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The Effect of NADPH in the Oxidization of Benzidine in the H_2O_2 / Peroxidase System

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Abstract
The carcinogenic chemicals are divided into two group:
The first group: it is effective in cancer and does not require pre-activation, such as alkaline agents.
The second group: their number is larger than the first group and need to be activated before they become carcinogenic, such as aromatic amines.
When entering the organism, the chemical compounds are subject to the effect of enzymes, especially oxidation enzymes such as oxygenases and Peroxidases. In the Peroxidase oxidation of aromatic amines are oxidized
And the amino group is transformed into an imino group.

$$H_2N\,C_6H_4.C_6H_4\,NH_2 \xrightarrow[-2e^- \quad -2H^+]{H_2O_2\ /\ \text{Peroxidase}} (HN=)\,C_6H_4.C_6H_4\,(=NH)$$

The NADP(H^+) inhibitor was selected because of its high concentration in the cell on the one hand and its spread in all types of tissues on the other.
Keywords: benzidine, Peroxidase, inhibition, Nicotinamide adenine dinucleotide phosphate.

1-Introduction:
The carcinogenic chemicals are associated with DNA and cause a change in its structure and cells grow and multiply without control, causing cancerous growth. All our environment is contamination with substance that cause cancer. We touch them eat them breathe them. The number of known carcinogens is large and is constantly increasing and includes a wide range of inorganic compounds and organic compounds. Often carcinogenic factors in our environment are caused by human-made materials. But some carcinogens are the products of living cell metabolism Statistical studies highlight the relationship between the external Environment and the incidence ob cancer (smoking and lung cancer, radiation and leukemia) in our research benzidine and its derivatives were selected for several reasons:

First: these compounds are widely used in the chemical, pharmaceutical, industrial, biological and medical research, and therefore a wide range of people are in constant contact with the previous materials.

Second: the cancerous effect these compounds appears after metabolic activation.

Third: benzidine and its derivatives are similar structure and differ from each other by the number of groups associated with --CH_3 or -OCH_3.

Fourthly: benzidine and its derivatives are typical substrates hematoproteins.

Oxidation of benzidine and its derivatives the first stage in the formation of carcinogenesis [2].

The most common carcinogenic substances affecting human and animal DNA include the following compounds:

1-Poly aromatic hydrocarbons
Many of these compounds are known to be carcinogenic and are produced from incomplete fuel combustion such as cool, diesel, grease and tobacco.
3.4- benzopyrene plays a special role in the human environment: Remains of combustion of oil, traffic fumes, dust on the streets, fresh land in the field, smoke of cigarettes and even smoked products contain in some cases a significant amount of this carcinogenic hydrocarbon. Tar contains carcinogens, including 3,4-benzopyrene and 3-methylcholanthrene
2-Aromatic hydrocarbons heterogeneous such as:
Methyl-3,4,5,6-dibenocarbazole , 1,2,5,6-Dibenzoacridin(C13H9N)
3-Aromatic amines and amides: such as
aniline,benzidine,2-naphtylamine, acetylaminofluorene AAF such as 4-Amino azo compounds:
4-dimethylaminoazobenzene
5-Nitroso compounds: such as N-Nitrozo-N-methyl urea, Dimethylnitrozoamine
6-Aflatoxins: such as Aflatoxins B1
Ethylcarbamte, Urethane, 7-**Other compounds such as :**
Ethionine, Carbon tetrachloride CCl_4.
Be^{+2},CO^{+2},Pb^{+2},Ni^{+2} Carcinogenic minerals, Epoxides, Lactones, Plastics [3]. The products of metabolism of

aromatic amines are formed in living organisms as a result of:
a-The group of acetyl joined the amino group or its parties
b-Cut double bond in diazo compounds.
c-Insert the hydroxyl group into aromatic ring.
d-Interaction with glucuronic acid.
Benzidine and aromatic amines The aromatic amines were synthesized by W.Perkin in 1856 and It was shown to cause bladder cancer in 1895 by the world L.Rehn. Aromatic amines by themselves are not carcinogenic if the are not metabolized within living organisms. Georgiana Bonser came to this result through the experiment on p-Naphthylamine. Benzidine has been widely used since the middle of the XIX century as a basis for the production of a large number of dyes, especially azodyes, for wool, cotton and leather. In the past, benzidine and its salts have been used in forensics for blood detection, in the rubber industry, in the production of adhesives and plastics. Enzyme activating aromatic amines different methods are synthesized electrolytes (A^+) can interact with nucleophilic compounds inside the cell such as proteins and nucleic acids. nucleophilic compounds include a wide range of compounds that differ from one another in chemical structure ,function .Benzidine is one of the aromatic substances that are oxidized by the Peroxidase and represent a substrate in our research Figure (1)[4. 5]

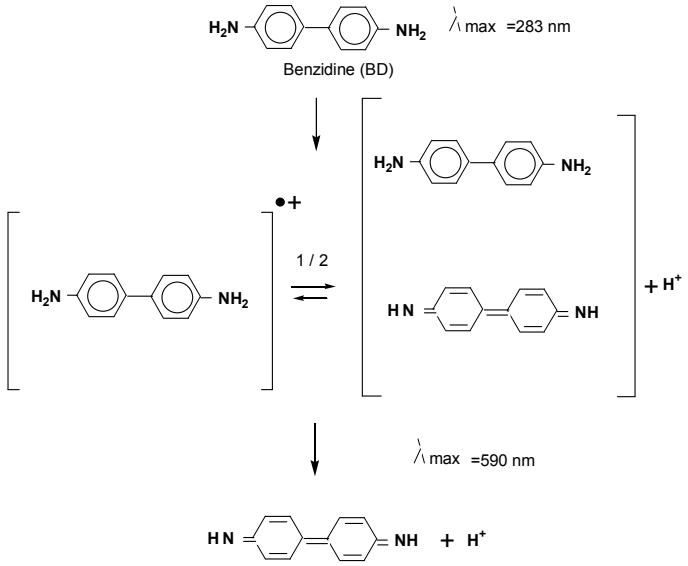

Figure (1): Mechanism of benzidine metabolism (in the absence of inhibitors)

Peroxidase enzyme (EC1.11.1.7) :

Peroxidase is one of the enzymes of oxidation and reflux it is crystalline, dark brown, its molecular weight is 44000. hematin is 1.48% of its weight it is widely found in plants such as radish and figs. Peroxidases consist of un – colored glycoprotein and a brown –red –Ferri porphyrin to it. Figure (2)

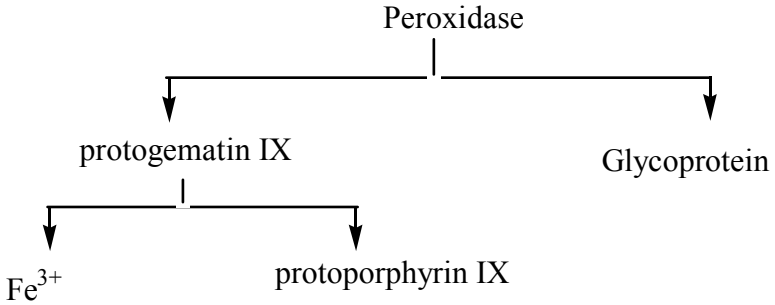

Figure (2): the chemical structure of Peroxidase enzymes

Protoporphyrin IX included in the active center forming a porphyrin ring of heme. Peroxidase is associated with H_2O_2 , forming a complex with different spectral properties benzidine (Bd) is the reaction substance of the enzyme Peroxidase, which oxidizes with the presence of H_2O_2
Figure (2) shows the chemical structure of NADP and NADPH [6 .7]

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

$$E_1 + S_2 \underset{K_{-2}}{\overset{K_2}{\rightleftharpoons}} E_1S_2 \xrightarrow{K_3} E_2P \xrightarrow{K_4} E + P$$

Figure(3) mechanism of reaction of Peroxidase enzyme with the interaction material S_1 and S_2.
E_1: Primitive enzyme,
S_1: is a complex of the primary enzyme and H2O2.
S_2: substrate (benzidine)

Nicotinamide adenine dinucleotide phosphate NADP$^+$:
A coenzyme composed of ribosylnicotinamide 5‘-phosphate coupled by pyrophosphate linkage to the 5'-phosphate adenosine 2',5' –bisphosphate.
In all live cells and its general chemical form $C_{21}H_{29}N_7O_{17}P_3$ its molecular
mass of 744.413g/mol dissolves in water . the nitrogen atom in the nicotine ring has an extra positive charge . Figure(4). The NADPH inhibitor was selected for its spread in all tissues and its high concentration in cell 10^{-3}M [8]. Figure (4) shows the chemical structure of NADP and NADPH

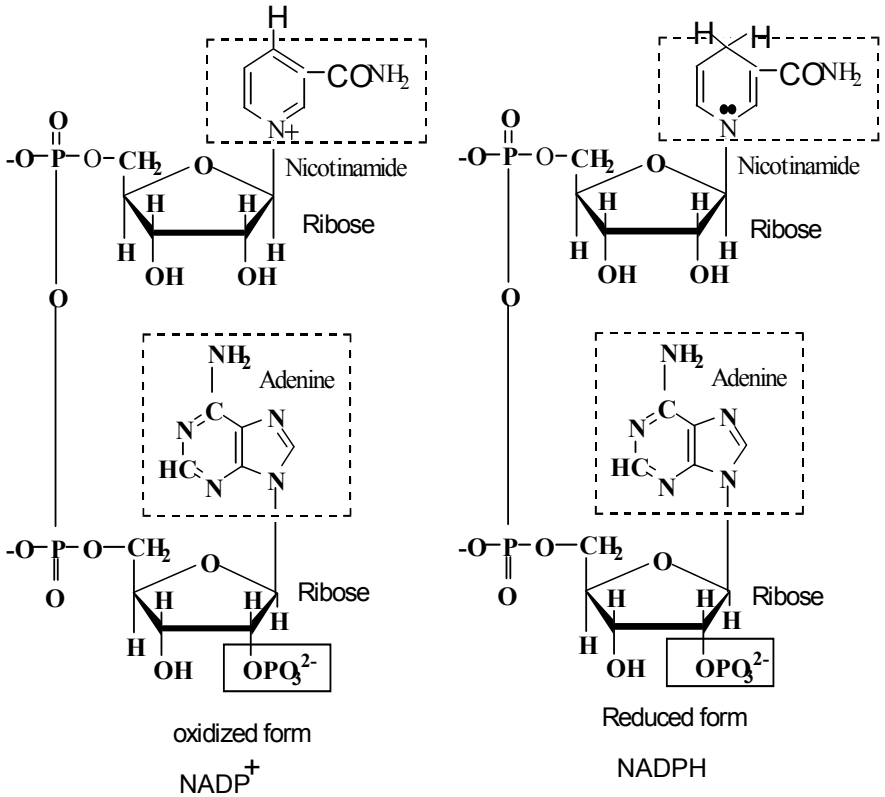

Figure (4) shows the chemical structure of NADP and NADPH

2-The aim of the research :
The research aims to Study the kinetic action of the Peroxidase enzyme in the presence of benzidine and H_2O_2 .
a- Effect of pH on benzidine oxidation speed
b-The effect of the concentration of benzidine on the rate of the enzyme's reaction.
c-Identification of inhibition
d-find the kinetics constants with or without the presence of inhibitors. and find the kinetics constants with or without the presence of inhibitors. Km , Vmax, (Kcat = Vmax / E),
(Kcat /Km) 50Find a concentration of the inhibitor NADPH which led to an find a enzymatic efficiency of

up to 50%

h-Find a constant inhibiting compound NADH

l-Comparison between: The constant inhibition of NADP(H+) is greater than that of the compound inhibition of the compound NAD (H+) by 5.6 times.

3-Methods of analysis

Methods of analysis used in research : kinetic methods , spectral analysis ,

chemical methods:

The concentration of H_2O_2 was determined using the iodometric calibration.

$$[H_2O_2] = \frac{\triangle V(Na_2S_2O_3) \times 0.01N}{2} \times 10^{-3}$$

1ml of hydrogen peroxide was taken and extended 1000 times , then 1ml of the previous sample was added .the following were added : 10ml of ice acetic acid and 1g of sodium bicarbonate (NaHCO3), five minutes later we added 1ml of saturated solution of potassium iodide (KI), dark place 25 minutes followed by calibration with the solution of sodium thiosulfate
($Na_2S_2O_3$) 0.01N .

Peroxidase from Horse Radish spectral analysis: 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-1Cm-1 from the following equation : [Peroxidase] = Δ A / ζ L

Where : ΔA = light density, L= the length of the UV instrument cell, ζ=Absorption Coefficient

Enzyme solution saved at grade (15°C) below zero kinetic 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(0.1N) were added . Different pH values of the pervious solutions were prepared. The pH value for buffer solution of phosphate-citrates was equal to 5,5 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

Table-1

Oxidation product		amin		reactant
$E.10^{-4}$ M-1.cm-1	λ max	$E.10^{-4}$ M-1.cm-1	λ max	
0.31	590	2.10	283	Bd

Preparation of benzidine solution

The preparation of substrate solution of 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.

4. Result and discussion

4-1-Factors affecting the speed of enzymatic reaction

4-1-1-Effect of pH on benzidine oxidation speed

Figure (5) shows the Effect of pH on the effectiveness of Peroxidase

This experiment shows the optimal pH for the action of Peroxidase enzyme which reached a value of 5.5

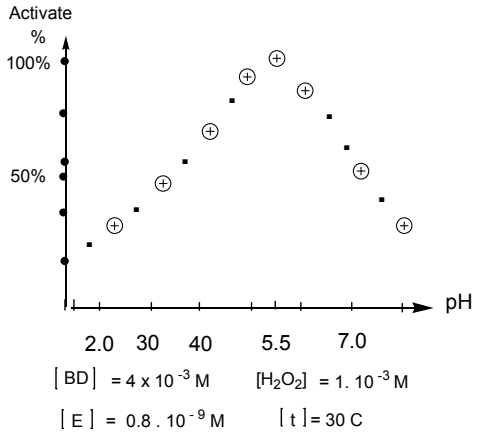

Figure (5) Effect of pH on the effectiveness of Peroxidase

4-1-2 effect of increased concentration of NADPH in enzymatic activity

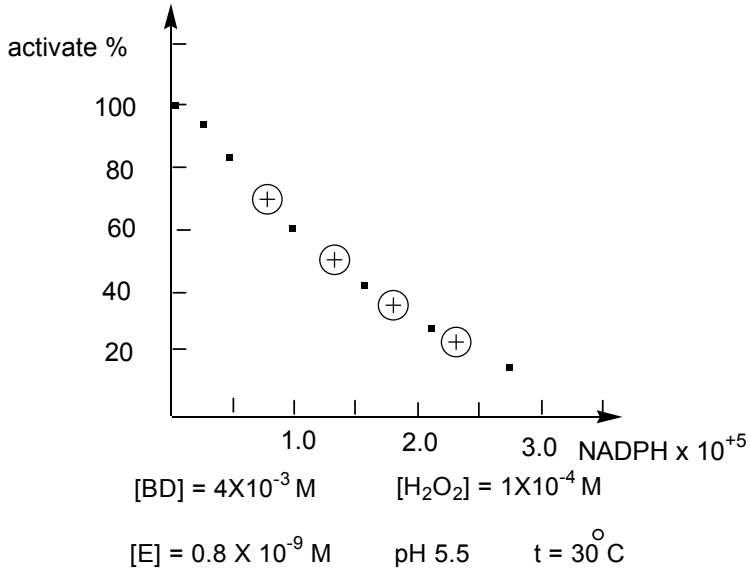

Figure (6)effect of increased concentration of NADPH in enzymatic activity

The value of the inhibitor concentration which led to reduced reaction speed reached 50%. 1.2 x 10^{-5} mol/l .Figure (6).

4-1-3-The effect of different concentration of NADH at the speed of interaction enzyme in inverted coordinates.

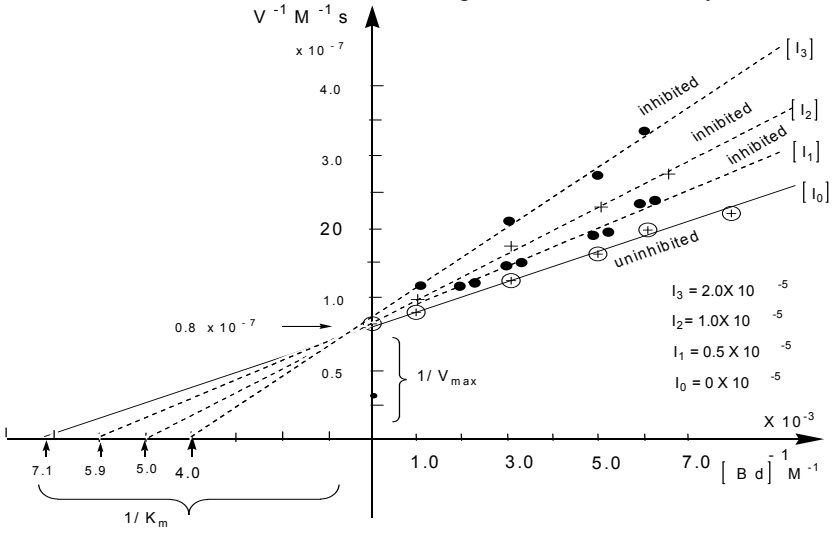

Figure (7)The effect of different concentration of NADPH at the speed of interaction enzyme in inverted coordinates;

Deduce from the figure (7) next: The compound NADPH inhibits oxidation reaction wholesale benzidine

H_2O_2/peroxidase in a competitive manner The maximum velocity of the enzymatic interaction was reached $1.37x10^{-}7MS^{-1}$ The value of the Michaels constant Km with a reactive material benzidine 1.37 x 10^{-4} mol/ l, and in the case not to use any inhibitor. The value of the Michaels constant with the presence inhibitor NADH has the following values: $1.7x10^{-4} \rightarrow 2.0x10^{-4} \rightarrow 2.5x10^{-4}$ mol/l When using the concentrations of the inhibitor respectively: $0.5x10^{-5} \rightarrow 1.0x10^{-5} \rightarrow 2.0x10^{-5}$ mol/l This means the a Michaels constant value in presence of the inhibitor NADH In creased almost twice $2.5x10^{-4}$ /1.4 x 10^{-4} = 1.78 Calculate enzyme concentration[E] in volume of 2 ml .

$$V_1 N_1 = V_2 N_2 \quad \Rightarrow \quad 0.04 \times 0.8 \times 10^{-9} = 2 \times N_2 \quad \Longrightarrow$$

$$N_2 = 0.016 \times 10^{-9} \Longrightarrow \quad N_2 = 1{,}6 \times 10^{-11} \quad \text{Eq/ liter}$$

The constant value of the catalyst kCat was 0,85x10+4 s-1

$$K_{Cat} = \frac{V_{max}}{[E]} = \frac{1.37 \times 10^{-7}}{1.6 \times 10^{-11}} = 0.85 \times 10^{+4} \quad S^{-1}$$

The value of the constant kCat / km which expresses the oxidation intensity value 0.61x10+8 $M^{-1}S^{-1}$

$$\frac{K_{Cat}}{K_M} = \frac{0.85 \times 10^{+4}}{1.4 \times 10^{-4}} = 0.61 \times 10^{+8} \ M^{-1} S^{-1}$$

4. Result and discussion

the pH Value of enzyme Peroxidase 5.5.Figure (50) The effectiveness of the enzyme decreased until half (50%) when the concentration of the inhibitor 1.2 x 10^{-5} mole/l. Figure (6)**.** The effect of different concentration of NADPH at the speed of interaction enzyme in inverted coordinates;

Deduce from the figure (7) next: The compound NADH inhibits oxidation reaction wholesale benzidine H2O2/peroxidase in a competitive manner The maximum velocity of the enzymatic interaction was reached $1.37x10^{-7}MS^{-1}$ The value of the Michaels constant Km with a reactive material benzidine $1.4X10^{-4}$ .mol/ l, and in the case not to use any inhibitor.

The value of the Michaels constant with the presence inhibitor NADH has the following values: 1.4 x 10^{-4} $\rightarrow 1.7x10^{-4} \rightarrow 2.0x10^{-4} \rightarrow 2.5x10^{-4}$ mol/l

When using the concentrations of the inhibitor respectively:

0.5x10-5 →1.0x10-5 →2.0x10-5 mol/l

This means the a Michaels constant value in presence of the inhibitor NADH In creased almost twice $2.5x10^{-4}$ /1.4 x 10^{-4} = 1.78

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

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

The value of the constant kCat / km which expresses the oxidation intensity value $0.61x10^{+8}$ $M^{-1}S^{-1}$

$$\frac{K_{kat}}{K_M} = \frac{0.85 \times 10^{+4}}{1.4 \times 10^{-4}} = 0.61 \times 10^{+8} \ M^{-1} S^{-1}$$

4-1-4-Find a constant inhibition of NADPH

The table (1) shows the values of inhibition constants (Ki) and the value of the (michales-Menton)Km

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

NADPH concentration	constants inhibiter KI
0.0 x 10 -5	1.4 x 10 - 4
0.5 x 10 -5	1.7 x 10 - 4
1.0 x 10 -5	2.0 x 10 – 4
2.0 x 10 -5	2.5 x 10 – 4

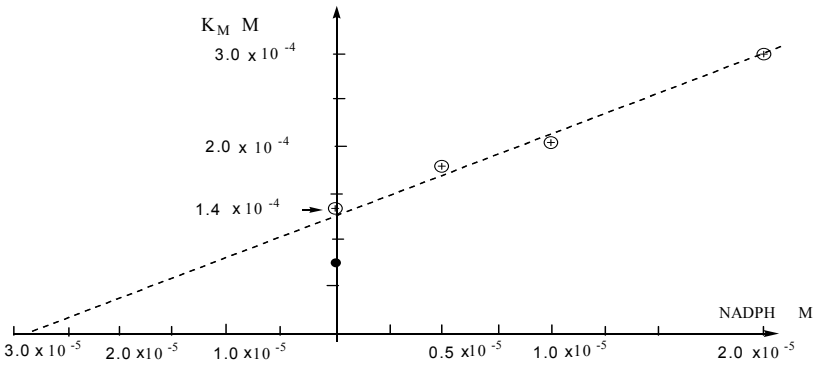

Figure (8) a constant inhibition of NADPH

The constant inhibition of the compound NADPHassociated with benzidine reached the value Ki = 28×10^{-6}mol/liter Figure (8)

Wile The constant inhibition of the compound NAD H associated with benzidine reached the value Ki = 5×10^{-6}mol/liter (previous search).

The result: The constant inhibition of NADPH is greater than that of the compound inhibition of the compound NAD H by 5.6 times.

5-Conclusions and suggestions

The ideal value of the enzyme Peroxidase was used with benzidine 5.5. Figures (5) From Figures (7) and (8) we conclude that:

1-The compound NADPH inhibits oxidation reaction of benzidine in presence of Peroxidase enzyme and hydro peroxide in a competitive way.

2- The maximum velocity of the enzymatic reaction value was

Vmax = 1.37×10-7 MS-1.

3-The value of the Michaels Km constant with the reaction material was benzidine 1,4 ×10-4 mol/l in the absence of inhibitor .

4-The value of the Michaels Km constant has increased with the presence of NADPH and has become as follows: 1.7×10-4, 2.0×10-4. and 2.5×10-4 mol/l.

The in creased value of the constant presence of the inhibitor

NADPH in the following amounts 1.21 , 1.43 , 1.78

this indicates that the affinity between the (Bd) material reaction and Peroxidase enzyme decreased. 2.5 ×10^{-4} / 1.37 × 10^{-4} = 1.78.

The constant inhibition of the compound NADPH associated with benzidine reached the value Ki = 28×10^{-6}mol/liter Figure (8)

Wile the constant inhibition of the compound NADP (H+) associated with benzidine reached the value Ki = 5×10^{-6}mol/liter

The final result: The constant inhibition of NADP(H+) is greater than that of the compound inhibition of the compound NAD (H+) by 5.6 times.

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