Dehydrogenation studies of dihydronicotinamide adenine dinucleotide (NADH) with methylene blue in the presence of the copper hexacyanoferrate(II) complex and light

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Abstract: The effects of copper ferrocyanide and light on the dehydrogenation rate of NADH by methylene blue is studied. The results suggest that the dehydrogenation rate of NADH with methylene blue is enhanced by copper ferrocyanide. Light also affects the reaction rate.

Keywords: Dehydrogenation, NADH, methylene blue, copper ferrocyanide, photosensitizer

1 Introduction

Whether bioorganic compounds were formed in a primitive terrestrial atmosphere or in an extraterrestrial environment, such as comets, remains controversial. Bioorganic compounds, such as amino acids form easily in a mixture of CH₄, NH₃ and H₂O in a ‘strongly’ reducing atmosphere, when using electrical discharge [1,2] or ultraviolet light [3] as energy sources. The abiotic synthesis of amino acids by X-ray irradiation suggests that the prebiotic formation of amino acids is possible in a primitive earth atmosphere with either X-rays or cosmic rays [4] present. The photolysis of CH₄ in primitive atmosphere during the late archean period may have produced a hydrocarbon smog layer that would have shielded the surface from solar UV radiation [5].

It is debatable as to how long the primitive atmosphere remained anoxic. Most authors agree that the prebiotic atmosphere was anoxic [6]. Cyanide forms easily under
prebiotic conditions [7], and cyanide may have formed stable complexes with the abundant transition metal ions present in the primeval sea. During the course of chemical evolution, cyanide could have formed metal ferrocyanides. Since metal ferrocyanides are usually insoluble in water, they are most likely settled to the bottom of the primitive ocean or were deposited at the seashore, where presence of metal ferrocyanides in the form $\text{M}_2[\text{Fe(CN)}_6] \cdot \text{H}_2\text{O}$, $\text{M} = \text{Fe, Zn, Cu, Mn, Mo, etc.}$ is highly probable. These metal ferrocyanides are good adsorbents [8-10] and ion-exchangers [11,12].

The $\text{NAD}^+ / \text{NADH}$ couple plays a significant role in biological systems (e.g. in glucose metabolism, in electron transport, respiratory control, as a cofactor in dehydrogenases, in the synthesis of ATP, etc.) [13-20]. The reversible redox behaviour exhibited by the $\text{NAD}^+ / \text{NADH}$ couple under physiological conditions is given as:

$$\text{NAD}^+ + \text{H}^+ + 2\text{e}^- \rightleftharpoons \text{NADH} \quad (1)$$

The single stage anodic oxidation of NADH involves the removal of two electrons and a proton to form NAD$^+$.

The structure of nicotinamide adenine dinucleotide (NAD$^+$) combines nicotinamide with two D-ribose molecules, two molecules of phosphoric acid, and one molecule of the purine base, adenine [21]. The reduced coenzyme NADH is characterized by a new absorption band at 340 nm that is not present in oxidized form, with a maximum absorption at 260 nm. The reduced form is air stable. Most NADH is formed in catabolic reactions and is oxidized in mitochondria, as part of the production of chemical potential energy in ATP [22]. The oxidized and reduced form of NAD$^+$ (and NADP) are shown in Figure 1.

Dehydrogenation studies of NADH are of interest, and some reports are available in chemical literature [23-31]. However, no reports are available on the dehydrogenation of NADH in presence of possible prebiotic photosensitizers. Thus, the present work describes the dehydrogenation of NADH by methylene blue (MB) in presence of copper ferrocyanide (CuFc) and light.

2 Experimental procedures

2.1 Chemicals

Potassium ferrocyanide and copper(II) chloride ($\text{CuCl}_2 \cdot \text{H}_2\text{O}$) were obtained from British Drug House (BDH India). Methylene blue dye and NADH were obtained from BDH (England) and Sigma (USA), respectively. All chemicals were of analytical reagent grade and used as such without further purification. Solutions were prepared in doubly distilled water.

2.2 Synthesis and characterization of copper ferrocyanide

Copper ferrocyanide was prepared by adding 167 mL of 0.1 M potassium ferrocyanide solution slowly to a copper chloride solution (500 mL; 0.1 M) with constant stirring [32].
Fig. 1 Oxidized and reduced forms of NAD (and NADP). The pyridine ring of NAD$^+$ is reduced by addition of a hydride ion to C – 4 when NAD$^+$ is converted to NADH (and when NADP$^+$ is converted NADPH). In NADP, the 2'$\text{-}$ hydroxyl group of the sugar ring adenosine is phosphorylated.

The reaction mixture was heated in a water bath at 100 °C for 2-3 h and then cured for 24 h. The precipitate was washed and dried at 60 °C. The final compound was ground and sieved to (100-150) BSS mesh size.

Copper ferrocyanide was characterized by elemental and spectral analysis. Copper ferrocyanide is a reddish, octahedral, amorphous solid with a face centered cubic lattice [33] that showed no X-ray pattern. Copper ferrocyanide is fairly stable in acids (HCl, H$_2$SO$_4$, HNO$_3$), bases (NaOH, KOH, NH$_4$OH) and salt solutions (LiCl, KCl, NaCl, CsCl, RbCl, NH$_4$Cl, CaCl$_2$, MgCl$_2$ and BaCl$_2$) at concentrations ranging from 1.0 to 2.0 M. Copper and iron were estimated by atomic absorption spectrophotometry on an IL – 751 spectrophotometer. Carbon, hydrogen and nitrogen analysis was performed on a CEST – 118, CHN analyzer. Copper ferrocyanide, Cu$_2$[Fe(CN)$_6$]·6H$_2$O, was found to contain Cu 28.30 %, Fe 12.90 %, C 16.03 %, H 2.56 % and N 18.32 % (calculated: Cu 28.43 %, Fe 12.49 %, C 16.12 %, H 2.68 % and N 18.80 %) (yield 99.50 %) by elemental analysis. Copper ferrocyanide has a broad infrared peak at 3700 cm$^{-1}$, which is characteristic of water and the OH group. The spectrum also has a peak at 1600 cm$^{-1}$ due to HOH bending. Two sharp bands at 2090 cm$^{-1}$ and 600 cm$^{-1}$ are characteristic of cyanide and Fe – C stretching, respectively. Another sharp band at 490 cm$^{-1}$ possibly indicates the presence of a copper – nitrogen bond, thus indicating a certain degree of polymerization in the product [34,35].

A detailed study on reaction of NADH with MB was carried out as follows:
(a) the dehydrogenation of NADH with MB without copper ferrocyanide or light,
(b) the dehydrogenation of NADH with MB using copper ferrocyanide only,
(c) the dehydrogenation of NADH with MB with both copper ferrocyanide and light, and
(d) the dehydrogenation of NADH with MB using light only.

A 100 mg sample of copper ferrocyanide (100-150) BSS mesh was used in steps (b) and (c) above. The reactions were observed at room temperature, 27 ± 1 °C. A 100 Watt incandescent lamp was used as a source of visible light; the lamp was kept vertically above the reaction mixture at a distance of 15 cm. Concentrations of NADH and MB were measured spectrophotometrically using a Perkin – Elmer (model 552) spectrophotometer at the corresponding \( \lambda_{\text{max}} \) for each compound (NADH = 263.8 nm; MB = 670.0 nm). The rate of the dehydrogenation reaction was measured with respect to the concentrations of both MB and NADH using a simple method of reaction kinetics.

3 Results

(a) The dehydrogenation of NADH with MB in the absence of copper ferrocyanide and light

The dehydrogenation reaction was followed by monitoring the concentration of both NADH and MB. Initial reaction rates were determined by plotting the substrate concentrations versus time (Figures 2 and 3). The observed initial reactions rates with respect to concentration of NADH and methylene blue are given in Table 1.

![Graph](image)

**Fig. 2** Reaction of NADH with methylene blue with respect to NADH concentration in absence of copper ferrocyanide and light.
The reaction studied in step (b) contained 100 mg of copper ferrocyanide (100-150) BSS mesh. The reaction rate was determined by comparing the decrease in NADH concentration to a blank, in which NADH was kept with 100 mg of copper ferrocyanide under similar conditions. The plots of concentration versus time for the dehydrogenation of NADH with MB in presence of copper ferrocyanide and the corresponding blank are shown in Figures 4 and 5. The reaction rates with respect to concentration of NADH and MB are given in Table 1.
Fig. 4 Reaction NADH with methylene blue with respect to NADH concentration in presence of copper ferrocyanide.
A = NADH + copper ferrocyanide.
B = NADH + methylene blue + copper ferrocyanide.

Fig. 5 Reaction of NADH with methylene blue with respect to methylene blue concentration in presence of copper ferrocyanide.
A = Methylene blue + copper ferrocyanide.
B = Methylene blue + NADH + copper ferrocyanide.

(c) The Reaction of NADH with MB in presence of copper ferrocyanide and light

The dehydrogenation reactions in step (c) were studied in presence of visible light, and reaction rate was determined by comparing the rate of decrease in substrate concentration with the corresponding blank, as shown in Figures 6 & 7. The observed initial reaction rates with respect to concentration of NADH and MB are given in Table 1.
Fig. 6 Reaction of NADH with methylene blue with respect to NADH concentration in presence of copper ferrocyanide and light.  
A = Methylene blue + copper ferrocyanide + light.  
B = Methylene blue + NADH + copper ferrocyanide + light.

Fig. 7 Reaction of NADH with methylene blue with respect to methylene blue concentration in presence of copper ferrocyanide and light.  
A = NADH + copper ferrocyanide + light.  
B = NADH + methylene blue + copper ferrocyanide + light.

The reaction of NADH and MB in the presence of light only

No further change in the reaction rate of NADH and MB was observed when the reaction in step (a) was run in visible light but in the absence of copper ferrocyanide. The observed initial rates are identical to those observed in absence of copper ferrocyanide and light (step a).
4 Discussion

When measured with respect to concentration of MB, the initial rates of reaction of MB with NADH with copper ferrocyanide is $9.16 \times 10^{-7}$ moles L$^{-1}$ min$^{-1}$. The rate increases to $9.83 \times 10^{-7}$ moles L$^{-1}$ min$^{-1}$, when light and copper ferrocyanide are both added. From these data, it is clear that initial rate for disappearance of MB concentration due to reaction with NADH is not much different than the initial adsorption rate of MB on copper ferrocyanide. This observation is valid whether in the presence or the absence of light. When measured with respect to the MB concentration, the reaction rate is almost unaltered whether MB is alone, adsorbed on copper ferrocyanide, or reacting with NADH in presence of copper ferrocyanide or copper ferrocyanide plus light.

When measured with respect to concentration of NADH, the net initial reaction rate of NADH and MB is $3.90 \times 10^{-7}$ moles L$^{-1}$ min$^{-1}$ with copper ferrocyanide and $4.33 \times 10^{-7}$ moles L$^{-1}$ min$^{-1}$ using both copper ferrocyanide and light. These rates are remarkably greater than the rate of $0.83 \times 10^{-7}$ moles L$^{-1}$ min$^{-1}$ found when neither copper ferrocyanide nor light are used. Thus, the presence of copper ferrocyanide and light both enhance the reaction rate between NADH and MB. It is also clear that light enhances the reaction rate between NADH and MB only in the presence of copper ferrocyanide.

The following tentative reaction mechanisms is proposed for the dehydrogenation of NADH with methylene blue in presence of copper ferrocyanide.

$$\begin{align*}
2 \text{CuFc(II)} & \rightarrow h\nu \quad \text{CuFc(II)* + CuFc(II)**} \quad (1) \\
\text{CuFc(II)*} & \rightarrow \quad \text{CuFc(II) + * (low energy)} \quad (2) \\
\text{CuFc(II)** + SH} & \rightarrow \quad \text{SH** + CuFc(II)} \quad (3) \\
\text{SH** + MB} & \rightarrow \quad \text{S + MBH + ** (high energy)} \quad (4)
\end{align*}$$

Scheme 1

$$\begin{align*}
\text{CuFc(II)} & \rightarrow h\nu \quad \text{CuFc(III) + e}\quad (1) \\
\text{SH + MB} & \rightarrow \quad \text{SH + MB}^- \quad (2) \\
\text{SH}^+ - \text{MB}^- & \rightarrow \quad \text{S}^- + \text{MBH}^+ \quad (3) \\
\text{MBH}^+ + \text{e}^- & \rightarrow \quad \text{MBH} \quad (4) \\
\text{CuFc(III) + S}^- & \rightarrow \quad \text{CuFc(II) + S} \quad (5)
\end{align*}$$

Scheme 2

Where CuFc(II) = copper ferrocyanide; CuFc(III) = copper ferricyanide; $h\nu$ = visible light; SH = hydrogenated substrate (NADH); S = dehydrogenated substrate (NAD); MB = methylene blue (coloured); MBH = reduced methylene blue dye (colourless); * = lower energetic state; ** = higher energetic state.

In Scheme 1 of the proposed reaction mechanism, CuFc(II) molecules are converted to
lower and higher energetic states by visible light in step 1. In step (2), the lower energetic CuFc(II)* molecule returns to the ground state, releasing energy without undergoing any reaction. In step (3), the more highly energetic CuFc(II)** molecule transfers its energy to the substrate and returns to ground state. In the last step, the highly energetic, hydrogenated substrate, SH** is oxidized and dehydrogenated by MB to give S and the reduced MBH with the release of energy.

In Scheme 2 of the proposed reaction mechanism, CuFc(II) is oxidized to CuFc(III) by visible light in step 1. In steps 2 and 3, the hydrogenated substrate, SH, and MB react to form S− and MBH+ with the formation of an intermediate species SH+-MB-. In step (4), MBH+ is reduced to MBH by the electron produced in step (1). In the last step, S− is oxidized by CuFc(III) to give the dehydrogenated substrate, S and the reduced CuFc(II).

Both mechanisms are presented as photocatalytic, emphasizing the photo-sensitizing activity of copper ferrocyanide. The experimental error of ± 0.1% in the results may be due to variations in temperature or may be due to an adsorptive interaction of the substrate to copper ferrocyanide, but results reported here are fairly reliable.

5 Concluding remarks

Copper ferrocyanide acts as photocatalyst for the dehydrogenation of NADH by MB. It is therefore possible that prebiotic minerals, such as transition metal ferrocyanides, acted as photosensitizers during the course of chemical evolution on the primitive earth [36].

The dehydrogenation of NADH with methylene blue in presence of copper ferrocyanide is more economical and rapid, indicating a significant advantage over other studies reported in chemical literature for the dehydrogenation of NADH. In my view, this report on the photosensitizing activity of copper ferrocyanide is a new finding in the research area of chemical evolution and the origins of life.

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