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The Effect of NADH in the Oxidization of Tetra Methyl Benzidine in the H_2O_2 /Peroxidase System

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Abstract
More than 1000 chemical material, which used in deferent categories, such as (cosmetic, detergent, pesticides, etc) may cause cancer to humane as will as animal, were recognized .
There carcinogens can be divided into two group:
The first group: material causes cancer without enzyme affection.
The second group: chemically inert materials, which exhibit its Carcinogens action after the effect of enzyme during metabolism inside the living body. And such materials are aromatic amines. Metabolized aromatic amines under the effects the enzymes by different path way and the Peroxidase path is one of these path way, in Which aromatic amines changed from non active into active form, Which can interact with the larges bio molecules as nucleic acids DNA and inhibited it. The aromatic amines metabolized under the effects the enzymes by different path way. and the Peroxidase path is one of these methods, in which aromatic amines changed from non –active into active form ,which can interact with larges biological molecules as nucleic acids DNA and inhibited it, Intra cellular components performed multi- functional such as Glutathione GSH, NADH. It was chosen because of the high damper NADH concentration in the cell (10-3M) and its spread in all types of tissues NADH(H+) inhibit large number of products of oxidation of the Aromatic amines cancerogenus compounds, such as benzidine Substrate and its derivatives such as3,3',5,5' tetra methyl benzidine in Competitive path way. NADH: nicotine amide adenine dinucleotide (reduced form).
Keywords: tetra methyl benzidine, Peroxidase, inhibition, nicotine amide adenine dinucleotide.

1- Introduction

The American scientist shows F. Rocher Cancer Institute director said that 90% of the causes of cancer caused by the surrounding environment [1]. Smoking causes 40% of the disease deaths and causes 30% nutritional factors and 10% occupational factors. The Russian researcher Georgina Bouncer carrid out experiments on the beta –naphthel amine, and she has been discerned that: The direct injection of beta –naphthel amine into dogs and rabbet did not cause tumor. However, taking this substance by mouth caused liver cancer. The researcher suggested that: the substance transformed into carcinogen form as the rustle of passing through the animal's organs (mouth, stomach, intestine), that is during metabolism [1]. The aromatic amines used widely in the syntheses of many dye materials, include cosmetic and hair coloring. For an example, the 1,2-phenylenediamine is used in the syntheses of heterocyclic chemical compounds and as hair coloring material. The absorption of this compound from the living organ in the presence of enzymes causes bladder cancer. In spite off the contact is through the head's skin, which is relativity far from the bladder .Further more, excessive use of medicine and long time incurrence to aromatic amines may cause this illness [2]. NADH inhibit large number of products of oxidation reactions of the amino compounds with enzymes such as tetra methyl benzidine in competitive path way.

Tetra methyl benzidine(T-MB) (FW =240.35): is one of aromatic compounds aromatic amines which is oxidized in two phases . At each phase of the loss of one electron and a turn as a result of this amino group turned into imino group and turn it Tetra methyl benzidine material to the active form ,which can interact with DNA (Figure3)[3].

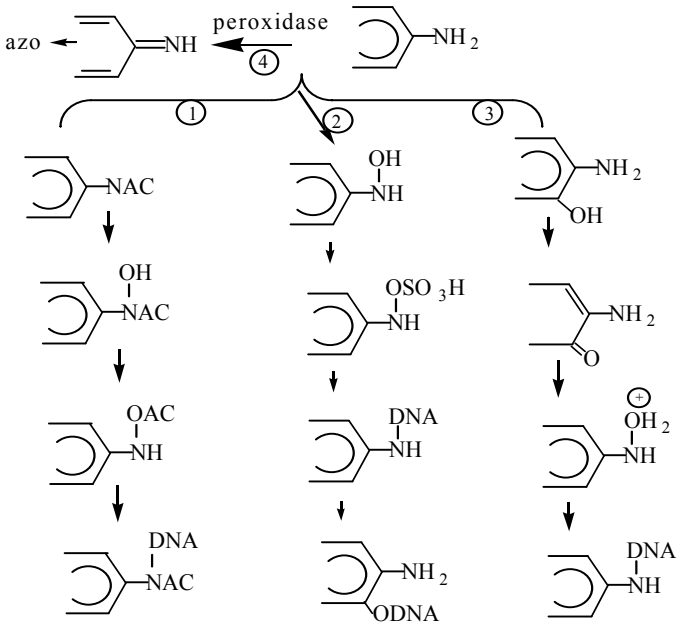


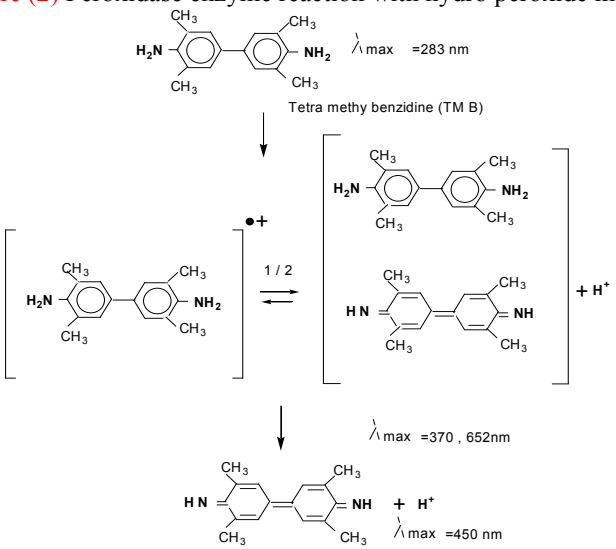
Figure (1): Method of metabolism of aromatic amines.

The figure (1) shows ways metabolism of aromatic amines witch indicates the number (1) in the interaction (2) of acillation atom of nitrogen and then enter. A group hydroxyl, or enter cligue hydroxyl atom nitrogen directly figure(2) and can last for a compound that protonation (H^+)and turns into a electrophilic compound doting electrons(3) which can easily interact with DNA disincentive his movement in the transfer of genetic traits. Of the ways of metabolism tetra methyl benzidine the Peroxidase road (4) which amino group($-NH_2$) tarned into imino group (=NH) and to cancerogenus azo compounds [4].

NADH: Nicotine amide adenine dinucleotide: a chemical compound found in all living cells and plays a role coenzyme composite molecular structure $C_{21}H_{27}N_7O_{14}P_2$ (663.44 g/mol),and dissolved in water. And consists of two units of oligonucleotid; the first contains a loop adenosine and contains a second amide nicotine, linked through a set phosphate. Have a nitrogen atom in the ring nicotine amide additional positive charge. Was chosen damper NADH due to its spread in all types of tissues and high concentration in the cell 10-3M .

Peroxidase enzyme (EC 1.11.1.7): it is one of oxidation and reduction ,are widely spread in plants (Horserodish, figs,…).it has a molecular weight of 44000 and the hematin represents 1.84% of weight. It is properties of reaction the enzyme is its ability to bind with hydro peroxide to composition of an intermitted compound is characterized by the properties of different spectral and discovered four types of complexes, and found to be the previous four complexes regard to the concentration of hydro peroxide. Figure (3) shows of Peroxidase enzyme reaction mechanism with of S_1 and S_2 [5-8]

Complex I consists directly sell add hydro peroxide a complex weak green and soon changes to complex II red color. illustrated in Figure (2) Peroxidase enzyme reaction with hydro peroxide mechanism.



Figure(2): The different metabolic forms of tetra methyl benzidine

Figure: The different metabolic forms of tetra methyl benzidine
The figue (3) shows the method of metabolism of tetra methyl benzidine according the Peroxidase way in which give up two electrons of the reactive material (-2e) and the transformation of reactive material to form active through amino group ($-NH_2$) tarned into imino group (=NH)
The effect of Peroxidase /H_2O_2 in aromatic amines (AA) [4]

$$E + S_1 \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} E_1 + H_2O \quad (1)$$

$$E_1 + S_2 \xrightarrow{K_2} E_2 + \tilde{P} \quad (2)$$

$$E_2 + S_2 \xrightarrow{K_3} E + \tilde{P} \quad (3)$$

$$2\tilde{P} \xrightarrow{K_4} P \quad (4)$$

Figure(3): The effect mechanism of the enzyme Peroxidase
S_1: hydro peroxide , E: enzyme , K: the fast constant of reaction.
S_2: of tetra methyl benzidine, P: the reaction product.
E_2: the second complex.

2- The aim of the research:
Study the kinetic action of the Peroxidase enzyme in the presence of tetra methyl benzidine and oxidizer substance. The effect of the concentration of material tetra methyl benzidine and [H_2O_2] on the rate of the enzyme's reaction, and find the kinetics constants with or without the presence of inhibitors. The kinetics constants contain the following: Maximum velocity Vmax, Michaelis constant Km, enzyme's activity constant K_{kat}= E/V, The constant which signify the catalysis intensity K_{kat} / km.
Find the kinetic constants in the absence and presence of inhibitors

Oxidation product		**Amine**		**substrate**
$E.10^{-4}$ $M^{-1}.cm^{-1}$	λmax	$E.10^{-4}$ $M^{-1}.cm^{-1}$	λmax	
3,90	655	2.37	287	TMB

3- EXPERIMENTAL
3-1- Materials and methods
The preparation of buffer solution of phosphate citrates. In a flask contains 0.5 liter of distilled water, the following materials were added: 3.2 liter of ortho phosphoric acid. 11.9 g of citric acid, 3.4 g of boric acid and 343ml of sodium hydroxide were added. Different pH values of the pervious solutions were prepared.
Determined the concentration of Peroxidase enzyme by using UV Instrument.
A-Zero the UV instrument in the wave length 405 nm was achieved by adding 2ml of distilled water in the instrument cell.
B-Then 2ml of glycerin 75% was added, and the instrument was zeroed again.
C-The sample was prepared by adding 1 ml from the solution in section B with 1ml of Peroxidase enzyme; the light density ΔD was measured.
D- The concentration of the Peroxidase enzyme was calculated in the wave length 403 nm by using the molar absorption coefficient $109000M^{-1}Cm^{-1}$ from the following equation:

$$[\text{Peroxidase}] = \Delta A / \zeta L$$

Where: ΔA = light density, L= the length of the UV instrument cell, ζβδλ=Absorption Coefficient
The enzyme solution was saved at grate (-15°C) below zero.
The pH value for buffer solution of phosphate-citrates was equal to 5.5 The preparation of substrate solution of 3,′3,5,′5- tetra methyl benzidine (0.01M) In 50 ml 50% of methyl form amide and 50 ml of distilled water,24mg of the substrate was dissolved.
In the preparation of the inhibitors solution the methyl form amide 50% was Used, and the buffer solution was used in preparation of water peroxides. The volume of the mixture reaction was 2 ml, with or without the presence of the inhibitors and contains the following materials:
A-In the absence of the inhibitors: substrate, enzyme, buffer solution and H_2O_2.
B- In the presence of the inhibitors: substrate, enzyme, inhibitor buffer, solution and H_2O_2.
The micro pipette was used to obtain the required volumes.
The tubes, which contain the mixture of reacted materials, were incubated in thermostat at the temperature of 30 °C. The rate if the reaction indication by the rate of the formation of the oxides products was expressed by using

the equation: V= + d C / d A. Where:
A: The reactor material, B: The product, Peroxidase enzyme from horseradish Japan.

4- Result and discussion

Work was done at the ideal pH Value of the Peroxidase enzyme pH 5.5 the effect of different concentration of NADH at the speed of interaction enzyme in inverted coordinates; Deduce from the figure (4) next: The compound NADH inhibits oxidation reaction 3, 3', 5, 5'- tetra methyl benzidine H_2O_2/peroxidase in a competitive way The maximum velocity of the enzymatic interaction was reached 8.4 $x10^{-6}MS^{-}$ The value of the Michael's constant Km with a reactive material 3, 3',5, 5'- tetra methyl benzidine $1.33x10^{-4}$. mol/ l, and in the case not to use any inhibitor. The value of the Michael's constant with the presence inhibitor NADH has the following values:

$1.4\ x10^{-4} \rightarrow 1.6\ x\ 10^{-4} \rightarrow 1.7\ x\ 10^{-4} \rightarrow 2.3\ x\ 10^{-4}$ mol/l

When using the concentrations of the inhibitor respectively:

$0.5\ x\ 10^{-5} \rightarrow 1.0\ x\ 10^{-5} \rightarrow 1.5\ x\ 10^{-5} \rightarrow 2.0\ x\ 10^{-5}$ mol/l

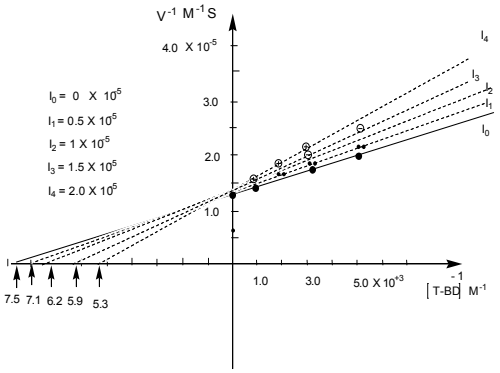
This means the a Michael's constant value in presence of the inhibitor NADH In creased

The constant value of the catalyst k_{kat} was $0.85x10^{+4}\ S^{-1}$

$$K_{kat} = \frac{V_{max}}{[E]} = \frac{8{,}46\ X\ 10^{-6}}{1\ X\ 10^{-9}} = 0.85\ X\ 10^{+4}\ \ S^{-1}$$

The constant value of k_{kat} / k_m expressed the intensity of oxidation value $6.4\ x\ 10^{+7}\ M^{-1}S^{-1}$

$$\frac{K_{kat}}{K_M} = \frac{0.85\ x\ 10^{+4}}{1.33\ x\ 10^{-4}} = 6{,}4\ x\ 10^{+7}\ \ M^{-1}\ S^{-1}$$



1 - NADH = 0,0 X 10^{-5} M

2 - NADH = 0.5 X 10^{-5} M

3 - NADH = 1.0 X 10^{-5} M

4 - NADH = 1,5 X 10^{-5} M

5 - NADH = 2.0 X 10^{-5} M

Figure (4) The effect of different concentration of NADH at the fast of interaction enzyme in inverted coordinate.

Tab (1): constants inhibiter of compound NADH at different concentrations

NADH concentration	constants inhibiter K_I
0.0 x 10^{-5}	1.33 x 10^{-4}
0.5 x 10^{-5}	1.4 x 10^{-4}
1.0 x 10^{-5}	1.6 x 10^{-4}
1.5 x 10^{-5}	1.7 x 10^{-4}
2.0 x 10^{-5}	1.9 x 10^{-4}

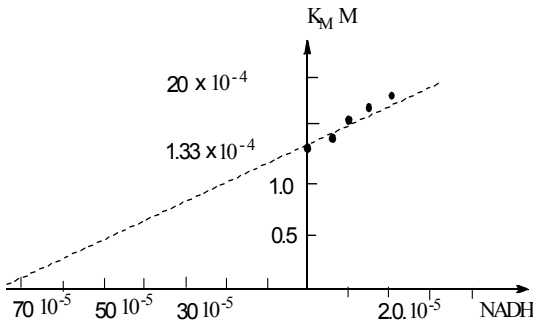


Figure (5) a constant inhibition of the existence of NADH

5- Conclusions and suggestions

The ideal value of the enzyme Peroxidase was used with aromatic amines (AA) 5.5.

From Figures (4) and (5) we conclude that:

1-The compound NADH inhibits oxidation reaction of 3, 3′,5, 5′ tetra methyl benzidine in presence of Peroxidase enzyme and hydro peroxide in a competitive way. (Figure 4).

2- The maximum velocity of the enzymatic reaction value was 8.4×10^{-6} MS^{-1}.

3-The value of the Michaelis K_m constant with the reaction material was 3, 3′, 5, 5′tetra methyl benzidine 1.33 $\times10^{-4}$ mol/l in the absence of inhibitor.

4-The value of the Michaelis K_m constant has increased with the presence of NADH and has become as follows: 1.4×10^{-4}, 1.6×10^{-4}, 1.7×10^{-4}, and 1.9×10^{-4} mol/l.

The increased value of the constant presence of the inhibitor Michaelis NADH in the following amounts 1.05, 1.20, 1.28, 1.43

This indicates that the affinity between the TMB material reaction and Peroxidase enzyme decreased by approximately one and a half times. 1.9×10^{-4} / $1.33.10^{-4}$ =1.5.

Recommendation: study of the effect of phosphate groups on the products of oxidation of aromatic amines with the presence of Peroxidase.

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