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12 PERSONAL AUTHOR(S)
Dr. Paulter

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The phototoxic effect of blue light on isolated pigment epithelium will be investigated. The emphasis will be on functional changes rather than a description of the pathology. The pigment epithelium is analogous to the blood-brain barrier; therefore, the principal functions to be investigated are the integrity of the barrier system and the transport system and the transport systems known to operate in the pigment epithelium.

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ANNUAL TECHNICAL REPORT FOR AFOSR-87-0189

THE PHOTOTOXICITY OF 'BLUE LIGHT' ON THE FUNCTIONAL PROPERTIES OF THE RETINAL PIGMENT EPITHELIUM

ORIGINAL STATEMENT OF WORK

The phototoxic effect of blue light (435 nm) on isolated pigment epithelium will be investigated. The emphasis will be on functional changes rather than a description of the pathology. The pigment epithelium is analogous to the blood-brain barrier; therefore, the principal functions to be investigated are the integrity of the barrier system and the transport systems known to operate in the pigment epithelium.

Since the damaging effect of blue light on the pigment epithelium is known to be mediated by a photodynamic action, an oxygen-dependent process, the bathing solutions will be aerated with 95% O_2, 5% CO_2. An established ionic solution rather than a media with a serum supplement will be employed to avoid the possible sensitizing effect of molecules in the media/serum.

The tissue will be mounted in a Ussing chamber which permits measurements of the transepithelial potential and short circuit current. From these electrical parameters the specific resistance can be calculated which is a measure of the "leakiness" of the epithelium. Significant reductions in the specific resistance indicate a failure of the barrier system either by a loosening of the tight junction between epithelial cells or physical damage to the membrane systems. The barrier system will be further analyzed by the employment of radioactive tracers of passive diffusion such as L-glucose. (See Fig. 1)

The unidirectional fluxes in both the retina to choroid (R C) and choroid to retina (C R) direction will be determined for transport systems representative of: 1) facilitated diffusion, 2) Na-dependent active transport systems requiring ATP, and 3) Na-independent concentrating systems which do not require ATP. A minimum of five experiments in each direction (R C and C R) will be performed. The transport of D-glucose will be used as an example of facilitated diffusion and glutamate transport for a Na-dependent active transport system. The L transport system for leucine has been identified in the pigment epithelium and serves as a model for an Na-independent, concentrating transport system with at most a minimal requirement for ATP.

These studies will be first conducted under exposure conditions to blue light which have been established by other investigators to be at or near threshold for pathological changes to occur. It is anticipated that functional changes can be detected before pathological events are observed. If changes in barrier or transport properties are noted at this threshold, the studies will be replicated at a lower intensity of blue light. In any event, an intensity one order of magnitude above the described threshold will also be studied.

When an intensity of blue light has been established to significantly alter any or all of the measures of pigment epithelium function, four agents will be investigated for possible protective or therapeutic effects. This will require pilot studies to ascertain the pharmacological dose to be investigated. The agents to be utilized are alpha tocopheral, ascorbic acid, melatonin and

querecetin. These will be utilized under two conditions. First, they will be administered only during the exposure period, and then the barrier and transport properties measured. Second, they will be administered immediately after the exposure to blue light to ascertain if any therapeutic action is in evidence.

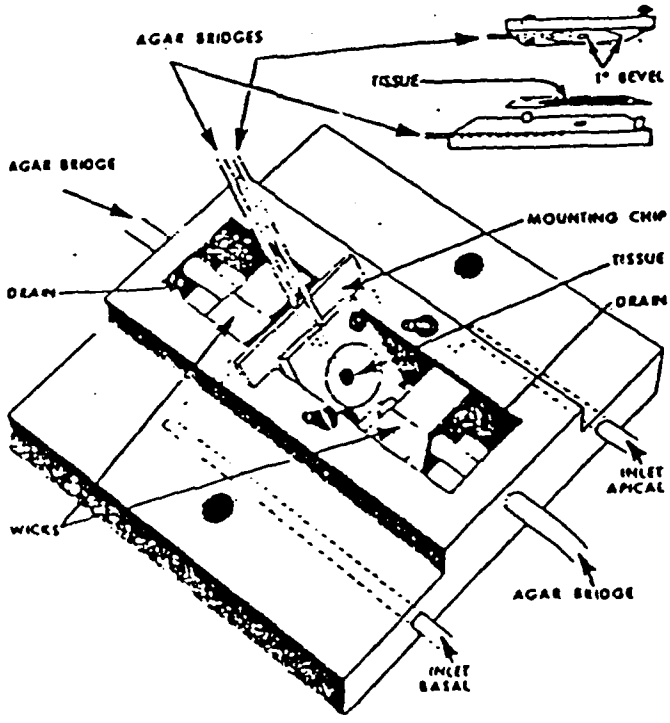

FIG. 1 Schematic view of the Ussing chamber which does not include the thermostatic heating elements.

Summary

The effects of blue light (430 nM, 20 nM/cm^2) on leucine (10 M) transport across the isolated bovine retinal pigment epithelium (RPE) have been continued to determine if Vitamin E and melatonin provided any protective action. Similar studies on the transport of glutamate (10 M) in the retina to choroid direction were also completed.

It was found that blue light diminished the transport of glutamate and that melatonin (20 nM) provided significant protection against blue light for both leucine and glutamate in terms of the transport rates. Vitamin E did not provide any protection against the adverse effects of blue light on the transport of either amino acid. However, it was noted in control studies that Vitamin E succinate inhibited the transport of leucine in the R-C direction when applied only to the basal (choroidal) side of the RPE whereas glutamate transport was not affected by similar treatment. It seems likely that incorporation of Vitamin E into the lipid phase of the basal membrane selectivity diminishes the leucine efflux transport system of the RPE.

Further studies on the change in the transepithelial potential (TEP) elicited by blue light were initiated. It now appears there are two components involved in the potential changes and that the second component tends to nullify the initial depolarizing act[illegible] of blue light. It is hypothesized that ethanol selectively interferes with the second component. The manifestation of the second component is being analyzed in terms of the membrane "cross talk" characteristic of polarized epithelial cells.

[illegible] physiology; radiation [illegible]
[illegible] biology; (K⁺)

Table I

Effects of Melatonin and Vitamin E Succinate on the Transport of Leucine and Glutamate

Condition	Unidirectional flux of Leucine
Control	1.64 ±.11
Blue light - 430 nM	1.33 ±.16
(20 mW/cm_2)	
Melatonin	1.67 ±.14
Melatonin with Blue Light	1.60 ±.15
Vitamin E succinate	1.24 ±.05
Vitamin E succinate with Blue Light	1.09 ±.09

Condition	Unidirectional flux of Glutamate
Control	1.76 ±.17
Blue Light -430 nM	1.31 ±.15
(20 mW/cm^2)	
Melatonin	1.87 ±.18
Melatonin with Blue Light	1.80 ±.16
Vitamin E succinate	1.73 ±.06
Vitamin E succinate with Blue Light	1.45 ±.12

During the first year, as outlined in the previous technical report, we determined that potentially toxic intensities of blue light reversibly reduced the transepithelial potential and short circuit current of the retinal pigment epithelium (RPE). The action spectrum revealed a Soret band thus indicating a hemoprotein is the sensitizing agent. From the spectral characteristics, cytochrome oxidase is considered a likely candidate. Concomitant with changes in the electrical parameters there is a reversible change in chloride transport during exposure to blue light. Leucine transport is also inhibited but with a delay of 10-40 minutes after the onset of illumination. The flavonoid, morin, was found to provide some protection against the inhibitory effect of blue light on leucine transport. Ascorbate was not effective at the concentrations employed. Concentrations of ethanol as low as .12% was found to enhance the effects of blue light on the electrical and transport properties of the RPE.

During the second year the possible protective effects of melatonin and Vitamin E succinate were assessed on leucine and glutamate transport. The results are shown in Table I. First, irradiation of isolated bovine RPE with 20 mW/cm^2 of blue (430 nM) light reduced the transport of both leucine and glutamate. Melatonin (20 nM) provided significant protection against the effects of blue light on the transport of both amino acids. We had previously observed that this concentration of melatonin mobilized the melanin granules of the RPE to the apical surface and with actual entry into the apical processes. The protective effective against blue light probably results from melanin acting as a light filter and reducing the intensity of light impinging on the intracellular organelles and constituents. Vitamin E (α8μg/ml0) did not provide any protection against the effects of blue light on the transport of either leucine or glutamate.

While conducting control studies with Vitamin E TPGS 6-α tocopheryl polyethylene glycol 1000 succinate at 8 μg/ml, it was observed that leucine transport was reduced in the R-C direction. Subsequent studies revealed that leucine transport was inhibited only when Vitamin E was administered to the basal side of the RPE (Fig. 2). Application to the apical side was ineffective in altering transport. Control studies with polyethylene glycol 1000 alone indicated that inhibition could be attributed to Vitamin E succinate. The selectivity of this inhibitory effect on transport was established by showing that glutamate transport was not impaired by the presence of the vitamin preparation. The observation that vitamin E can modulate leucine transport adds to the growing body of knowledge suggesting that vitamin E serves as a significant regulator of biological processes in addition to its well established role as an antioxidant and free radical scavenger.

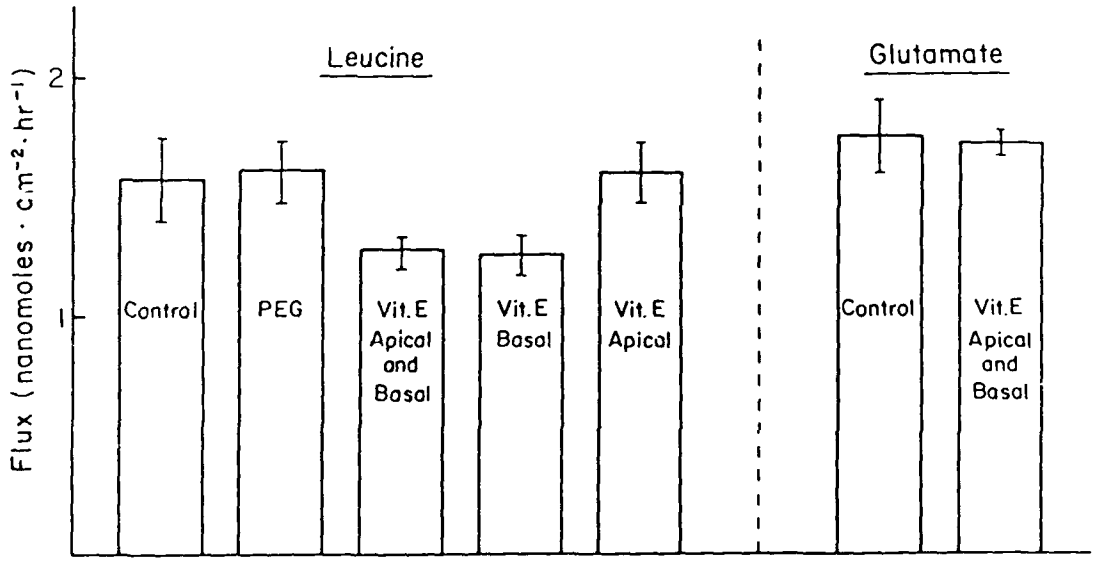

Fig. 2 The effects of Vitamin E succinate on the transport of leucine and glutamate in the retina to choroid direction. Control studies with the vehicle were without effect on transport whereas application of Vitamin E TPGS (8μg/ml) to the basal (choroidal) side significantly reduced leucine transport but had no effect on the transport of glutamate at the same carrier concentration (10^{-5} M). Data is present as mean $\pm$ SD with $N \geq 5$.

In addition the studies directly focused on the specific aims of the grant, we compared our action spectrum with absorption spectra ofsuspected sensitizing molecules of the RPE, and, as previously reported, found a close correspondence with cytochrome oxidase (Fig. 3) and were able to exclude riboflavin retinal/retinol and melanin, all of which have been considered as possible sensitizers. Ham (1) has published an action spectrum based on minimal lesions produced by UV-A and visible comparing our action spectrum with that of Ham's (Fig. 4), we can see a very close correspondence between 400 and 500 nM but a large discrepancy exists in the UV-A region. This immediately suggests that damage to the RPE and retina by UV-A is mediated by mechanisms distinct from those involved with the phototoxicity of blue light. One possibility is suggested by the work of Cook (2) on the photohemolysis of red blood cells which are much more sensitive to hear UV-A than visible light. Cook established that photohemolysis by visible light required a photodynamically active pigment in the cell whereas the lesions produced by near UV were oxygen independent.

Further studies on the depolarizing effect of blue light on the TEP indicates there is a second component involved in the potential change. A control recording for the effects of ethanol alone is shown in Fig.5. Administration of 0.5% ethanol results in an initial decrease in the TEP followed by a more prolonged increase. There is no sustained diminution of the TEP under control conditions. This is similar to the effects of ethanol on the steady potential of the eye studied by Skoog et al (3). The response shown is typical of most isolated bovine RPE although there is considerable variability in the magnitude of the potential changes elicited by ethanol. The effects of ethanol in conjunction with blue light irradiation are illustrated in Fig. 6 and 7. In Fig. 6, the onset of blue light results in an initial decline in the TEP but starts to recover about half way through the period of irradiation. After

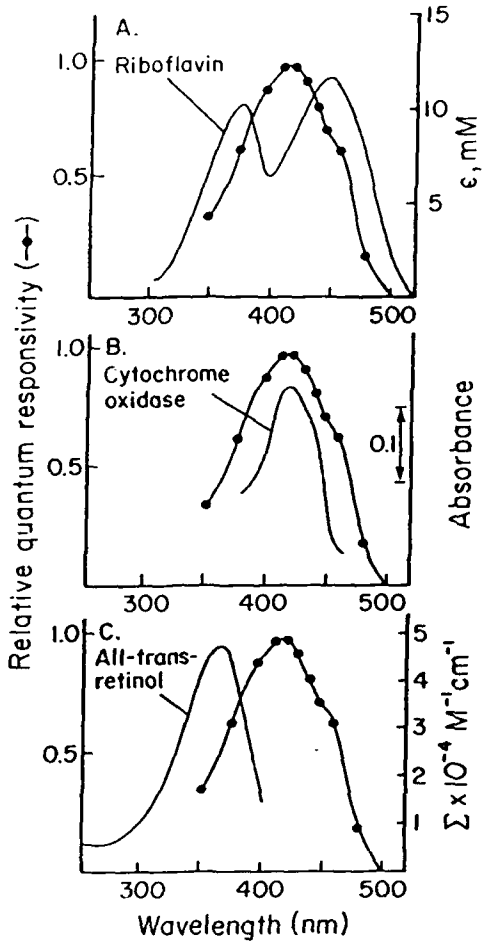

Fig. 3 The action spectrum derived from the depolarizing effect of blue light on the TEP of the bovine RPE is compared to the absorption spectra of possible sensitizing molecules. The relative quantum responsivity refers to the action spectrum whereas the extinction coefficients and absorbance scales are related to the absorption spectra. The cytochrome oxidase spectra compares well with our action spectra which precludes the other molecules as mediators of blue light damage.

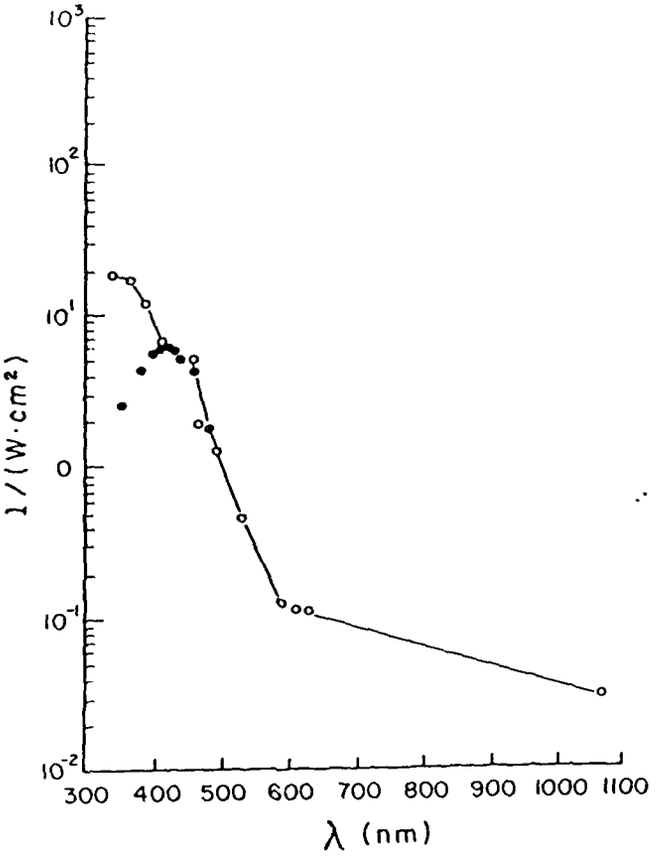

Fig. 4 The action spectrum based on minimal damage to the retina and RPE obtained by Ham (-O-) is compared to our action spectrum (●). There is very close agreement between 400-500 nM but a serious departure exists in the UV-A region suggesting a different mechanism.

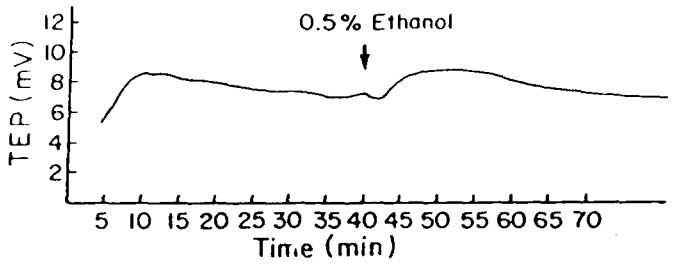

Fig. 5 A control recording to illustrate the effects of 0.5% ethanol alone on the TEP of the bovine RPE. Ethanol results in a slow biphasic potential change but does not impair the TEP when administered alone.

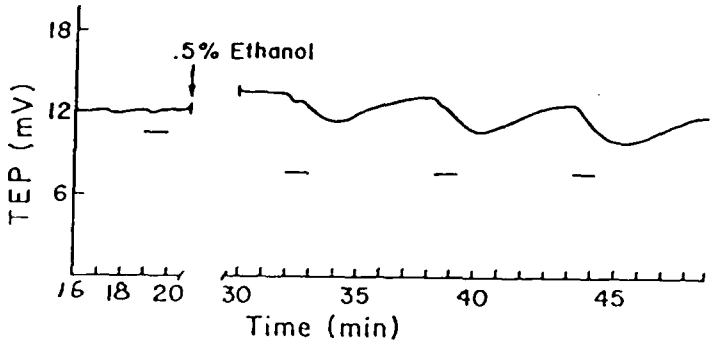

Fig. 6 A recording of the TEP to illustrate the effects of ethanol on the potential elicited by blue (430 nM) light of 20 mW/cm^2. In the control recording, with irradiation indicated by the bar, there is a brief depolarization followed by a recovery of potential during the irradiation period. After ethanol treatment, the first response to blue light reveals a "notch" or tendency to recover which disappears in subsequent exposures to blue light.

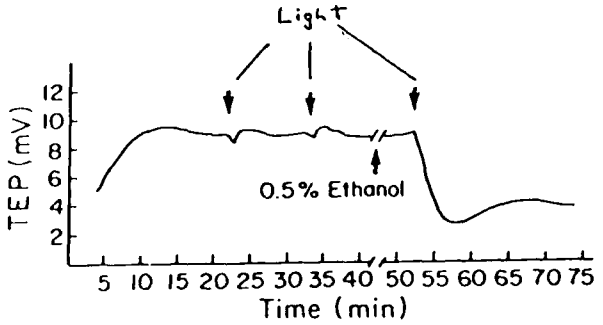

Fig. 7 Two minute irradiation periods with intense white light results in the dominance of the second component. After ethanol treatment, the TEP is greatly reduced by intense light with no evidence of the second component.

ethanol treatment, the first response still shows a "notch" or tendency to recover during the first period of irradiation but disappears with repeated exposures as ethanol exerts its full affect which takes about 20 minutes. The disappearance is not a mere bleaching phenomenon since, without ethanol, it will persist for either repeated or prolonged exposures. With intense white light, the positive component can dominate as shown in Fig. 7. After administration of ethanol, irradiation only produces a large depolarization as if the positive component had been eliminated. This is somewhat similar to Granit's classic method of analyzing the ERG into components.

In order to analyze this apparent compensatory response one must recognize that epithelia are polarized tissues in which the apical and basal membranes are provided with different ionic and substrate transport properties. The TEP is fundamentally the difference between the apical and basal membrane potential with adjustments for shunt pathways. Diamond (4) has reviewed the transcellular cross-talk between epithelial cell membranes and discussed how inhibiting the basolateral pump reduces ionic permeability of both basolateral and apical membranes. Other examples of cross-talk are noted when apical sodium channels are blocked with amelioride, the basolateral potassium conductance is decreased as rapidly as apical sodium conductance. Also increased chloride transport is associated with increased transepithelial conductance which apparently involves increases in basolateral potassium and in apical chloride conductance. In terms of physiological mechanisms, more recent studies (5) have shown that during secretion, apical chloride and basolateral potassium conductance increases in order to prevent a prolonged depolarization which would prevent chloride exit through the apical membrane and to avoid accumulation of potassium which could lead to increases in cell volumes. Diamond speculates that calcium may function to integrate these conductance changes; however, intracellular pH (pHi) could

also serve as a regulator. Both of these signals are intimately involved with the metabolism of the cyclic necleotides and phosphoinosides. The major point is that compensatory membrane changes are well established in epithelia and the events we observe with blue light irradiation are not unique.

Pilot studies have been conducted seeking out possible factors which mediate the depolarizing and recovery effects of blue light on the TEP. Although very preliminary, the following observations were made.

1. The decrease in the TEP with blue light appears to be dependent on Cl^- in the incubating solution whereas the recovery of TEP after cessation of light seems to required sodium.
2. The calcium ionophore at 5 μM concentrations appears to enhance the second component.
3. Administration of trimethyl amine (5 mM) which presumably alkalinizes the cells may depress the second component although not nearly as effectively as ethanol.
4. With the α-1 stimulator, .1 mM phenylephrine, applied to the basal (choroidal) side of RPE, a remarkable stabilization of the TEP extending for two hours is seen. This pharmacologically induced potential is very sensitive to ethanol which is similar to that of the second component elicited by blue light irradiations (Fig. 8). These pilot studies reveal the complexity of the regulatory mechanisms and suggest that such variables as pHi and calcium levels should be investigated and the involvement of the phosphoinotides be considered.

The hypothesis that ethanol interferes with compensatory cellular responses to stress has some interesting implications. It is well recognized that a leading cause of death from drugs is alcohol "in combination". The above hypothesis suggests that the toxicity of alcohol "in combination" may not result

from an enhancement (synergistic or additive) of the direct effect of another drug such as a barbiturate but, instead, eliminates a homeostatic response which is required to maintain the integrity of the cell under stress.

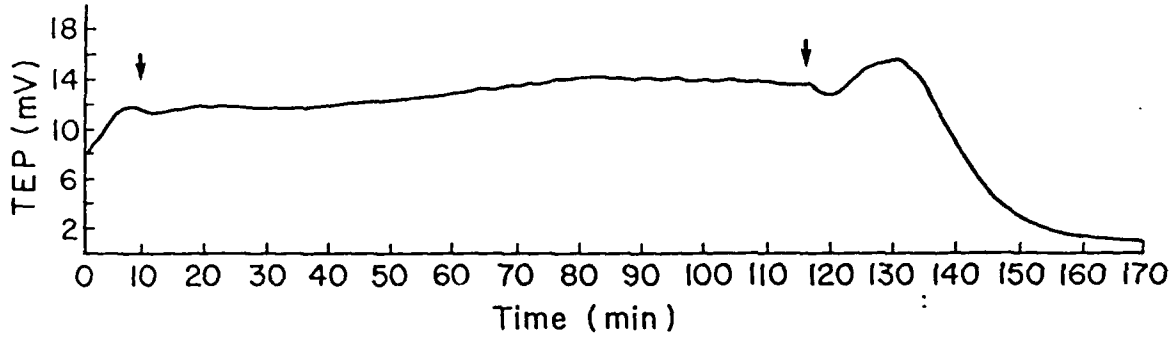

Fig. 8 When the bovine RPE is treated with .1 mM phenylephrine only on the basal (choroidal) side (first arrow), the TEP is remarkably well maintained with some increase during a two hour period. This pharmacologically induced TEP reveals a sensitivity to 0.5% ethanol (second arrow) not exhibited in untreated tissues.

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Personnel

1. E. L. Pautler, Ph.D.

   Professor, Department of Physiology

2. M. Morita, Ph.D.

   Research Scientist, Department of Anatomy

3. D. Beezley, Ph.D.

   Research Associate, Department of Physiology

4. Paul Skutches

   Hourly Labor

5. Peter Ingrassia

   Hourly Labor

6. Joseph Beyer

   Hourly Labor

Publications

1. Paper presented by invitation

   a. Reversible and irreversible blue light damage to the isolate, mammalian pigment epithelium. 8th International Congress of Eye Res., San Francisco, CA, 1988.

   b. Phototoxicity of blue light on the retinal pigment epithelium: effects of ethanol. 6th International Neurotoxicology Conference, Little Rock, AR, 1988.

2. Journals and Books

   a. Pautler, E. L., Mortia, M., and Beezley, D.

   Reversible and irreversible blue light damage to the isolated, mammalian pigment epithelium.

   In International Symposium on Retinal Degenerations, LaVail, M., Anderson, R. E. and Hollyfield, J. (eds.). Alan R. Liss, Inc. (in press).

   b. Pautler, E. L., Tengerdy, C., Beyer, J. and Beezley, D.

   Modification of leucine transport across the bovine pigment epithelium by metabolic stress. Amer. J. Physiol. (accepted with rewrite).

   c. Pautler, E. L., Morita, M. and Beezley, D.

   Cytochrome c-oxidase is a mediator of blue light damage to the retinal pigment epithelium. Submitted to Science.

   d. Pautler, E. L. and Beezley, D.

   Inhibition of transepithelial transport of leucine by Vitamin E succinate. Submitted to Nature.