be of interest to label the protozoa and observe label emission as a function of time. Again the time course of any changes could be correlated with the course of the contraction process.

Spin Labeling

It is a natural extension of the above work to consider the spin labeling²⁸ of visual materials. Some work of this nature has already been carried out by C. M. Lang who expects to be here for the summer of 1973. Under the auspices of an NSF Research Participation grant Dr. Lang studied several spin probes-2,2,6,6,-tetramethylpiperidine-1-oxyl (TEMPO) and 2,2,6,6,-tetramethylpiperidine-1-oxyl-4-ol (TEMPOL) and incorporated these labels into membranes constructed from ferri- and ferrochrome C and PE. It should not be difficult to incorporate these labels either into the rod-outersegment itself or into membranes made from rhodopsin. Again we will seek information on environment changes as a function of time and temperature that occur after a flash of light.

Publications Resulting From This Work

1. "Visual Pigment Fluorescence," A. V. Guzzo and G. L. Pool, *Science* 159, 312 (1968).
2. "Fluorescence Spectra of the Intermediates of Rhodopsin Bleaching," A. V. Guzzo and G. L. Pool, *Photochem. Photobiol.*, 9, 565 (1969).
3. "Energy Transfer to the Triplet State of All-trans Retinal," A. V. Guzzo and G. L. Pool, *J. Phys. Chem.*, 73, 2512 (1969).
4. "Fluorescence Properties of Visual Pigments," by A. Guzzo, G. L. Pool and C. Leman, in "Photochemistry of Macromolecules," (1970) Plenum Press, N.Y., P. 105.
5. "Singlet-Triplet Absorption Spectrum of All-trans Retinal," by R. Raubach and A. Guzzo, *J. Phys. Chem.*, 75, 983 (1971).
6. "The Radical Anions of Vitamin A Aldehyde and Related Schiff Bases," by C. Lang, J. Harbour and A. Guzzo, *J. Phys. Chem.*, 75, 2861 (1971).
7. "ESR Spectroscopy of Non-Cyclic Enones," by J. Harbour and A. Guzzo, *Mol. Phys.*, 20, 565 (1971).
8. "Anion Radicals of Non-Cyclic Enones; An Example of Non-planarity in a Pi radical," by J. Harbour and A. Guzzo, *Mol. Phys.*, 23, 151 (1972).

Articles Submitted

"Light Induced Changes in the Rhodopsin Protein Fluorescence," by A. V. Guzzo, to *Photochem. Photobiol.*

"Photoisomerization Pathways in the Visually Important Polyenes," Parts I and II, by R. Raubach and A. Guzzo to *J. Phys. Chem.*

Training Aspects

I. Graduate Students Trained and Degrees Granted From the Period August 15, 1969 to August 14, 1972.

1. Calvin B. Leman, Ph.D., August 1969, now at St. Joseph's College, West Haven, Connecticut.
2. C. Marvin Lang, Ph.D., August 1970, now at Wisconsin State University, Stevens Point, Wisconsin.
3. John Harbour, Ph.D., June 1971, now postdoctorate, U. of Arizona, Tucson.
4. Rodger Raubach, Ph.D., June 1972, now postdoctorate, U. California, Santa Cruz, California.
5. Nui Oi Cheng, M.S., August 1970.

II. Post Doctorate Tenures.

None.

Present Division of Federal Support

Work in this area has been entirely supported by the AEC Contract No. AT(11-1)-1627. No other source of federal funds was available.

Needed Future Investigation

Much now seems to be known concerning the basic isomerization mechanism and spectroscopic properties of the retinals and of the protonated Schiff base model compounds. It would, however, seem that the latter compounds are not well characterized with regard to the question of what is

protonation and how does the anion affect the properties of the compound.

More work should be carried out on cellular systems and on basic excitable membrane phenomena.

Further studies then should possibly include:

- 1) ORD and CD studies on intact, excitable receptor cells and excitable membranes.
- 2) Synthetic membrane studies using membranes constructed from proteins and lipids. The proteins might have certain known structural changes with external variable and these could be correlated with the physical properties of the membrane, i.e., synthetic excitable membrane studies.
- 3) Studies on the state of water in excitable tissue via NMR and NMR spin echo.
- 4) Labeling studies on excitable cells to probe for structural events related to excitation.

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