Pyridine nucleotide levels in the corneal epithelium were measured using redox fluorometry, a noninvasive method for monitoring the metabolic status of corneal tissue, and a sensitive bioluminescent assay and an improved extraction procedure that allows the simultaneous extraction and measurement of both NADH and NAD. The same corneas were measured using each of the two methods to enable comparison of both chemical analysis and fluorometric monitoring of metabolic status. A plot of the fluorescence and analytical data for each cornea showed a positive correlation between the two methods, with a correlation coefficient (r value) of 0.80. The correlation was improved but was not dependent on the high values of the KCN treated corneas; an r value of 0.73 was obtained for the non-KCN treated corneas alone. Additional measurements of the temperature dependence of the fluorescence intensity of an NADH solution and the cornea gave a decrease in intensity of 17% from 25°C to 35°C for the NADH solution and 11% (P = 0.0004) for the reduced pyridine nucleotide fluorescence in the cornea over the same temperature range. Invest Ophthalmol Vis Sci 30:2274-2278, 1989

Pyridine nucleotides (PN) are cofactors in many enzymatic reactions and play important roles in regulating various physiological functions. In the cornea, both chemical analysis and fluorometric monitoring have been used to measure PN.1-8,11 The direct fluorometric methods have advantages over chemical analysis in that they are noninvasive, thus enabling repeated measurements of the same tissue sample and avoiding possible artifacts due to extraction. Additionally, they allow optical longitudinal and transverse spacial sectioning. We have been measuring autofluorescence from reduced PN in cornea and lens using the method of ocular redox fluorometry.7-9,11 Although it has generally been assumed that changes in the redox fluorometry signal reflect changes in tissue PN, and all of the experimental measurements to date are consistent with this assumption, the direct correlation of the fluorometric signals with the PN...
level in the tissue has not been firmly established experimentally for the cornea.

NADH and NAD are the primary pyridine nucleotides present in the corneal tissue and the NADH/NAD ratio is an important parameter of the cellular metabolic state. To demonstrate directly a correlation between the redox fluorometric measurement of PN and the PN tissue level requires a comparison of fluorometric and analytical measurements made on the same tissue under the same conditions. Until recently the measurement of NADH and NAD in the same small sample has been complicated by their radically different pH sensitivities; thus usually NAD has been extracted in acid (which destroys NADH) and NADH has been extracted in alkali (which destroys NAD). To determine either the NADH/NAD ratio or the absolute values of NADH and NAD then requires the determination of the protein content of the two samples and the scaling of the two values to give equal protein concentrations in the two parts of the same tissue. The experimental error of each of the steps involved is considerable and thus far such methods have been able to demonstrate only a qualitative correlation between the fluorometric signals and the analytically determined PN levels.

This report describes experiments in which we have used a recently developed alcohol extraction and bioluminescent assay of NADH and NAD that reduces the errors associated with the previously used methods. The results demonstrate the correlation between the redox fluorometric measurements and PN corneal epithelial tissue levels performed on the same tissue for normal and KCN treated rabbit corneal epithelium.

**Materials and Methods.** New Zealand albino rabbits weighing 3–4 kg were used. The animals were treated in accordance with the ARVO Resolution on the Use of Animals in Research. After intramuscular injection of ketamine and xylazine, they were sacrificed by an overdose injection of air via ear vein. The eyes were enucleated immediately and placed in a container with TC199 medium.

Sixteen corneas studied were maintained at 25°C during the experiment period. Five out of 16 corneas were bathed in a medium containing 1 mM KCN in order to attain a reduced redox state. After redox fluorometric measurement, each cornea was immediately frozen in liquid nitrogen and the central epithelium (approximately 4 mm in diameter) was scraped off using a surgical blade (Bard-Parker, No. 10, Becton Dickinson and Company, Rutherford, NJ). During the scraping process, the cornea and the instruments used were continually placed in liquid nitrogen to keep them from warming. The scraped epithelium was processed and assayed for NADH and NAD by minor modification of the procedure of Karp, Raunio and Lovgren. In brief, the PN were extracted in 0.05–0.2 ml of 70% ethanol, 10 mM potassium phosphate, pH 7, by 20 min of vortexing in a microfuge tube, followed by 5 min centrifugation in a Beckman microfuge E (Palo Alto, CA). An 0.01 ml aliquot of the supernatant was assayed sequentially for NADH and NAD using a bioluminescent NADH monitoring kit (Pharmacia LKB Nuclear, Inc., Gaithersburg, MD) and a Turner model 20E luminometer (Turner Designs, Inc., Mt. View, CA). After NADH was determined, lactate dehydrogenase was added to the reaction mixture to convert NAD to NADH. The readings were normalized with internal standards, and the fraction NADH/(NADH + NAD) was calculated directly. NADH and NAD for standards were purchased from Boehringer-Mannheim GmbH (Indianapolis, IN); stock solutions were calibrated spectrophotometrically at 340 and 260 nm, respectively. Since PN fluorescence intensity presumably reflects relative NADH levels, the analytical data are expressed as the fraction NADH/(NADH + NAD).

Statistical analysis was done using the Microstat statistics package. The correlation coefficient (r value) was determined by doing a regression of y on x (y = NADH/(NADH + NAD), x = PN signal). This analysis also gave a least squares fit line assuming no variance in x. A regression of x on y was then done and this gave a least squares fit line assuming no variance in y. The average slope and intercept of these two lines were calculated and used to plot the least squares fit line to the data that assumed variance in both x and y. The correlation coefficient is the same in both regressions. The paired and non-paired student t-test were used to determine the possible significance between values.
After the correlation studies were completed, an interest developed in the values of the PN fluorescence intensity and in the NADH/(NADH + NAD) fraction at 35°C. As a result, eight additional corneas from four rabbits were studied. One cornea of each animal was first measured with the redox fluorometer at 25°C after which time the temperature of that cornea was raised to 35°C and it was measured again. That cornea was then frozen as described above and stored in liquid nitrogen while the fellow cornea was measured at 25°C and then frozen. Both corneas of each animal were scraped and assayed bioluminometrically at the same time. To determine the fluorescence intensity change due to the inherent change of fluorescence with temperature, the fluorescence intensity of a 0.5 mM solution of NADH in Tri-ethanolamine-HCl buffer (Fisher) was measured at 25°C, and again at 35°C. Thirty three readings were taken at each temperature.

**Results.** For the corneas maintained at 25°C, the fraction NADH/(NADH + NAD) in corneal epithelium in the absence of KCN was 0.14 ± 0.06 (mean ± SD, n = 11). KCN treatment significantly increased the NADH/(NADH + NAD) fraction to 0.24 ± 0.03 (n = 5; P < 0.001). In the same samples the PN fluorescence intensity rose significantly from 2840 ± 605 to 5147 ± 738 (P < 0.0001).

Figure 1 shows a graph of the analytically measured NADH/(NADH + NAD) fraction and the fluorometrically measured PN signal for the corneas maintained at 25°C. The correlation coefficient (r value) for this data is 0.80. The correlation is improved but is not dependent on the high values of the KCN treated corneas; an r value of 0.73 is obtained for the non-KCN-treated corneas alone. The straight line shown is the least squares fit to the data points assuming variance in both the analytic and the fluorometric measurements.

The four corneas measured at two different temperatures had a PN fluorescence intensity of 2027 ± 171 (mean ± SD) at 25°C and 1802 ± 188 at 35°C. The mean decrease, calculated on a pairwise basis, of the fluorescence intensity was 11.2% ± 2.1%. This decrease was significant (P = 0.0004). These corneas, frozen from the 35°C state, had an NADH/(NADH + NAD) fraction of 0.14 ± 0.03. The four fellow corneas, measured only at 25°C, had a PN fluorescence intensity of 2054 ± 139 which was not significantly different (P = 0.41) from their fellows. These corneas, frozen from the 25°C state, had an NADH/(NADH + NAD) fraction of 0.12 ± 0.05, which was not significantly different (P = 0.20) from the fraction measured in the fellow corneas.

The PN fluorescence intensity of the 0.5 mM solution of NADH was 638 ± 13 (mean ± SD) (n = 33) at 25°C and 528 ± 13 at 35°C (n = 33). The average change of fluorescence with temperature was 1.7%/°C.

**Discussion.** This study, using an improved assay for NADH and NAD, has demonstrated the correlation between redox fluorometry and analytical measurements of PN in corneal tissue. A major advantage of the assay used in the present study, as compared with the assay used previously in this laboratory as well as by Masters et al., is that it enables the simultaneous extraction of both the oxidized and reduced forms of PN from the same sample. The measured fraction NADH/(NADH + NAD) is thus free from possible differential errors associated with scraping the tissue from the underlying stroma of two different corneas.

The method used to obtain the tissue sample was also an improvement on that used earlier by us and by Masters et al. In the current study the tissue and surgical blades used for scraping were kept at a much lower temperature than was used previously in order to minimize possible changes in metabolic state produced by the scraping process. It is likely that the mechanical damage would, if anything, increase the NADH/(NADH + NAD) fraction because of activation of anaerobic glycolysis. The lower NADH/(NADH + NAD) fraction obtained in this study compared with the results reported previously may thus be a result of the lower sample temperature that we used while scraping.

The least squares fit line to the data has an intercept that is about 12% of the value of the mean of the non-KCN-treated corneas. Since the NADPH level is 9% to 20% that of the NADH level in the normal rabbit corneal epithelium, this intercept can be accounted for as the NADPH level that was not assayed.
for but that is measured by the fluorometric method. These measurements show that there probably are no other unknown fluorochromes in the corneal epithelium that interfere materially with the reduced PN signal.

The correlation coefficient of 0.80 between the redox fluorometry measurements and the bioluminescent assay of PN demonstrates a strong correlation between the analytic and fluorometric measurements and shows that, at least for the corneal epithelium, the fluorometric PN signals are measuring the reduced PN levels. However, there is considerable data scatter especially at the lower values. Several factors may contribute to this scatter. First, although the scraping method used probably caused a smaller change in the metabolic state than that used previously, there was probably still some indeterminate and variable change due to the scraping. Second, since binding of NADH to mitochondrial proteins enhances its fluorescence by a factor of ten, variation in the amount of mitochondrial versus cytoplasmic NADH would preferentially affect the redox fluorometry signal. Finally, differences of the tissue volume sampled by each method may be a source of disparity due to presumed spacial variations of redox state. Redox fluorometry measured fluorescence from 20–30 cells, which was much smaller than the number of cells (about $1 \times 10^6$) processed for the bioluminescent assay. Hence, localized variations in metabolism might be seen by redox fluorometry but these would be averaged out in the larger sample taken for chemical analysis.

For the non-KCN-treated corneas, there was a considerable range of values for both fluorescence and chemical analysis of PN among different animals. Whether this apparent variation in metabolic state is a consequence of differences in physiological condition of the rabbits or of insults from anesthesia or enucleation is a question worthy of further investigation. The grouping of values was much tighter for the KCN-treated corneas, representing the extreme metabolic state of anoxia.

The measurements made to determine the values of the measured parameters at 35°C, showed that a difference in the NADH/(NADH + NAD) fraction from 25°C to 35°C could not be detected by the bioluminometric assay although the redox fluorometry method showed an 11% change that was highly significant ($P = 0.0004$) between these two temperatures. The measured difference in the redox fluorometry intensity between 25°C and 35°C of 17%, or 1.7% per degree C, is consistent with the reported value of 1.6% per degree C for NADH due to the large negative temperature coefficient which is characteristic of fluorescence in general. The variation between the expected 17% change and the measured 11% change is presumably due to the difference in biochemical state although additional experiments are required to support this conjecture.

**Key words:** pyridine nucleotide, corneal epithelium, redox fluorometry, bioluminescent assay, potassium cyanide

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