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SPECTROPHOTOMETRIC INVESTIGATION OF PRODUCTS FORMED FOLLOWING THE INITIAL ONE-ELECTRON ELECTROCHEMICAL REDUCTION OF NICOTINAMIDE ADENINE DINUCLEOTIDE (NAD^+)

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On electrolysis of NAD^+ in aqueous solution at a potential corresponding to the initial one-electron reduction of NAD^+ to a free radical, a greenish-yellow color appears which fades when electrolysis is complete. Literature ultraviolet absorption data for the resulting dimer show considerable variation. When the electrolysis is conducted in darkness, the colored product has ϵ_{340} of approx. 5700 $M^{-1} \cdot cm^{-1}$ and ϵ_{259} of approx. 31000 $M^{-1} \cdot cm^{-1}$. On ultraviolet and visible illumination, the color disappears, the 340-nm peak decreases and the 259-nm peak increases. On only visible illumination, the color disappears, both peaks increase, the dimer's polarographic oxidation wave decreases and the wave due to 1-substituted nicotinamide reduction increases. The data suggest that the dimer decomposes to NAD^+ and 1,4-NADH.

On electrolysis of the biologically important coenzyme, nicotinamide adenine dinucleotide (NAD^+) at a potential at which it undergoes a one-electron ($1e^-$) reduction to a free radical which rapidly dimerizes, a distinct greenish-yellow color appears, which is most intense when the electrolysis is 80 to 90% complete, and which fades to a pale yellow color when the NAD^+ is exhausted [1–4]. The linear time-log current plots obtained during electrolysis show a first-order electrolysis rate and the absence of mechanistic complications. The color changes indicated were studied by taking aliquots from the electrolysis solution, diluting them, and transferring the resultant solutions to spectrophotometer cuvettes for analysis. Since exhaustive electrolysis on a bulk scale (10 to 20 ml of 0.5 to 2 mM solution) requires three or more hours, it is difficult to monitor the transient species.

In recent years, spectroelectrochemistry, which is essentially the marriage of spectroscopic and electrochemical techniques, has proven quite effective for the investigation of inorganic, organic and biological redox reactions. The theory, instrumentation and applications of spectroelectrochemistry have been reviewed [5–8].

One of the more easily fabricated spectroelectrochemical assemblies is the Optically Transparent Thin-layer Electrode (OTTLE) which consists of a thin gold mesh sandwiched between two optical flats. The bottom of the apparatus is immersed in a cup which holds the analyte solution and the reference and auxiliary electrodes. About 0.1 ml is drawn up into the electrode apparatus, which is set in the optical path of a spectrophotometer, and exhaustive electrolysis occurs in minutes [9,10]. Such an assembly, along with bulk-scale electrolysis followed by conventional spectrophotometry, has been used to follow the color changes which accompany the $1e^-$ reduction of NAD^+.

Nature of the one-electron reduction product

As noted, $1e^-$ reduction of NAD^+ produces a radi-

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TABLE I

REPORTED ULTRAVIOLET ABSORPTION DATA FOR THE ONE-ELECTRON REDUCTION PRODUCT OF NAD^+

ϵ_{340} ($M^{-1} \cdot cm^{-1}$)	ϵ_{259} ($M^{-1} \cdot cm^{-1}$)	Method of preparation	Ref.
7 000	36 000	Radiolysis [a]	11
ca. 5 300	–	Radiolysis [b]	12
5 240 ± 100	–	Radiolysis	13
6 650	33 100	Electrolysis	14
6 890 ± 100	32 100 ± 1 300	Electrolysis	1,2
6 600	34 000	Electrolysis	4
6 900	32 000	Electrolysis	17

[a] Data estimated from a figure in the reference.
[b] Data for the nicotinamide adenine dinucleotide phosphate ($NADP^+$) radiolysis product.

cal which rapidly dimerizes. Table I contains the molar absorptivities (ϵ) reported for radiolytically and electrochemically generated dimers of NAD^+ and nicotinamide adenine dinucleotide phosphate ($NADP^+$); there is appreciable variation in the data. Since the spectra of $NADP^+$ and NADPH do not differ from those of NAD^+ and NADH, respectively, it is doubtful that ϵ values for the corresponding dimers differ.

Land and Swallow [11] prepared the dimer by irradiation of an NAD^+ solution with X-rays. The spectrum of the free radical, with λ_{max} at 400 nm, decayed rapidly, according to second-order kinetics, to the dimeric product for which spectral data are given in Table I.

The radiation-induced dimerization of $NADP^\cdot$ free radical has also been reported [12]. The dimer is less stable than 1,4-NADPH, acid-labile, insensitive to oxygen, and decomposes on addition of HCl or CO_2. These observations agree with those reported [1,2] for the $NAD^\cdot$ dimer (cf. subsequent discussion).

Bielski and Chan [13] have shown that $NAD^\cdot$ free radical, prepared by radiolysis, is stabilized by attachment to malate dehydrogenase. The molar absorptivity reported by them for the dimer is considerably less than many other reported values (Table I).

The ultimate product of the $1e^-$ cathodic reduction of NAD^+ was first established as a dimer by Burnett and Underwood [14]. Structural evidence for the dimer included molecular weight estimation by two methods and was supported by the polarographic diffusion coefficient. Proton NMR and ultraviolet spectrophotometry, although inconclusive, suggested that the monomeric units were joined at the nicotinamide C-4 position.

More recently, the dimeric structure was confirmed by ^{1}H-NMR data at 250 MHz and ^{13}C-NMR data at 62.8 MHz [15]. From splitting of the nicotinamide C-4 peak and the spectrum of the electrolysis product of NAD^+ enriched with deuterium at C-4, a 4-4′ dimeric structure was assigned.

The greenish-yellow color was reported [1,2] to reach maximum intensity when electrolysis was 80 to 90% complete; on continuation of electrolysis for 0.5 h after the current decayed to background level, the color diminished in intensity, yielding finally only a faint yellow solution; spectral data for the final product are given in Table I. The absorbance at 430 nm of 0.62 mM NAD^+ increased from zero to 0.052 and fell to 0.029 during the course of electrolysis. The color was more intense for 2.15 than for 1.25 mM NAD^+, appeared in the presence of several different buffer systems, and disappeared on oxidation of the dimer back to NAD^+. Nicotinamide mononucleotide underwent similar changes on electrolysis, which rules out involvement of the adenine moiety of NAD^+ [1].

Cyanide does not add to the dimer, as it does to the NAD^+ nicotinamide ring at C-4; the dimer reacts with $HgCl_2$. The electrochemical behavior of the dimer has been characterized [1,2], e.g., it is oxidized to NAD^+. Oxygen had little effect on the stability of the dimer, whose decomposition is accelerated upon decrease in pH.

Based on the appearance of a new band at 290 to 300 nm, Hanschmann [16] proposed that the acid-catalyzed decomposition products of 1-substituted nicotinamide dimers were the corresponding 1,4,5,6-tetrahydronicotinamide derivatives. He noted that the monomeric 1,4- and 1,6-dihydronicotinamides (including 1,4-NADH) are converted to the 1,4,5,6-tetrahydro derivatives under acidic conditions with disappearance of the primary absorption band and appearance of the 290 to 300 nm maximum.

In a study of the decomposition of the $NAD^\cdot$ dimer, Jensen [4] reported that half of the amount decomposed went to either nicotinamide or a 1-substituted nicotinamide, i.e., cleavage of the dimeric bond forms an oxidized, polarographically reducible

nicotinamide moiety; the other half is not polarographically active.

Czochralska, Szweykowska and Shugar [17] have recently shown that illumination of the electrochemically prepared NAD dimer at 254 nm or at wavelengths exceeding 320 nm leads to loss of the 340-nm band and regeneration of NAD^+. They reported some decomposition of NAD^+ at 254 nm. The molar absorptivities reported by them for the dimer (Table I) were calculated on the assumption of quantitative reduction.

Experimental

Reagents and solutions. NAD^+ (P-L Biochemicals; Chemalog) solutions were prepared immediately before use. The base electrolyte was 0.4 M KCl/0.05 M K_2CO_3/0.05 M $KHCO_3$; the pH was adjusted to 9.1 with HCl. All spectra were obtained in this solution.

Spectroelectrochemistry. The optically transparent thin-layer electrode was similar to that used by Heineman, Norris and Goelz [18]. The working electrode was a 500 lines per inch gold wire mesh (Buckbee Mears Co., St. Paul, MN). After assembly, it was amalgamated according to the method of Meyer et al. [19]; pretreatment in the plasma cleaner was not necessary. The active mesh area was 10 mm high by 9 mm wide. Suprasil-2 quartz flats (Amersil Inc., Sayreville, NJ) were cut into 13 X 37-mm pieces for optical windows. Stacks of two 1.5-mm wide strips of No. 1 microscope cover glass served as spacers, giving an optical path of 0.0236 cm, which was determined by measuring the absorbance of NAD^+ at 259 nm (ϵ = 17000 $M^{-1} \cdot cm^{-1}$). For electrical connection a wire was attached to the mesh, which extended outside the electrode apparatus, with conducting epoxy (Technology Inc., Billerica, MA). Household epoxy was used to insulate the conducting epoxy from the solution and to hold the apparatus together.

The reference and auxiliary electrodes were a saturated calomel (SCE) and a Pt gauze, respectively; potentials cited are referred to the SCE. The potentiostat was a Princeton Applied Research Model 174. Due to the resistance of the electrode, electrolysis of NAD^+ was carried out at −1.3 to −1.4 V. Oxygen was not removed from solution; although oxygen reacts with the $NAD^\cdot$ free radical produced on pulse radiolysis, this reaction is impossible when $NAD^\cdot$ is generated electrochemically, since oxygen is reduced at less negative potential than that required for the initial $1e^-$ reduction of NAD^+. The presence of the oxygen did not alter the observed spectrophotometric behavior, e.g., the spectra obtained on the electrode apparatus were identical within experimental error to the spectra obtained on bulk-scale electrolysis during which oxygen was carefully excluded.

Product spectra were recorded on a Cary 14 spectrophotometer at constant potential after the current decayed to a negligible level. Absorbance at constant wavelength vs. time was recorded during electrolysis and for several minutes after the current decayed to a negligible level.

Bulk-scale electrolysis. Controlled potential electrolysis with coulometry on a bulk scale was done in a thermostat-controlled (25°C) three-compartment cell. The SCE reference and Pt gauze auxiliary electrodes, immersed in base electrolyte alone, were separated from the working electrode compartment by glass frits. The working electrode was a 3-cm diameter Hg pool. The working solution was de-aerated with purified nitrogen prior to electrolysis; a nitrogen atmosphere was maintained over the solution during electrolysis. Magnetic stirring with a Teflon-covered bar was used. Typically, 15 ml 0.4 to 0.8 mM NAD^+ was electrolyzed to at least 80% completion, based on both coulometry and polarographic determination of residual NAD^+. The electrolyzed solution was immediately removed from the working compartment to avoid reaction of dimer with any Hg^{2+} present [1]; aliquots were then taken for polarography and for dilution (typically 1 : 10) for spectrophotometry.

Spectra for the bulk-scale electrolysis products were obtained on a Cary 219 spectrophotometer.

Results and Discussion

On electrolysis of NAD^+ in the electrode apparatus, the peak at 259 nm decreases and a peak appears at 340 nm (ϵ at 340 nm is given in Table II). Numerous electrolyses were followed at fixed wavelength in the 340 to 500 nm region where the absorbance causing the color occurs. The expected decrease following the initial increase in absorbance, such as that previously reported [1,2] at 430 nm and observed visually in the bench-top experiment, was not seen with this electrode. The behavior in the appara-

TABLE II

ULTRAVIOLET ABSORPTION DATA FOR THE ONE-ELECTRON REDUCTION PRODUCT(S) OF NAD^+

ϵ_{340} ($M^{-1} \cdot cm^{-1}$)	ϵ_{259} ($M^{-1} \cdot cm^{-1}$)	Method of preparation
5530	– [a]	Spectroelectrochemistry in darkness
5600	30000	Bulk electrolysis in darkness
5800	32100	Bulk electrolysis in darkness
6300	32100	Bulk electrolysis in darkness followed by illumination with incandescent light

[a] Not measured.

tus could not be explained until the effect of light on the electrolysis product was discovered (cf. subsequent discussion).

Since the greenish-yellow color is most intense when electrolysis is 80 to 90% complete and fades when the NAD^+ is exhausted, formation of a charge-transfer complex between NAD^+ and the dimer was considered as a possible cause for the color, since the coenzyme is known to form such complexes with absorption bands in the 300 to 500 nm region [20, 21]. If a charge-transfer complex did exist, it would be possible to generate it by mixing solutions of NAD^+ and the dimer. Stock solution of the dimer, obtained by exhaustive electrolysis of NAD^+ solutions, were used to prepare mixtures which duplicated the concentrations of the colored electrolysis solutions. Unlike the latter, the mixtures were colorless and showed only the spectral bands of the components. Since no charge-transfer complex bands were obtained, it was concluded that this was not responsible for the color observed on electrolysis.

Effect of light on the electrolysis product. During the course of the preceding experiments, it was discovered that an electrolyzed solution on exposure to daylight lost its color in several minutes, while over the same period of time another portion of the same solution in the spectrophotometer sample chamber, not exposed to light, remained yellow. Apparently a photochemical reaction is responsible for the loss in color. The rate of this reaction in normal room light is such that it proceeds on the same time-scale as the electrolysis and, for that reason, was not discovered in the past. Since light causes, or at least hastens, the color loss, it is understandable that no color loss was seen in the optically transparent thin-layer electrode, which was always kept in the darkness of the spectrophotometer sample chamber.

A three-compartment bulk-electrolysis cell, painted flat black, was used to run the electrolysis in the dark; the spectrum of the product is shown in Fig. 1; the molar absorptivities are given in Table II. When this solution was illuminated with ultraviolet and visible light, the greenish-yellow color disappeared; the absorbance decreased at wavelengths higher than 325 nm and increased at lower wavelenghts (Fig. 1). These changes are similar to those reported as due to photo-oxidation of the dimer to NAD^+ [17].

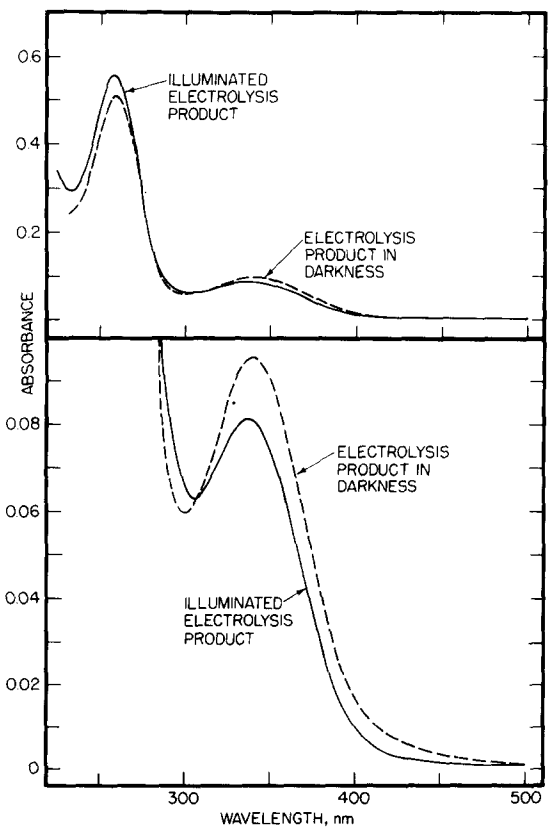

Fig. 1. Effect of illumination of a solution of the NAD dimer with ultraviolet and visible light. Expanded scale spectra are shown in the lower panel.

In another experiment an electrolyzed solution, prepared in the dark, was then illuminated with a household light bulb for 30 min; the greenish-yellow color disappeared, and the absorbance decreased at wavelengths higher than 365 nm and increased at lower wavelengths (Fig. 2). The increase in the 340-nm peak was not observed on illumination with ultraviolet light here or in the previously reported photochemical study [17]. The effect of visible light on the dimer spectrum accounts for the wide variation in spectral data reported for the dimer (Table I).

Throughout the last experiment, the variations in NAD^+ and dimer concentrations were monitored polarographically. Of the initial 0.431 mM NAD^+, 92% was reduced when the electrolysis was stopped. After illumination, the NAD^+ concentration increased from about 0.034 to 0.049 mM and the dimer concentration decreased slightly from 0.199 to 0.194 mM. The initial dimer concentration was calculated based on the assumption that all reacted NAD^+ went to dimer. Within experimental errors, this assumption is valid based on coulometry during electrolysis. However, the value for unreacted NAD^+ may be inaccurate by as much as 50% due to experimental uncertainly at the low concentration levels involved. Molar absorptivities for the dimer, based on the polarographic concentrations, are given in Table II.

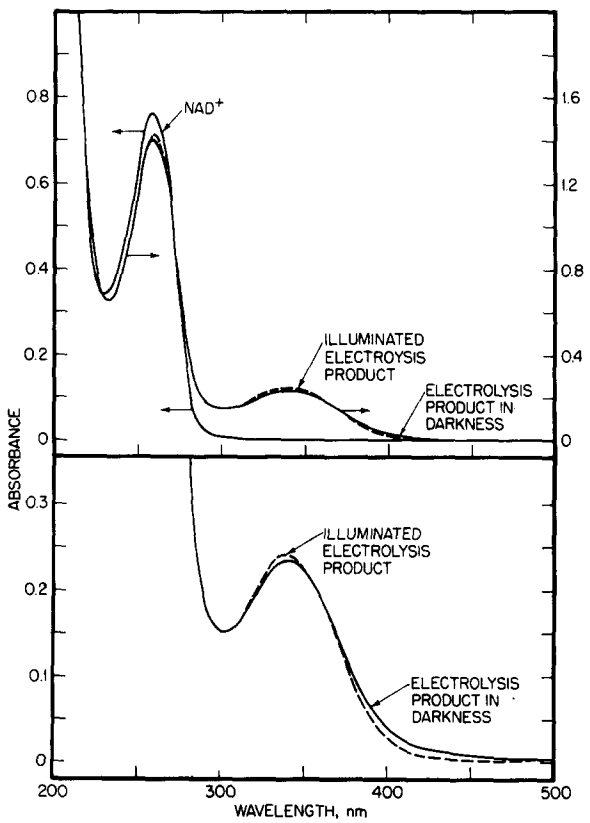

Fig. 2. Effect of illumination of a solution of the NAD dimer. Spectrum of 0.0431 mM NAD^+ is prior to electrolysis. After electrolysis in the dark, the residual NAD^+ was 0.0034 mM and the dimer was 0.0199 mM in the cuvette. After illumination with visible light for 30 min, NAD^+ was 0.0043 mM and the dimer was 0.0194 mM in the cuvette. Concentrations in the electrolysis cell were 10-times those in the cuvette. Expanded scale spectra are given in the lower panel.

Photochemical dimer reaction. Jensen [4] showed that half of the decomposition products of the NAD dimer was a polarographically reducible nicotinamide or 1-substituted nicotinamide, and that the other half was not polarographically reducible. Eisner and Kuthan [22] reported that, on heating, dimers of pyridine and pyridinium salts break down into a 1 : 1 mixture of the corresponding 1,4-dihydropyridine and pyridinium ion. For the NAD^+ dimer the corresponding reaction would be:

$$(NAD)_2 + H^+ \rightarrow NAD^+ + 1,4\text{-NADH} \qquad (1)$$

The products of Eqn. 1 fit the description given by Jensen for dimer decomposition [4] and are in harmony with the spectrophotometric and polarographic results.

Since ϵ for 1,4-NADH at 340 nm is 6220 $M^{-1} \cdot cm^{-1}$ and that calculated for the dark dimer is between 5500 and 5800 $M^{-1} \cdot cm^{-1}$, conversion of dimer to NAD^+, which does not absorb at 340 nm, and to 1,4-NADH would lead to an increased absorbance at 340 nm, which is observed. Since the sum of the molar absorptivities of NAD^+ and 1,4-NADH at 259 nm is $17000 + 14400 = 32200$ $M^{-1} \cdot cm^{-1}$, which is slightly larger than that of the dimer, 32100 $M^{-1} \cdot cm^{-1}$, a small increase in the 259-nm peak is predicted on illumination, which is born out in Fig. 2. The increase in NAD^+ and the decrease in dimer seen polarographically are also in agreement, at least qualitatively. Unfortunately the formation of NADH was not confirmed enzymatically.

Decomposition of the dimer to 1,4-NADH would also account for the report of Burnett and Underwood [23] that the dimer is slowly reduced to 1,4-

NADH; this reduction could not be confirmed [1,2].

Many paths can be proposed for decomposition of the dimer; e.g., decomposition of the coenzyme itself, such as conversion to the tetrahydro-derivatives as proposed by Hanschmann [16], should be considered. Operationally, Eqn. 1 represents the proton-assisted disproportionation of the dimer, analogous to the disproportionation of a semiquinone into the oxidized and reduced species, e.g.,

$$QH\text{-}QH \rightarrow Q + QH_2 \qquad (2)$$

which species can form on uptake or loss of an electron (plus necessary proton transfer) the free radical whose dimerization produced the semiquinone.

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