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**Effect of Ultraviolet Radiation on
the Energy Metabolism of the Corneal Epithelium
of the Rabbit**

(Reprint)

By

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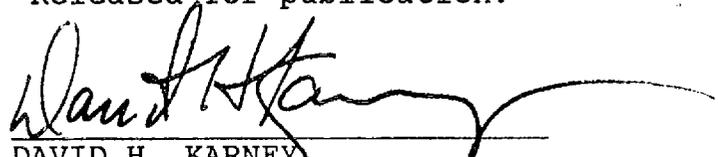
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EFFECT OF ULTRAVIOLET RADIATION ON THE ENERGY METABOLISM OF THE CORNEAL EPITHELIUM OF THE RABBIT

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Abstract—The present research was directed at quantifying possible alterations in corneal epithelial metabolic activity secondary to *in vivo* exposure to ultraviolet radiation (UVR). Microfluorometric energy metabolite assays on microgram (μg) sized, freeze-dried tissue samples were used as an *in vitro* means of assessing overall metabolic activity in the epithelium of control rabbit corneas and in the epithelium of UVR-exposed rabbit corneas 2 min after discontinuation of exposure. The specific assays were for glucose, glycogen, adenosine triphosphate (ATP), and phosphocreatine (PCr). The radiant exposures were kept constant at 0.05 J cm^{-2} for all UVR wavelengths utilized (290, 300, 310 and 360 nm). Experimental UVR exposure conditions served to increase epithelial glucose and glycogen concentrations. Although the epithelial ATP concentrations were unchanged, the epithelial PCr concentrations (a high energy phosphate bond reservoir) decreased as a result of UVR exposure. Overall, the data demonstrate a decrease in corneal epithelial metabolic activity, which may be wavelength-dependent, as a result of UVR exposure. It is suggested that immediate metabolic stress can be responsible for the pattern of epithelial cell loss seen in photokeratitis.

INTRODUCTION

Many investigations into the effects of ultraviolet radiation (UVR)* on the corneal epithelium have concentrated on morphological evaluations utilizing the biomicroscope, the light microscope, and/or the electron microscope (Verhoeff and Bell, 1916; Cogan and Kinsey, 1946; Pitts and Tredici, 19971; Ringvold, 1980; 1983). Such studies have provided information concerning the delayed structural changes characteristic of UVR damage that occur 4 to 12 h after exposure. As a result of the histological detection delay, information concerning either immediate or functional effects of UVR cannot be probed by such studies.

Delays in structural changes secondary to UVR exposure can imply that there is a similar delay in alteration of tissue function or in metabolic activity. Clinically, this has outwardly been suggested by the presence of a delay or latency in the onset of both the subjective and objective symptoms associated with excessive UVR exposure (i.e. conjunctivitis, erythema, foreign body sensation, photophobia, epiphora, and blepharospasm). However, Millodot and Earlam (1984), in their investigation of this phenomenon, revealed the presence of a period of decreased corneal sensitivity immediately following

exposure to UVR. Their finding appears to signify an immediate effect of UVR upon the sensory neurons subserving the corneal epithelium. If such is the case, and knowing that these axons appear deep within the basal cell layer of the corneal epithelium and within the anterior stroma, it would be reasonable to assume that there might also be an immediate effect of UVR on the corneal epithelium itself. Knowledge of these immediate effects might help reveal the underlying processes or chain of events that eventually cause epithelial cell death and delayed tissue loss characteristic of excessive UVR exposure of the cornea.

The purpose of this study was to evaluate the immediate effect of exposure to UVR on the energy metabolism of the corneal epithelium in the rabbit. This approach is not novel; a number of studies have investigated corneal metabolism and UVR (Reim *et al.*, 1977; 1978). However, earlier studies were conducted 30 min after UVR exposure and utilized bulk tissue extraction methods, which prevented the performance of multiple energy metabolite assays on the same cornea. In this study, specific microfluorometric assays (utilizing techniques detailed by Lowry and Passonneau (1972) and modified by McCandless (1985)) were performed for the epithelial energy metabolites glucose, glycogen, adenosine triphosphate (ATP), and phosphocreatine (PCr). The radiant exposure was kept constant at 0.05 J cm^{-2} for all UVR wavelengths utilized (290, 300, 310, and 360 nm).

The four experimental wavelengths were chosen based on an interest in maintaining an environmental relevance, since 290 nm UVR and above can be found at the earth's surface (Petit, 1932). An

*Abbreviations: ATP, adenosine triphosphate; H_c , corneal thresholds for histological damage; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NBS, National Bureau of Standards; NIH, National Institutes of Health; PCr, phosphocreatine; UVR, ultraviolet radiation; Xe-Hg, xenon-mercury.

additional factor was the intention of creating a distinctive span of effects; the corneal thresholds for histological damage (H_c) vary considerably. The corneal radiant exposure H_c in the rabbit ranges from 0.012 J cm^{-2} at 290 nm, to 0.022 J cm^{-2} at 300 nm, to 0.05 J cm^{-2} at 310 nm, to near 65 J cm^{-2} at 360 nm (Pitts, 1970). By varying the wavelength and maintaining a constant level of radiant exposure at 0.05 J cm^{-2} it was anticipated that metabolic effects would vary from severe at 290 nm, to moderate at 300 and 310 nm, to minimal or non-existent at 360 nm. Lastly, the source output happens to peak in these regions, thereby helping minimize some of the time differences inherent in a noncoherent source exposure.

MATERIALS AND METHODS

Experimental animals. Healthy, adult, Dutch-belted, pigmented rabbits were used as the experimental animals. The animals were housed in NIH-approved quarters under controlled, normal lighting conditions. The animals were maintained and the experiments conducted in accordance with procedures outlined in the *Guide for Laboratory Animals Facilities and Care* of the National Academy of Sciences-National Research Council. Anesthesia was maintained throughout the course of the experiment with intramuscular injections of Ketamine (10 mg/kg) and Rompun (5 mg/kg). The UVR-exposed animals were sacrificed by cervical dislocation 2 min after exposure was discontinued. The eyes were then immediately removed and immersed in liquid nitrogen in order to prevent significant change in metabolite levels. Control animals, after a mock-exposure period under the same level of anesthesia as the experimental animals, underwent the same tissue preparation procedures.

Processing procedure. The rabbit eyes were transferred from the liquid nitrogen container into a -80°C freezer for storage until tissue processing could be accomplished. The cornea was removed from the globe by dissection under -30°C conditions in a Wedeen cryostat. The isolated cornea was cut into halves, which were mounted on sectioning buttons by immersion in a dry ice-cooled hexane solution. The corneal button-mount then was transferred to a cryostatic microtome where tissue sectioning was performed. The resulting central cornea cross sections were approximately $20 \mu\text{m}$ in thickness. The sectioned tissue samples were placed in a metal tissue holder, covered with glass slides, and inserted into a vacuum tube. The tube was placed in a -20°C freezer and attached to a vacuum pump. The tissue was then freeze-dried for a 24 h period. After the freeze-drying process was completed the tissue was kept at -20°C until assayed.

Samples needed for assay were thawed under vacuum for 1 h to prevent condensation-stimulated enzyme action. The different layers of the cornea were defined clearly, which permitted easy isolation of the corneal epithelium under a $3\times$ binocular dissecting microscope. Tissue size was determined by dry weight, rather than by tissue section dimensions, which permitted the analysis of very small and irregularly shaped specimens. The tissue samples immediately were weighed on a quartz fiber fishpole balance possessing μg sensitivity. After weighing and recovery, the samples were placed in an oil well rack for specific metabolite assay.

Underlying principles. The cycling system contains several enzymes which catalyze specific inter-related reactions yielded a 'net reaction'. A byproduct of this multi-step reaction is reduced nicotinamide adenine dinucleotide phosphate (NADPH), which fluoresces light of 460 nm

wavelength when excited with UVR of 340 nm wavelength. By measuring the amount of this reduced pyridine nucleotide fluorescence, the original concentration of the assayed metabolite can be inferred by calculation (Lowry and Passonneau, 1972). Appropriate blanks and standards were employed to monitor the reliability of the assays. Individual enzymatic cycling and incubating techniques permitted isolation of the specific metabolite being analyzed.

Source measurement. Source calibration and radiometric quantification followed the procedures described by Pitts *et al.* (1977). The UVR source was a 5000 W Xe-Hg high pressure arc lamp, powered by a 10 kW direct current power supply regulated to $\pm 0.5\%$ and capable of delivering from 0 to 80 amps at 25 to 65 V to the arc electrodes. The lamp housing was cooled by a double-blower system. The radiation from the source was focused at a monochromator entrance slit by the housing optics. A 10 cm quartz-enclosed water chamber was placed between the focusing lenses and the monochromator in order to remove infrared radiation. The desired UVR waveband was obtained with a Czerny-Turner double grating monochromator with gratings blazed at 300 nm and grooved with 1180 grooves/mm, allowing approximately a 5 nm bandpass. The linear dispersion equated to a value of 0.82 nm/mm . Entrance, intermediate, and exit slits were set to pass a nominal full bandpass of 6.6 nm. The system was aligned with a helium-neon laser and the wavelength counter was calibrated with a mercury source. Exposure durations were set with a Gerbands electronic shutter controlled by a Hewlett Packard preset counter. The preset counter allowed exposure durations of any desired length with millisecond accuracy.

The exit optical beam was focused by a quartz lens with a beam size of 1.6 cm by 1.8 cm at the plane of the experimental animal's cornea. An Eppley 16-junction thermopile, traceable to a National Bureau of Standards (NBS) standard source, was used to characterize the spectral irradiance of the UVR source. When taking the spectral irradiance readings, the thermopile was placed in the same position relative to the monochromator exit port as the rabbit's cornea was to be situated during UVR exposure. The irradiance (E_c), in watts per square centimeter (W cm^{-2}), incident on the thermopile was determined by the following relationship:

$$E_c = kV_t$$

The value k represents the thermopile calibration constant in microwatts per square centimeter per microvolt ($\mu\text{W cm}^{-2} \mu\text{V}^{-1}$), while the value V_t represents the average (mean) of three thermopile-voltmeter readings in μV . The calibration constant for the thermopile used in this experiment was $5.131 \mu\text{W cm}^{-2} \mu\text{V}^{-1}$. The radiant exposure (H), in Joules per square centimeter (J cm^{-2}), was calculated by the formula:

$$H = E_c t$$

The value t is simply time in seconds; it should be kept in mind that a Joule is a Watt-second. Therefore, for a given irradiance E_c the exposure duration t can be varied to obtain different values of radiant exposure H as desired. Conversely, a radiant exposure can be kept constant, even though wavelength irradiance may differ, by varying the time of exposure. This means of output characterization and source calibration was estimated to have a $\pm 10\%$ accuracy. The spectral irradiance data for the source at the four experimental wavelengths is provided in Table 1.

The variation of t in order to obtain a constant H , in the context of the wavelengths used in this experiment, creates an outcome that is somewhat dependent upon the

Table 1. Spectral irradiance data of UVR source providing a constant radiant exposure of 0.05 J cm^{-2}

Wavelength (nm)	Average V_i (μV)	E_c (W cm^{-2})	Time (s)
290	4.66	2.391×10^{-5}	2091
300	20.00	1.026×10^{-4}	487
310	25.00	1.283×10^{-4}	390
360	14.30	7.337×10^{-4}	682

validity of the principle of reciprocity (i.e. the biological effects or endpoints are independent of exposure time and irradiance). Corneal effects of a krypton-ion laser, with simultaneous output at 350.7 and 356.4 nm (3:1 ratio) illustrates that the product of threshold intensity and the pulsewidth is a constant; the thresholds for multi-pulse exposures have been shown to be in agreement with those for single-pulse exposures (Zulich and Connolly, 1976). A similar corneal damage pattern can be elicited from helium-cadmium laser data at 325 nm (Ebbers and Sears, 1975). Based on the literature, it would not be unreasonable to assume that reciprocity holds for all four UVR wavelengths utilized in this experiment.

RESULTS

The energy metabolite assays were performed on 20 to 32 different tissue samples from 3 different rabbit eyes for each of the experimental groups (290 nm, 300 nm, 310 nm and 360 nm) and the controls. Figure 1 provides a bar-chart illustration of the epithelial metabolite concentration as a function of the wavelength of UVR exposure. An analysis of variance of the data demonstrated a highly significant overall, between groups effect ($P < 0.0001$) for glucose, glycogen and PCr. An analysis of variance of the ATP data failed to demonstrate a significant effect ($P > 0.65$).

Individual wavelength comparisons revealed each glucose and glycogen group to be significantly different from every other ($P < 0.05$). However, at a 0.01 level of significance, the 300 and 310 nm glycogen data are not significantly different from one another, nor are the 310 and 360 nm data; the glucose data hold at those wavelengths at the 0.01 level. These differences may be due, in part, to the delicate nature of the glycogen assay causing a greater variability.

None of the ATP groups were significantly different from one another. Specific PCr comparisons revealed only certain groups to have statistical significance ($P < 0.05$). The 290, 300, and 310 nm groups differed significantly from the 360 nm and control groups. However, individual members of the above two subgroups did not differ from one another.

DISCUSSION

Glucose and glycogen

The data from the epithelial glucose assay reveal increasing accumulations of glucose as a function of decreasing UVR wavelength (Fig. 1), with the shorter wavelength exposures creating greater accumulations. This increase in glucose concentration demonstrates a decrease in epithelial glucose utilization, the magnitude of which appears to be related to the specific wavelength of exposure. Similarly, the data from the epithelial glycogen assay reveal increasing accumulations of glycogen as a function of decreasing UVR wavelength (Fig. 1), with the shorter wavelength exposures creating greater accumulations. Since glycogen is a complex

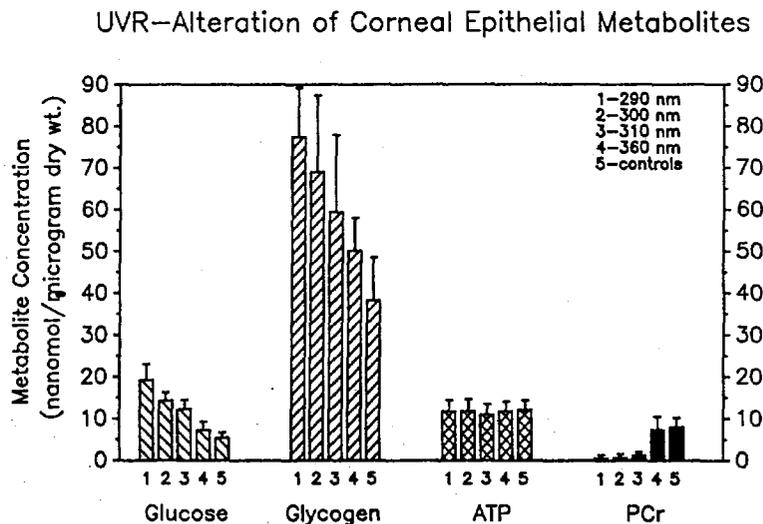


Figure 1. illustrates the mean metabolite concentration \pm one standard deviation. Experimental UVR exposure conditions serve to increase corneal epithelial glucose and glycogen concentrations. Although the corneal epithelial ATP concentrations are unchanged, the PCr concentrations (a high energy phosphate bond reservoir) decrease as a result of UVR exposure. The data demonstrate an apparent wavelength-dependent decrease in corneal epithelial metabolic activity as a result of UVR exposure.

aggregate of glucose molecules, the increased glycogen concentration further demonstrates the decrease in epithelial glucose utilization that appears to be related to the specific wavelength of UVR exposure. It should be noted that the control glycogen values are quite high, exceeding those documented by other researchers (Thoft and Friend, 1977). Documented values in the literature were obtained by bulk tissue extraction techniques, which may not isolate all glycogen present.

ATP and PCr

The ATP data reveal no changes in ATP concentration regardless of the UVR exposure conditions (Fig. 1). The data from the epithelial PCr assay reveal a decrease in PCr as an apparent function of UVR wavelength. Although there is a sparing of ATP, the decrease in PCr concentration highlights a decrease in high energy phosphate bond formation that is related to UVR exposure. The ATP results are in agreement with Foulkes *et al.* (1978), who were the first to observe that corneal epithelial ATP concentrations are unchanged within 30 min after UVR exposure. This constancy in the ATP concentration previously has been taken to suggest that UVR does not have an effect on corneal epithelial metabolism. However, the data for PCr, which serves as a high energy phosphate bond reservoir, argue against that interpretation. Therefore, it is recommended that both ATP and PCr be taken into consideration when assessing UVR metabolic effects.

Transmittance issues

Corneal transmittance in the rabbit has been shown to vary considerably depending upon the incident wavelength: at 290 nm the cornea has a transmittance of about 1%, at 300 nm transmittance is near 2%, at 310 nm it is approximately 15% and at 360 nm corneal transmittance approaches 43% (Barker, 1979). However, the published data is for the cornea as a whole; there is no transmittance data available for the corneal epithelium alone. Since the rabbit cornea as a whole is approximately 50 μm thick (the epithelium accounting for only about 10% of total thickness), and if one assumes uniformity of corneal tissue and absorption of incident radiation in accordance with 'Lambert's Law', then the absorbed 'dose' of each wavelength can be approximated. Using this logic 9.9% of incident 290 nm radiation would be absorbed by the corneal epithelium. Similarly, 9.8% of 300 nm radiation would be absorbed by the corneal epithelium, 8.5% at 310 nm, and 5.7% at 360 nm. It can be seen here that transmittance/absorbance differences could have affected the data outcome by nearly a factor of two, when comparing 290 nm data to 360 nm data.

However, counter to the above process is the likelihood that the necrotic superficial epithelial cells and the stable intermediate cells are less metabolically active than the mitotic basal cells. As a result, the potential metabolic targets of UVR are in much greater supply in the very deeper portions of the epithelium. It is likely that absorption takes place only at the susceptible target(s), consequently UVR metabolic effects documented here may represent influences imposed primarily on the basal layer. Therefore, transmittance issues may not be a significant factor for this corneal epithelial data.

On the other hand, if the anterior layers of the epithelium differentially absorb or 'filter out' more short wavelength radiation, then actual wavelength differences might be even greater than the present data demonstrate. It should be noted that histological data have documented a spatial filtering effect in the epithelium (Friedenwald *et al.*, 1948). This may be pertinent to the metabolic data, since the Friedenwald study documents a loss of basal cell mitotic activity after UVR irradiation.

Significance

Since UVR exposure acts to decrease glucose utilization, increase glycogen accumulation, and decrease high energy phosphate bond availability, the implication is that overall epithelial metabolic activity decreases secondary to UVR exposure. Speculation regarding the possible mechanism responsible for this alteration in metabolic activity would have to take into account certain conditions:

1. The change in metabolite concentration was registered 2 min after the exposure was discontinued.
2. The glucose accumulation was paired with an accumulation of glycogen.
3. The stabilized ATP concentrations were paired with a PCr depletion.

Current damage mechanism theories involve DNA structural alteration (Murphy, 1975; Ramabhadran, 1975; Grabner and Brenner, 1981), generalized changes in enzymatic activity (McLaren and Luse, 1961; Augenstein and Riley, 1964; Vladimirov *et al.*, 1970; Grossweiner, 1976 and 1984), and/or changes in mitochondrial activity (Kashket and Brodie, 1963; Ninneman *et al.*, 1970; Ninneman, 1974; Werben *et al.*, 1974; Crocket and Lawwill, 1984). Because the metabolic alteration evidence in this study is essentially immediate, it would be unlikely that DNA structural alteration is responsible. A generalized change in overall enzymatic activity can also be ruled out since glycogen synthesis and PCr conversion, both enzymatically mediated processes, appear to continue despite the UVR exposure. It is possible that a change in mitochondrial activity is responsible for the UVR-induced decrease in corneal epithelial metabolic activity. However, isolated enzymatic modifications

could be responsible for this pattern of energy metabolite changes, as well.

Histological damage secondary to excessive UVR exposure could be explained on the basis of immediate metabolic stress. The UVR-exposed tissue initially may be in a state of suspended or reduced metabolic activity; the effect would induce an enforced state of reduced glucose availability, and/or hypoxia. Functional recovery and viability would depend on the capacity of each cell to overcome the insufficiency.

This process would account for the morphological observation made by some investigators concerning the presence of isolated dying cells amid large regions of normal epithelium at the radiant exposure levels in this study (Cullen, 1980; Pitts *et al.*, 1987). Conversely, work at higher fluences has shown isolated cell survival amid large regions of cell death (Pitts *et al.*, 1988). Subsequent research should seek to differentiate between mitochondrial and enzymatic theories.

A final notation should be made concerning the range of results from 290 to 360 nm. The data do not correspond to predictions based on histological damage threshold studies. The 290 nm data are less catastrophic than might have been expected, while the 360 nm data are indicative of a greater disruption of tissue function than would reasonably be anticipated. It is possible that anterior filtering effects could have influenced the 290 nm results; the 360 nm results were unexpected, and represent a cause for concern. Until now, radiation in the wavelength range of 340–400 nm (UV-A) has generally been regarded as 'harmless' at fluences even greater than that used here, although UV-A is capable of causing histological damage by way of drug interaction at low radiant exposures. The prudent use of UV-A sources should be encouraged until this anomaly can be resolved.

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