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PHOTO-INITIATED PROCESSES IN VISION

Technical Progress Report

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ABSTRACT

Our investigation of the photo-initiated processes in vision have proceeded along two main lines--changes in the retinal system detectable by emission spectroscopy and changes detectable by ESR techniques. Preliminary work from both areas seems encouraging in that the data obtained reflect the presence of systems whose function could relate to visual excitation mechanisms. Our present effort is devoted to the study of these systems and their possible relationship to the scheme of transformations described by Wald.

TABLE OF CONTENTS

- I. Abstract
- II. Results obtained in the ESR investigation of retinal components
- III. Results obtained via emission spectroscopy of retinal components
- IV. Significant differences between present project level and project originally proposed
- V. Bibliography

I. Results obtained in the ESR investigation of retinal components

The motivation behind the present ESR measurements on components of the visual system stems from the work of M. A. Ostrovskii^{1, 2, 3}. His original studies showed that an ESR absorption could be observed by the visible light irradiation of frog retinas. Although he attempted to show that the pigment epithelium was not involved in the generation of this signal, the work was not conclusive. The epithelium has an ESR signal of its own and has been investigated extensively by Cope and co-workers^{4, 5} who claim that the observed ESR absorption in the retina must be due to remnant fragments of epithelium adhering to the retina.

Our initial studies were carried out on cattle retina (not dark adapted, but isolated and used under dark conditions) and the light sensitive narrow line that Cope and Ostrovsky observe can easily be observed. In order to see if this signal remained in any of the retinal components, about 50 retina were fractionated to obtain, according to Wald's procedure⁶, relatively pure rod outer segments. The ESR signal detected previously appears to remain but diminished greatly in these cell fragments. The persistence of this signal in the rod outer segments might be interpreted as evidence of the intracellular origin of the free radicals responsible, rather than a physical adherence of epithelium to the fragments. The localization of ubiquinone in rod outer segments would support this conclusion⁷, since the photo-production of semiquinones as is postulated in epithelium melanin might occur in the visual cell also.

In addition to the narrow line there is also observed a broad underlying absorption, which may be due to the presence of metal ions. In order to verify this, several large (1 gm) samples of rod outer segments were ashed (using both porcelain and platinum crucibles), dilute HCl added and the ESR from such solutions observed. The signal observed clearly indicated the presence of copper and manganese⁸. Chemical spot tests verify this conclusion indicating roughly one part in 10^5 in the original sample of both metals. It is not known at the present time what the function of the metals is but it is assumed that they are present as co-factors in some as yet unknown enzyme systems.

The data gathered in experiments with albino rat retinas and cattle retinas are listed in Table 1. In general the lack of positive results indicates that clearly a radical mechanism in visual excitation has not been substantiated; however, our work is not extensive enough to rule out the mechanism. If the metals mentioned above have a function in the excitation pathway, one might not detect any change in their ESR signal at room temperature since the whole process of excitation would be over in a few milliseconds. At low temperatures (-176°), on the other hand, the process, if enzyme controlled, might not occur at all. One suspects that some optimum temperature might exist where the radical, if generated, might live for several seconds. We are presently examining the ESR absorptions of intact cattle retinas at intermediate low temperatures with this in mind.

If we allow a preparation of rod outer segments to dry and insert a section of the resulting compact into the EPR cavity, the signal observed is anisotropic. This signal is illustrated in Figure 1 for two orientations of the sample in the magnetic field. This observation apparently indicates that a certain amount of orientation of the rods has occurred in drying and in so doing has caused an alignment of some metal complex, probably a copper-protein complex. Further, if the same procedure is carried out with the digitonin-rhodopsin complex, we again obtain an anisotropic EPR signal. The digitonin micelles in this case have probably aligned to produce the anisotropy. Mixing of the sample disrupts the alignment and only a very weak EPR signal Figure 1c can be observed.

The EPR cavity utilizes the rectangular TE₁₀₂ mode and in some cases anisotropies can be introduced into the output signal if the sample shape or placement is improper. We are currently investigating this point using paramagnetic standards pressed into various shapes. In addition we are attempting to show the possible alignment of the rods and digitonin micelles in the dried samples using light and electron microscope techniques.

ESR of ROD COMPACT
a, b) Two Orientations 90° Apart
c) Mixed

Figure 1

II. Results obtained via emission spectroscopy of retinal components.

There are several possible pathways of study that could be provided by an examination of the emission spectroscopy of components of the visual system. Proteins containing any of the aromatic amino acids fluoresce and phosphoresce and this property may be applied in the determination of configuration changes in the protein induced by selected treatments. Since one of the mechanisms proposed to account for the onset of visual excitation involves a structural change in rhodopsin, it seemed worthwhile to investigate the emission characteristics of this protein. Secondly, the concentration of DPNH and DPN is known to be quite high in the retina and since DPNH fluorescence can be easily observed, a study of the changing concentration of this compound with various stages of bleaching of the retina should be possible. This information should be valuable in that such measurements should verify whether DPNH is utilized only in the enzymic regeneration of rhodopsin or has an additional function.

Our studies so far have been of a cataloging nature in that we have recorded most of the observable emissions and excitations from retinal components, i.e. rod outer segments, and retinas. The data collected so far is given in Table 2 along with similar information on DPNH and DPN.

We are presently engaged in completing the table and in a close examination of those emissions which appear photo-sensitive. The peak at $455 \text{ m}\mu^*$ seems to be due to DPNH since its pH fluorescence dependence follows that given for DPNH in vitro. Qualitatively the intensity of the peak increases after the sample, rods or retina, is exposed to a flash of white light; after a few seconds the peak is observed to decrease in intensity to a level somewhat below its initial value. Clearly the action spectrum of this effect will be highly informative.

The emission at $350 \text{ m}\mu^*$ appears photo-sensitive however our initial results indicate the effect is variable. This peak arises in the protein and changes in intensity may reflect environment changes influencing the emitting chromophore. Such changes may be directly related to possible excitation mechanisms.

Recent work utilizing interference filters to cut down on scattered light has given evidence of fluorescences at longer wavelengths. These peaks, possibly at $525 \text{ m}\mu$ and $550 \text{ m}\mu$, must be examined carefully under more precise conditions.

* Uncorrected wavelengths

Sample	Observable EPR	Conditions
Rat Retina-2	no	Dark adapted, at 25°C or -176°C Samples too small.
Cattle retina-1	yes narrow line photo-sensitive	Retina as obtained from Armour, 25°C or -176
Cattle Rods	weak narrow line weak broad line	prepared According to Wald, 25°C and -176°C
Rhodopsin-Cattle	broad line	Freeze dried only, solution in digit- onin is too dilute

Table I. Samples used in the investigation of possible EPR activity in the visual system.

Sample	Excitation maxima - mμ	Emission maxima F or P	Conditions	Source
Rat retina	250, 290	347F	Rm.T	protein emission
Rat retina	310, 338	445F	Rm.T	DPNH?
Rod outer segments	same as above			
Rat retina	250, 290	395P, 410P, 440P, 455P, 495P	-176°C	protein phosphorescences
Retinol	349, 375	470F	ethanol- methanol mixture	
Retinal	320, 400, 440	530F	same	
Retinol	325, 338	455F	water- ethanol mixture	
Dph	340	470		

Table 2. Emission and excitation Data for components of the visual system. All wavelengths are in mμ and are uncorrected for spectrometer non-linearity. F denotes fluorescence and P phosphorescence.

Significant Differences between Original Proposal and Present Project

The specific aims of the original proposal were the following:

a) To investigate the photo-induced electron spin resonance absorption of components of the eye and determine the lifetimes of the radicals produced, their action spectra, temperature dependence, and under ideal conditions, the nature of the radical.

b) To investigate the photochemistry of the derivatives of vitamin A and the carotenes, using E.S.R. and spectroscopic techniques to attempt a clarification of what species and states are generated and the possible quenching effects of donors or acceptors.

c) To investigate the photoconduction properties of the vitamin A and carotene donor and acceptor complexes and determine the nature and properties of the charge-carrying species.

Of these topics, project a) has received the major effort while initial work on b) has begun. A fourth related area was proposed which appeared to have great promise, that of the emission spectroscopy of retinal components. The one graduate assistant requested was given this project, since it seemed most suitable for him. The level of activity of the project has been that originally proposed although funds to support the graduate assistant, the technician, and the principal investigator's summer salary were not required since these were funded from other sources. Approval was obtained from the AEC to divert some of these funds to the purchase of a spectrofluorometer. Aside from this major purchase, the level of expenditure was essentially that proposed.

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