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PHOTOLYTIC OXIDATION OF REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE

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ABSTRACT

A photo-reaction between thionine dye and NADH has been observed which leads to the oxidation of the co-factor. Thionine (fluorescent), which is converted into semi/leuko thionine (non-fluorescent) during light reaction, fully recovers during the dark reaction. Analytical applications of this discovery are discussed. This reaction opens the door to the measurement of NADH using the fluorescence quenching of thionine and to the construction of several optical biosensors.

1. INTRODUCTION

The measurement of reduced nicotinamide adenine dinucleotide (NADH) is of importance in several bio-analytical procedures, particularly those in clinical and bio-reactor applications. The amount of NADH which is produced or consumed during a cofactor-dependent enzymatic reaction provides a means of monitoring such reactions. This forms the basis of several immunoassay schemes and a variety of biosensors [1]. Further, for several years there has been an increasing interest in the development of miniaturized fluorescence probes for use in biotechnology and in clinical applications. These probes have been based on the measurement of the intrinsic fluorescence of NADH (ca. 460 nm) or on a bio/chemiluminescent reaction involving NADH as one of the reactants [1-3]. Several applications of such probes have been explored including studies of enzymatic reactions, suspended-cell metabolism, suspended cell biomass concentrations and detection of cell-toxic compounds [3-8]. Another important application of particular interest is the measurement of NADH in the whole blood matrix. Due to the strong fluorescence and absorption properties of the endogenous compounds in the blood, this is currently, not possible. It is therefore highly desirable to have a sensing scheme which can overcome these difficulties. One technique which can be adopted to enable the construction of an optical sensor with minimal interference from the background is fluorescence quenching (provided the indicator is chosen carefully) [9-10]. The requirement of full reversibility can be met by selecting an indicator which undergoes dynamic quenching by the analyte. However, up to now, the selection of a suitable indicator could only be achieved by a large scale screening of numerous fluorophores. Another possibility is to utilize the process of reversible exciplex formation between an analyte and the possible photo-excited indicator molecule for which a more defined theoretical basis exists. A novel photolytic reaction is presented here between excited thionine and NADH which can be employed to monitor NADH concentrations. Experimental evidence is presented which confirms the formation of NAD^+ as the product of this reaction. The discovery of this photolytic reaction opens the door to the construction of a novel fibre optic sensor for NADH based on fluorescence quenching.

2. MATERIALS AND METHODS

All the fluorescence measurements were made with a Perkin Elmer LS 5 Spectrophotofluorometer which is equipped with a pulsed (8.3 W Xenon lamp) light source and a Hamamatsu R928P photomultiplier as the detector. The absorption spectra were recorded using a Perkin-Elmer 15 spectrophotometer. The thionine was purchased from Aldrich Chemical and was used as obtained. NADH, NAD^+, glucose and glucose

dehydrogenase were from Sigma Chemical Co. Further, double distilled deionized water was employed. Thionine was chosen as an indicator because of its well known photophysical behaviour (see refs. 11-18) and favourable optical properties such as visible excitation in green-red region. Further, its chemical structure suggested possibilities of a charge transfer reaction with NADH.

The stock solutions of thionine and NADH were prepared by dissolving appropriate amounts of these materials in a suitable quantity of a 0.1 M, pH 7.0 phosphate buffer (buffer). The concentrations of these stock solutions were for NADH and thionine 1.0 mM and 0.5 mM respectively. The standard solution of thionine was prepared by mixing the desired amount of stock solution and diluting it with the buffer. For thionine-NADH interaction studies a suitable amount of the stock solutions of NADH and thionine were mixed together before being diluted with the buffer to obtain a final concentration of 25 μM in NADH and 1 μM in thionine (the mixture). All the experiments were made in a standard quartz cell (1x1 cm^2) at room temperature of 22 +2 °C.

3. RESULTS AND DISCUSSION

The absorption spectra of thionine, NADH, NAD^+ and the thionine+NADH mixture were studied separately before and after irradiation with white light. Various absorption spectra obtained before irradiation are shown in Fig. 1.

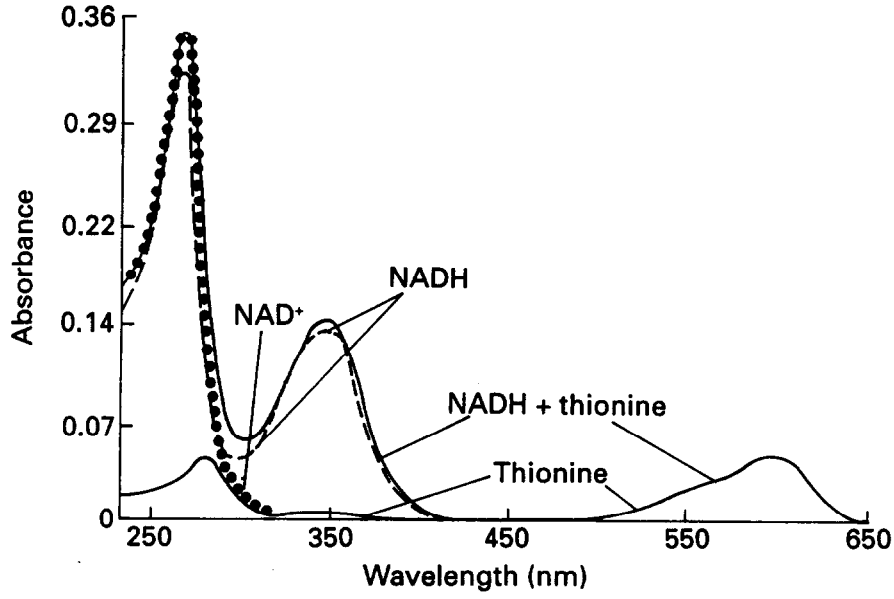

Figure 1. Absorption spectra of thionine (a), NAD^+ (b), NADH (c) and thionine+NADH mixture (d).

No spectral changes are seen in the thionine or NADH spectra when mixed together. The spectra of the thionine+NADH mixture shows merely the sum of the thionine and NADH spectra taken independently, suggesting that thionine does not undergo any reaction with NADH in the ground state. In a separate experiment the mixture (5 ml) was irradiated by white light from a 200 W mercury vapour lamp (which was kept at a distance of 50 cm from the cell containing the mixture) for a period of 1 h. No collimating optics was used for this purpose. Fig. 2 shows the absorption spectra of the mixture before and after irradiation. It is clear that the band ca. 340 nm which

corresponds to NADH disappears in the latter case. The absorption band at 270 nm and the other spectral features remain unchanged. This confirms that a photoreaction had taken place between thionine and NADH during which the NADH was converted into a form which absorbs at ca. 270 nm. A comparison of this band with that of the absorption spectra of NAD^+ suggests that NADH is oxidized during the photoreaction. Further, since no changes in the absorption spectra of thionine could be observed, it is suggested that the concentration of the thionine did not change or that it was fully recovered, i.e. thionine acted only as a catalyst in the photo-reaction.

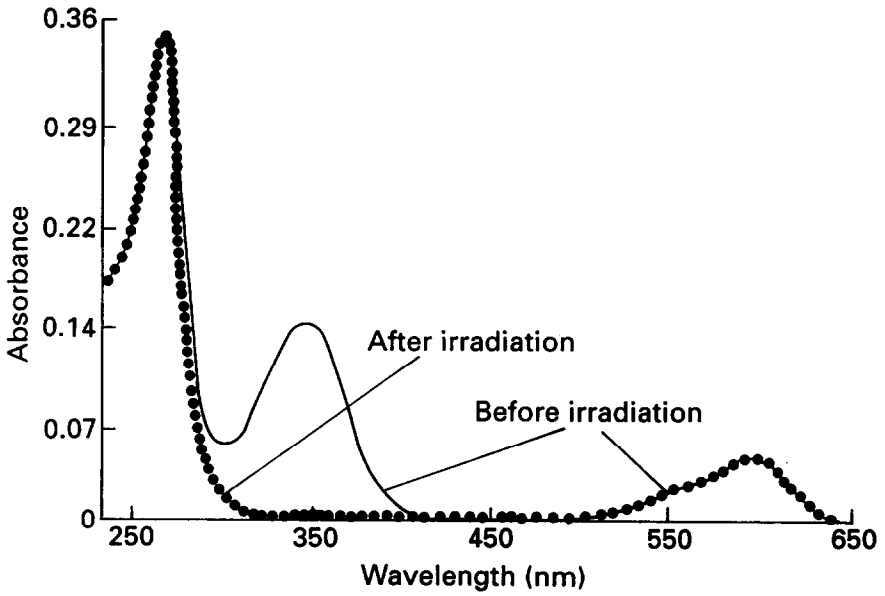

Figure 2. Absorption spectra of the mixture of thionine + NADH before and after irradiation with white light.

In order to further confirm the photo-oxidation of NADH by thionine, the fluorescence spectrum of the mixture of thionine and NADH was studied before and after irradiation. When excited at 340 nm (wavelength of maximum absorption of NADH), the irradiated mixture of thionine+NADH resulted in the characteristic fluorescence of NADH ca. 460 nm. A weak thionine fluorescence ca. 630 nm also resulted. This is caused by the fact that thionine, the fluorescence of which is strongly quenched by NADH, has a very weak absorption at 340 nm. However, the excitation of the irradiated solution revealed interesting results: the NADH fluorescence was not seen and the thionine fluorescence remained almost unchanged, suggesting decomposition of NADH into a non-fluorescent species in the presence of excited thionine. In order to confirm this, the fluorescence spectrum of the thionine+NADH mixture was recorded by selectively exciting thionine only (i.e., at 580 nm) before and after irradiation. A comparison of these spectra with that of the fluorescence spectrum of thionine, recorded in similar conditions, (i.e. same band pass and excitation wavelength of 580 nm) showed no appreciable changes, neither in intensity nor in shape. This suggests that thionine undergoes a photo-reaction leading to the quenching of its fluorescence, and with time the fluorescence recovers back to the original value. This

indicates that the excited thionine molecules continuously cycle the NADH to a product until all the NADH is consumed. Further, the increased fluorescence of thionine is indicative of the reduced NADH concentration and it also suggests that the product formed during the photo-reaction effects neither the ground nor the excited states of thionine.

Thionine is known to undergo upon excitation by visible light a cyclic photochemical reaction with several molecules and ions [11-18]. Both this behaviour and the possible resonance structure of the thionine molecule prompted the suggestion that the excited thionine is reacting with NADH resulting in short lived semi/leuko-thionine (Fig. 3) and NAD^+.

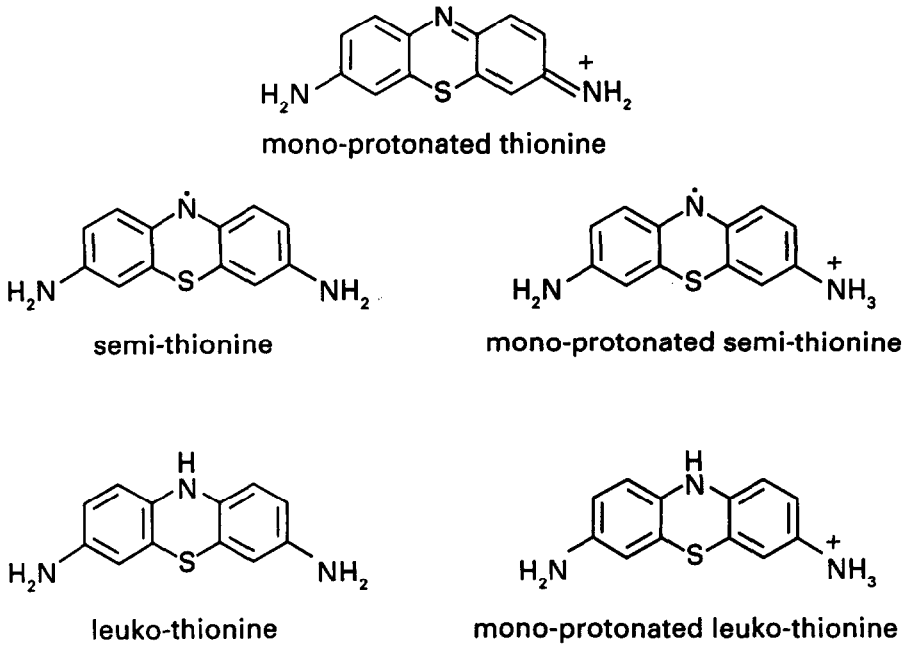

Figure 3. Various forms of thionine.

The above experiments support this reflection and it seems logical to conclude that in the presence of NADH thionine undergoes a cyclic photo-reaction, which results in the oxidized form of the co-factor and ground state thionine. Although only a detailed photophysical study can reveal the actual kinetics of the reaction, the most probable path of the reaction can be summarized in the following reaction scheme:

(1) $TH + H_3O^+ = = = = = TH^+ + H_2O$
(2) $TH^+ \text{---}^{h\nu}\text{---}> TH^{*+}$ excitation
(3) $^*TH^+ \text{--------}> {}^{*3}H^+$ triplet formation
(4) $^*TH^+ + NADH \text{-------}> TH^+ + NAD^+$ fluorescence quench.
(5) $^{*3}TH^+ + NADH \text{-------}> TH^+ + NAD^+$ phosphorescence quench.
(6) $^*TH^+ + NADH \text{-------}> TH_2^+ + NAD^+$ semithionine formation
(7) $^{*3}TH^+ + NADH \text{-------}> TH_2^+ + NAD^+$ semithionine formation
(8) $2TH_2^+ + NADH \text{--------}> TH^+ + TH_4^{2+} + NAD^+$ leukothionine formation
(9) $TH_4^{2+} + NADH + H_3O^+ \text{--------}> TH_2^+ + NAD^+ + 3H_2O$
(10) $TH_2^+ + H_2O \text{--------}> TH^+ + H_3O^+$

Reactions (4) and (5), which represent the collisional quenching, are probably not operative in the present case. This conclusion is supported by the unusually high fluorescence quenching rate constant of 1.8×10^{-14} M^{-1} s^{-1} for the present fluorophore

quencher combination [19]. The photo-reactions described in (6) and (7) are most likely to be the principle mechanism leading to fluorescence quenching. The reaction scheme consists therefore of two major steps; (a) a light reaction during which thionine is converted into semithionine and leukothionine and (b) a dark reaction during which it recovers. A similar behaviour is expected in the case of co-enzyme NADP(H).

The presence of NAD^+ was further confirmed by adding a small amount of glucose and glucose dehydrogenase to the irradiated mixture of thionine and NADH. Glucose undergoes an enzymatic reaction resulting in gluconolactone and NADH. The production of NADH was followed by the recovered absorption band ca. 340 nm and also by the re-appearance of the fluorescence band ca. 460 nm. Finally, the reduction in the intensity of thionine fluorescence confirmed the regeneration of NADH. It is also important to note that the thionine fluorescence was not effected by glucose.

The discovery of this reaction during which NADH is oxidized will provide a new direction for the researchers involved in the development of NADH assays or sensors and their applications. The significance of this discovery is the potential to develop fluorescence assay procedures for NADH and possibly a NADH sensor based on the fluorescence quenching of thionine. Furthermore, numerous secondary optical biosensors based on the measurement of NADH production or consumption can be developed. It is hoped that several other molecules will soon be identified to make this new method more versatile. Related work on (1) the study of the photo-kinetics of this reaction, (2) the search for other molecules showing similar behaviour and based on this finding (3) the development of an optical NADH sensor is in progress.

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