A NEW RELATIONSHIP BETWEEN CYTIDINE DEAMINASE ACTIVITY AND CANCER VIA OXIDATIVE HYPOTHESIS

A Thesis

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By

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بسم الله الرحمن الرحيم

الله لا اله إلا هو الحي القيوم لا تأخذه سنه ولا نوم له ما في السموات وما في الأرض من ذا الذي يشفع عنده إلا بأذنه يعلم ما بين أيديهم وما خلفهم ولا يحيطون بشي من علمه إلا بما شاء وسع كرسيه السموات والأرض ولا يؤده حفظهما وهو العلي العظيم ۞

صدق الله العظيم

سورة البقرة الآية 255

الخلاصة

تم في هذه الدراسة تحديد الظروف المثلى لقياس فعالية إنزيم السايتيدين دي أمينيز في أمصال دم أشخاص أصحاء ظاهريا (27 ذكر، 25 أنثى) بطريقة طيفية. ثم قيست فعالية إنزيم السايتيدين دي أمينيز في أمصال دم مرضى مصابين بالمرضِ اللمفاوي الهودجكني (13 ذكر، 8 إناث)، الورم اللمفاوي اللاهودجكني (13 ذكر، 10 إناث)، سرطان الثدي (38أنثى) ، ابيضاض الدم اللمفاوي الحاد (5 ذكور، 5 إناث)، سرطان المثانةِ (8 ذكور)، ابيضاض الدم النقوي الحاد (4 إناث)، سرطان المشيمةِ (3 إناث)، سرطان المبيضِ (3 إناث)، سرطان القولونِ (3 ذكور)، سرطان الكبدِ (ذكرين), والداخلين إلى وحدة الأورام في مستشفىَ مرجان التعليمي في مدينةِ الحلة للمدة من تشرين الأول2001 إلى كانون أول2002،لتحديد دور هذا الإنزيمِ بصفته مؤشرا كيميائيا حياتيا لمرض السّرطانِ، والتحقّق فيما إذا كان يعمل إنزيما مضادا للأكسدة.

تم كذلك قياس تركيز بعض المتغيّرات التي تعمل مضادات للأكسدة مثل فيتامين ج والكلوتاثايون والألبومين والعناصر النزرة مثل الخارصين و السلينيوم و النحاس والحديد ،لقياس الإجهاد ألتا كسدي المرافق للسّرطانِ.

وَجدتْ فعالية إنزيم السايتيدين دي أمينيز بأنها تزداد معنويا لدى المرضىِ المصابين بالورم اللمفاوي اللاهودجكني وسرطانات الثدي و المشيمة ، و الكبدِ ،عند مُقَارَنتها بالأشخاص الأصحاء. كما ازدادت فعالية إنزيم السايتيدين دي أمينيز بين الذّكور المصابين بابيضاض الدم اللمفاوي الحادِ والإناث المصابات بابيضاض الدم النقوي الحاد والإناث المصابات بسرطانِ المبيضِ.بينما وجد بان فعالية إنزيم السايتيدين دي أمينيز تقل لدى المرضىِ المصابين بالمرضِ اللمفاوي الهودجكني،والذكور المصابون بسرطانِ المثانةِ، الإناث المصابات بابيضاض الدم اللمفاوي الحاد ، والذكور المصابين بسرطانِ القولونِ.

مع وجود بِضْعَة إستثناءاتِ ، وَجد أنْ تركيز كل من فيتامين ج و الكلوتاثايون وبروتين الدم الكلي والألبومين و نسبة الألبومين /الكلبيولينات والخارصين والسلينيوم تقل في المرضىِ عند مقَارنتها بتلك النتائج المستحصلة من الأشخاص الأصحاء.بينما وجد أن تركيز الكلبيولينات والنحاسِ يزداد مقَارنة بالأشخاص الأصحاء. وتفاوت تركيز الحديد بين الزّيادةِ والتّناقصِ عند المرضى المصابين بالأنواعِ المختلفةِ من السّرطانِ.قد يعزى النقصان في تركيز مضادات الأكسدة إلى اشتراك هذه المواد في تفاعلات للوقاية منِ الإجهاد ألتا كسدي.

ربما يسبب ا لإجهاد ألتا كسدي المرافق للسّرطانِ إطلاق إنزيم السايتيدين دي أمينيز من الخلايـاِ المختلفةِ التي يكون فيها غشاء البلازمـاِ متضررا بفعل فصائل الأوكسجينِ الّفعالةِ وفصائل النتروجينِ الّفعالةِ المسببة للإجهاد ألتا كسدي ,وتلك الخلايـاِ التي تعرّضت إِلى مستوياتِ عاليةِ من الإجهاد ألتاكسدي قد تظهر مستويات عالية من فعالية إنزيم السايتيدين دي أمينيز.

من الممكن أن تعد فعالية إنزيم السايتيدين دي أمينيز مؤشرا كيميائيا حياتيا للدلالة على وجود الورم اللمفاوي اللاهودجكني وسرطانات الثدي و المشيمة، و الكبدِ.وقد تعد مؤشرا كيميائيا حياتيا للتمييز بين الورم اللمفاوي الهودجكني والورم اللمفاوي اللاهودجكني.

علاقة جديدة بين فعالية إنزيم السايتيدين دي أمينيز ومرض السرطان عبر فرضية الأكسدة

رسالة مقدَّمة إلى

مجلس كليةِ العلوم – جامعة بابل

وهي جزء من متطلبات نيل درجة الماجستير في

علومِ الكيمياء الحياتيةِ

مِن قِبل

عبدالسميع حسن حمود الطائي

بكالوريوس علوم كيمياء

جامعة البصرة 1991م

تشرين الأول 2003م

CERTIFICATION

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To:

The memory of my father,

My mother,

My wife, and

My lovely sons:

Yaser,

Osama, and

Hareth

Abdulsamie

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ABSTRACT

The optimum conditions of cytidine deaminase (CDA) activity measurement, in sera of healthy controls (27 males, 25 females) have been done. Then, CDA activity in sera of patients with Hodgkin’s disease (HD) (13 males, 8 females), non-Hodgkin’s lymphoma (NHL) (13 males, 10 females), breast cancer (38 females), acute lymphoblastic leukemia (ALL) (5 males, 5 females), bladder cancer (8 males), acute myeloid leukemia (AML) (4 females), placenta cancer (3 females), ovary cancer (3 females), colon cancer (3males), liver cancer (2 males) admitted to Oncology Unit in Merjian Teaching Hospital in Hilla city between October 2001 and December 2002 were measured, using spectrophotometric method to evaluate the role of this enzyme as a biochemical marker of cancer and to investigate whether it acts or not as an antioxidant enzyme.

The concentration of antioxidant variables such as vitamin C, glutathione, albumin, and trace elements zinc, selenium, copper, and iron were also assayed to measure the oxidative stress associated with cancer.

Compared with healthy controls, CDA activities were found to significantly increase in patients with NHL, breast, placenta, and liver cancers. CDA activities were increased in males with ALL, females with AML and females with ovary cancer, whereas, CDA activities were found to decrease in patients with HD, males with bladder cancer, females with ALL, and males with colon cancer.

With few exceptions, vitamin C, glutathione, total serum protein, albumin, albumin /globulin ratio zinc, and selenium concentrations were found to reduce when compared with those of healthy controls, whereas globulins and copper concentrations were found to increase, when compared with healthy controls. Iron concentrations were fluctuated between increase and decrease in patients with different types of cancer. The depletion in antioxidant concentrations may be due to their protective role against oxidative stress.

The oxidative stress associated with cancer may be due to CDA release from different cells in which plasma membrane is damaged by reactive oxygen species (ROS) and /or reactive nitrogen species (RNS). The cells exposed to high levels of oxidative stress may reveal high CDA activities.

CDA activities could be considered biochemical markers for NHL, breast, placenta, and liver cancers. They may also be considered biochemical markers for the differentiation between HD and NHL.

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LIST OF ABBREVIATIONS

AA	Ascorbic Acid
AAS	Atomic Absorption Spectrophotometer
ACF	APOBEC-1 Complementation Factor
ACP	Acid Phosphatase
A/G ratio	Albumin / Globulins Ratio
AIDS	Acquired Immune Deficiency Syndrome
Ala	Alanine
ALL	Acute Lymphoblastic Leukemia
ALP	Alkaline Phosphatase
AMI	Acute Myocardial Infarction
AML	Acute Myeloid Leukemia
AP	Angina Pectoris
apoB	Apolipoprotein B
APOBEC-1	ApoB mRNA Editing Catalytic Subunit 1
APOBEC-2	ApoB mRNA Editing Catalytic Subunit 2
ARA-C	Cytosine Arabinoside
Asn	Asparagine
ASP	APOBEC-1 Stimulating Protein
A. thaliana	*Arabidopsis thaliana*
5-AZA C	5-Azacytidine
Bis	Methylene Bis Acrylamide
B. subtilis	*Bacillus subtilis*
CDA	Cytidine Deaminase
CDAR	Cytidine Deaminase Active on RNA
CPK	Creatin Phosphokinase
Cys	Cysteine
2,6-DCIP	2,6-Dichlorophenolindophenol
DHA	Dehydroascorbic Acid
DNPH	2,4-Dinitrophenyl Hydrazine
DTNB	5,5`-Dithio Bis (2-Nitrobenzoic Acid)
EDTA. $2H_2O$	Ethylenediaminetetracetate Acid Dihydrate
ESR	Electron Spin Resonance
E. coli	*Escherichia coli*
5-FU	5-Fluorouracil
GC-MS	Gas Chromatography-Mass Spectroscopy
Glu	Glutamic Acid
GOT	Glutamic Oxaloacitic Transaminase
GPT	Glutamic Pyrovic Transaminase
GSH	Glutathione
GSSG	Oxidized Glutathione
α -HBDH	α -Hydroxy Butyrate Dehydrogenase

HD	Hodgkin’s Disease
HDL	High Density Lipoprotein
H. influenzae	*Haemophilus influenzae*
His	Histidine
HIV	Human Immune Deficiency virus
HPLC	High Performance Liquid Chromatography
IDL	Intermediate Density Lipoprotein
kDa	Kilo Dalton
LDL	Low Density Lipoprotein
LDH	Lactate Dehydrogenase
m-HPO_3	Meta-Phosphoric Acid
m-RNA	Messenger Ribonucleic Acid
NHL	Non-Hodgkin’s Lymphoma
NAA	Neutron Activation Analysis
8-OHG	8-Hydroxyguanine
8-OHdG	8-Hydroxydeoxyguanine
PAGE	Polyacrylamide Gel Electrophoresis
PMNC	Polymorphonuclear Cells
RA	Rheumatoid Arthritis
R_f	Retention Factor
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
R_t	Retention Time
SDS	Sodium Dodecyl Sulfate
SF	Synovial Fluids
SLE	Systemic Lupus Erythematosus
SOD	Superoxide Dismutase
TCA	Trichloroacetic Acid
TEMED	N,N,N,N`-Tetramethylethylene Diamine
THU	Tetrahydrouridine
Tris	Tris (Hydroxymethylene) Aminomethane
VLDL	Very Low Density Lipoprotein

CHAPTER ONE

1. INTRODUCTION

1.1 General Introduction

Without enzymes, our life would not be possible. [1] Life requires the action of several enzymes to provide the building blocks to make new cells and to furnish energy in order to live and to maintain health. [2]Enzymes are proteins that enhance the rate of a wide variety of biochemical reactions that occur in the biological systems and they are considered to be biological catalysts [2-4].

As with inorganic catalysts, enzymes accelerate that rate of chemical reaction without altering the equilibrium, [4,5] and have extraordinary catalytic power, which is far greater than that of synthetic catalysts. They have a high degree of specificity for their substrates, and acts in dilute aqueous solutions under mild condition of temperature and pH. [6-9] Enzymes catalyze reaction by stabilizing transition state, [9] therefore, they may become temporarily bound to their substrate during intermediate stages of the reaction, but it will be regenerated in the end to its original form .[8]

Nearly all known enzymes are proteins. However, it is now recognized that proteins do not have a monopoly in catalyzing reaction in living system. Certain catalytically active ribonucleic acid (RNA) molecules, called ribozymes, may catalyze the chemical modification of RNA in the absence of protein. [3,9]

Some enzymes require only polypeptides for activity, whereas, other enzymes, require for activity an additional chemical component called a cofactor. The cofactor may be either inorganic, which is called an activator, or it may be a complex organic molecule, called a coenzyme. A complete, catalytically active enzyme together with its coenzyme or metal is called a holoenzyme, whereas the protein part of an enzyme is called the apoenzyme. [5-8]

The different forms of enzyme that catalyze the same reaction are called isoenzymes (also called isozyme), which may originate from genetic or non genetic causes, and may be differentiated from each other base on certain physical properties, such as electrophoretic mobility and solubility, or resistance to inactivation. [3,5,6,10]

In the clinical laboratory, enzymes are used either as specific reagents for the quantitative determination of different constituents in tissue sample, or as an index in the diagnosis of disease, as well as they may be use as therapeutic agents.[11,12]

Amylase was the first enzyme to be determined for diagnostic purpose in body fluids by Wohlgemuth in 1908. Determination of enzyme activity in serum began in the 1920s and 1930s with the studies by Key, King, Bodansky, and Roberts on alkaline phosphatase (ALP) in bone and liver disease. Kutsher and Wolbergs as well as Gutman and Gutman, shortly afterward, recognized the value of acid phosphatase (ACP) activity in serum to diagnose prostatic cancer. [11,13]

Enzymes exist throughout the body and their activity measurement can provide valuable diagnostic information. [14] Most of them are present in cell at much higher concentrations than in plasma. Some occur predominantly in cells of certain tissues, where they may be located in different cellular compartments such as cytosol or bond to membrane or mitochondria.[2,15] If the cell is damaged, at first cytosolic enzymes leak into the surrounding tissue fluid. When cells become more damaged or destroyed, both cytosolic and membrane-bound enzymes are released into the surrounding tissue fluid. [12,15]

Enzymes in the blood serum or plasma are either normal or abnormal constituents [12]. One enzymes is considered normal serum enzyme; pseudo cholinesterase, where it fulfil its physiological function. [12,14] Abnormal serum enzymes (non-plasma, specific enzymes) may be divided into two groups; secretory enzymes such as α-amylase, and intracellular enzymes, such as ALP, ACP, creatin phosphokinase (CPK), glutamic oxaloacetic transaminase (GOT), glutamic pyrovic transaminase (GPT), lactate dehydrogenase (LDH) and α -hydroxy butyrate dehydrogenase (α-HBDH).[12]

Small amounts of intracellular enzymes are present in the blood as a result of normal cell turnover. [14] Tissue damage (as in myocardial infarction) and infection (as in hepatitis) cause release of tissue enzymes into the blood, and the concentration in the blood will rise. [12]

However, such increase is always due to tissue damage. Other possible causes include increased cell turnover, cellular proliferation, increased enzyme synthesis (enzyme induction), obstruction to secretion, and decreased clearance from the circulation. [14,15]

Changes in plasma enzyme activity may help to detect and localize tissue cell damage or proliferation, or to monitor treatment and progress of disease ,[14,15] as well as diagnosis of particular diseases or physiologic abnormalities. [5,14,15]

Recently, several important strategies are being developed to create improved enzymes to serve specific purposes. Immunoglobulin molecules that are naturally devoid of catalytic powers may be designed as abzymes to modify bound antigens. The use of gene cloning techniques permits the introduction of specific mutations into critical regions of the polypeptide chain and influences the enzyme functioning. This is called site-directed mutagenesis. Another advancing technique is the splicing of genes from different sources to code novel hybrid enzymes. These processes have been employed to modify the specificity of enzymatic catalysis or enhance reaction rates. [3]

Several enzymes and isoenzymes of clinical interest have some value in the diagnosis and management of various malignancies and diseases, therefore, serve as tumor and biochemical markers. [5,16] Enzymes are also frequently used as therapeutic agents, such as amylase and pepsin. [12] A complete understanding of enzymes involved in the biosynthetic of molecules precursor of RNA and DNA is very important in chemotherapy of cancer. [17,18]

In addition, many enzymes appear to occupy a central role against oxidative stress, and act as antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase. [1]

1.2.1 Cytidine Deaminase

Cytidine deaminase (CDA) or cytidine aminohydrolase, (EC 3.5.4.5) [20-22] is a cytoplasmic enzyme existing in many types of cell, [23] that catalyze the hydrolytic deamination of cytidine (CR) or deoxycytidine (CdR), to uridine (UR) or deoxyuridine (UdR), respectively. [24-26]

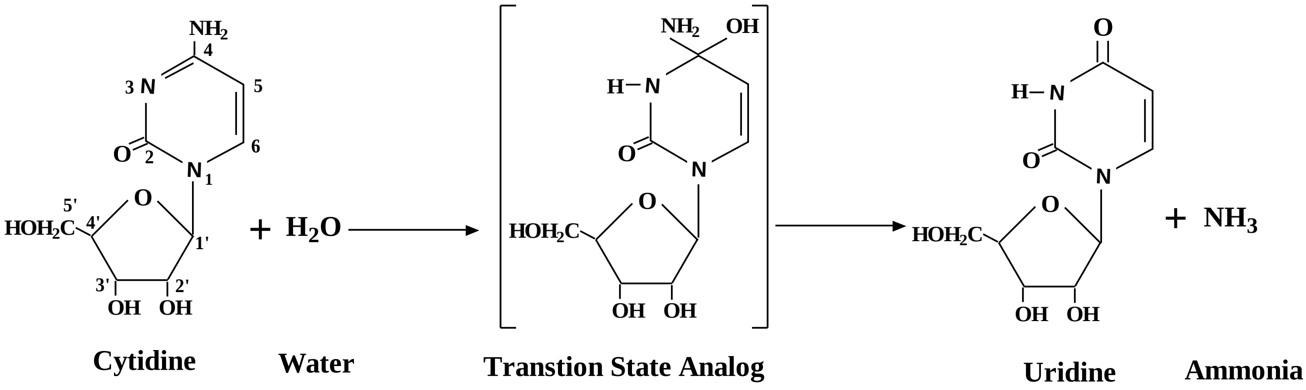


Figure 1-1 The Cytidine Deaminase Reaction (Figure taken from [41]).

The mechanism of reaction suggests that it may be considered a potential "transition state analog" [20] as shown in Figure 1-1. Other mechanistic information is consistent with the likehood that CDA may catalyze displacement of ammonia by water through an addition-elimination mechanism. [27,28]

CDA is present in most kind of cell and widely distributed among mammalian [25] and plant tissues, [29-31] as well as microorganism. [20] It was purified and studied from *Escherichia coli* (E. coli), [20-22, 32,33] baker's yeast, [34] and *Bacillus subtilis* (B. subtilis) .[35] Also, CDA was identified in fungus such as *Aspergillus niger*, [36] and in parasitic protozoa such as *Trypanosoma cruzi*, *Crithidia fasciculata*,[37] *Leishmania*, [38] *Entamoeba histolytica* [39] and *Toxoplsama gondii*. [40]

CDA was purified from human liver, [41] human placenta, [42,43] human spleen,[44] human granulocytes, [26,45] and mice spleen. [46] It has also partially purified from mouse kidney, [47,48] sheep liver, [49] and cow liver in our laboratory (unpublished data).

The molecular weight and size of CDA are variant, according to its sources, therefore, there difference are reflect different enzyme structure, while the short type of CDA work as homotetramers, the larger CDA enzymes are homodimeric, each monomer composed of two distinct core domains that have nearly the same tertiary structure. [50] Apparently, the large CDA evolved from the short CDA ancestor by sequence duplication and fusion. [30]

CDA is found to be homodimeric in several gram-negative bacteria such as E. coli and *Haemophilus influenzae* (H. influenzae), and in plant such as *Arabidopsis thaliana* (A. thaliana), as well as human APOBEC-1 (mRNA editing). On the other hand, CDA has been reported to be homotetramers in several gram-positive bacteria such as B. subtilis, and in yeast such as *Saccharomyces cerevisiae* and in human CDA.[22,30,43,50,51]

Determination of molecular weight of CDA depends upon its source and the technique used. Molecular weight of CDA from E. coli determined by gel filtration on Bio-Gel P-100 is 54 kDa.[52] Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) shows a single band at molecular weight 35 kDa, suggesting that the enzyme (which has molecular weight of 73 kDa) [20] may be comprised of two identical subunits. [20-22]

The molecular weight reported for CDA from other sources are, mouse spleen, 74 kDa,[46] yeast, 57 kDa,[53] B. subtilis, 59.2 kDa,[50] A. thaliana, 65 kDa,[30] human placenta, 48.7 kDa,[43] and human granulocytes, 51 kDa.[45]

CDA from E. coli is reasonably stable, retaining 47% of its activity after 3 months at 4°C when stored in the presence of mercaptoethanol. Freezing inactivates the enzyme, with less than 30% recovery of activity being obtained after one freeze-thaw cycle. [22]

CDA has been identified in a variety of biological sources, and plays a major role in pyrimidine nucleoside and nucleotide salvage, [54-56] as well as an important role in the transport and utilization of pyrimidine nucleoside. [32]

1.2.2 Other Types of Cytidine Deaminase

The cytidine deaminases belong to the family of multisubunit enzymes that catalyze the irreversible hydrolytic deamination of their substrates to corresponding uracil products, [57] and are involved in the maintenance of pyrimidine nucleotide pool in the cell. [58] Although the intracellular distribution of CDA and related enzymes has previously been considered to be cytosolic, it has recently been shown that human CDA is also present in the nucleus. [57]

Another type of proteins has been reported to have CDA activity, which is called apoB mRNA editing protein. [59]

RNA editing has been defined as a modification of RNA that changes its coding capacity. [60-61] RNA editing reaction changes only one or two nucleotides. [62] Apolipoprotein B (apoB) mRNA editing involves a site specific CDA reaction, which converts of codon - 2153 CAA, a glutamine codon to a UAA a translation stop codon at nucleotide 6666 of apoB mRNA. [63-68]

ApoB is an integral and non-exchangeable structural component of lipoprotein particle referred to as chylomicrons, very low density lipoprotein (VLDL) and low density lipoprotein (LDL). [69]

ApoB circulates in human plasma as two isoforms, apoB 100 and apoB 48. Unedited and edited mRNA are translated into full length apoB 100 and a truncated variant apoB 48, respectively, [65,70-72] the latter of which is associated with a reduced risk of atherogenic disease. [69,73,74] This editing event occurs in the small intestine but not in the liver [63,69] . The apoB 100 isoform is synthesized only in the liver and is used to assemble the VLDL that is necessary for the transport endogenously synthesized triglycerides and cholesterol. [61] VLDL is metabolized to intermediate density lipoprotein (IDL) and subsequently to LDL. Owing to the interaction of the carboxyl terminus of apoB 100 with LDL receptor, LDL is removed from the circulation. Such a functional interaction is medically important, as high levels of

LDL cholesterol is one of main risk factors for coronary heart disease. [61,69] Conversely, apoB 48, which lacks the carboxyl terminus of apoB 100, is generated in the small intestine and is necessary for the synthesis and secretion of chylomicrons. [61,75]

Under physiological conditions, apoB RNA editing is a nuclear event [63,76,77] and is a posttranscriptional process occurring primarily at the time of splicing or at a time after splicing but before nuclear export. [63,66,77]

The posttranscriptional deamination editing of apoB mRNA is catalyzed by APOBEC-1 (apoB mRNA editing catalytic subunit 1), [66] or by enzymes known as CDARs (cytidine deaminase active on RNA). [74]

Unlike the CDA, APOBEC-1 cannot edit mRNA without interaction with auxiliary proteins.[74] A 64 kDa human protein, cloned and identified as either ACF (APOBEC-1 complementation factor)[78] or ASP (APOBEC-1 stimulating protein) ,[74,79] together with APOBEC-1 function as a minimal editosome in vitro, [74,80] as shown in Figure1-2.

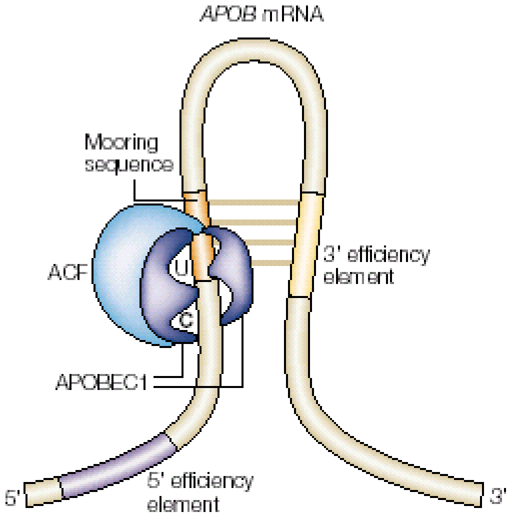


Figure 1- 2 APOBEC-1 binds to apoB mRNA in the presence of ACF (Figure taken from [61])

Several studies have suggested that apoB mRNA is edited by multiprotein holoenzyme complex called the C → U editosom. [66,81,82]

CDAs can be classified by their size and sequence as either homodimers or homotetramers. APOBEC-1 is found to be homodimer of two identical subunits [81].

In addition to its involvement in the reducing of atherogenic disease, [69,73,74] the conversion of C →U has been implicated in the disease processes because overexpression of ABOBEC-1 leads to dysplasia and hepatocellular carcinomas in the mouse and rabbit. [83,84] Furthermore C →U changed have been found in human immunodeficiency virus type 1 (HIV-1) transcripts. [85,86]

Recently, it is reported the cloning of APOBEC-2 (apoB mRNA editing catalytic subunit 2), is another member of the subfamily.[87] Human and mouse APOBEC-2 both contain 224 amino acid residues and are expressed exclusively in the heart and skeletal muscle.[61,87] APOBEC-2 does not display detectable apoB mRNA editing activity, but like other editing enzymes of the CDA super family, it has low, but definite, intrinsic CDA activity. [61,87]

1.2.3 Active Site of CDA and Catalytic Process

CDA family members are dimeric or tetrameric, with a unique and different fold. Both types are use similar mechanisms, involving the activation of the attacking water by catalytic zinc. [62] CDA from E. coli is a dimer of identical subunits, each containing a single zinc atom. [51,88,89] CDA from B. subtilis and human are a tetramer of identical subunits, and are found to contain a single atom of zinc per enzyme subunit [43,51]. A region of amino acid sequence homology, containing residues that are involved in zinc coordination in the enzyme from E. coli, strongly suggests that in the enzyme from B. subtilis, zinc is coordinated by the thiolate side chains of three cysteine residues (Cys-53, Cys-86, and Cys-89) .[90] This pattern of zinc coordination appears to be novel for a hydrolytic enzyme, and might be expected to reduce the reactivity of the active site substantially compared with that of the enzyme from E. coli (His-102, Cys-129, and Cys-132) [51].

The coordination of the active site metal is significantly different between CDA and other hydrolytic enzymes, such as carbonic anhydrase and adenosine deaminase (in which zinc atom is directly coordinated by three histidine residues), as the zinc atom in CDA from E. coli is bound in a nearly perfect tetrahedral arrangement of four ligands (two cysteine residues, one histidine residue, and a water molecule or hydroxyl group from the bound substrate analog). Neither of the cysteine residue in CDA has a functional or structural homolog in the other hydrolytic enzyme, [91] (Figure 1- 3).

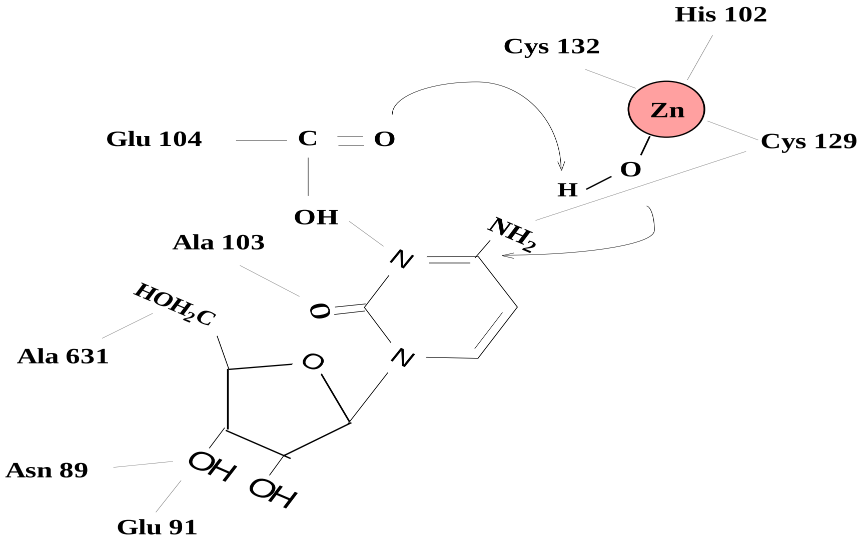


Figure 1-3 Schematic of E. coli CDA active site [51,62,91]

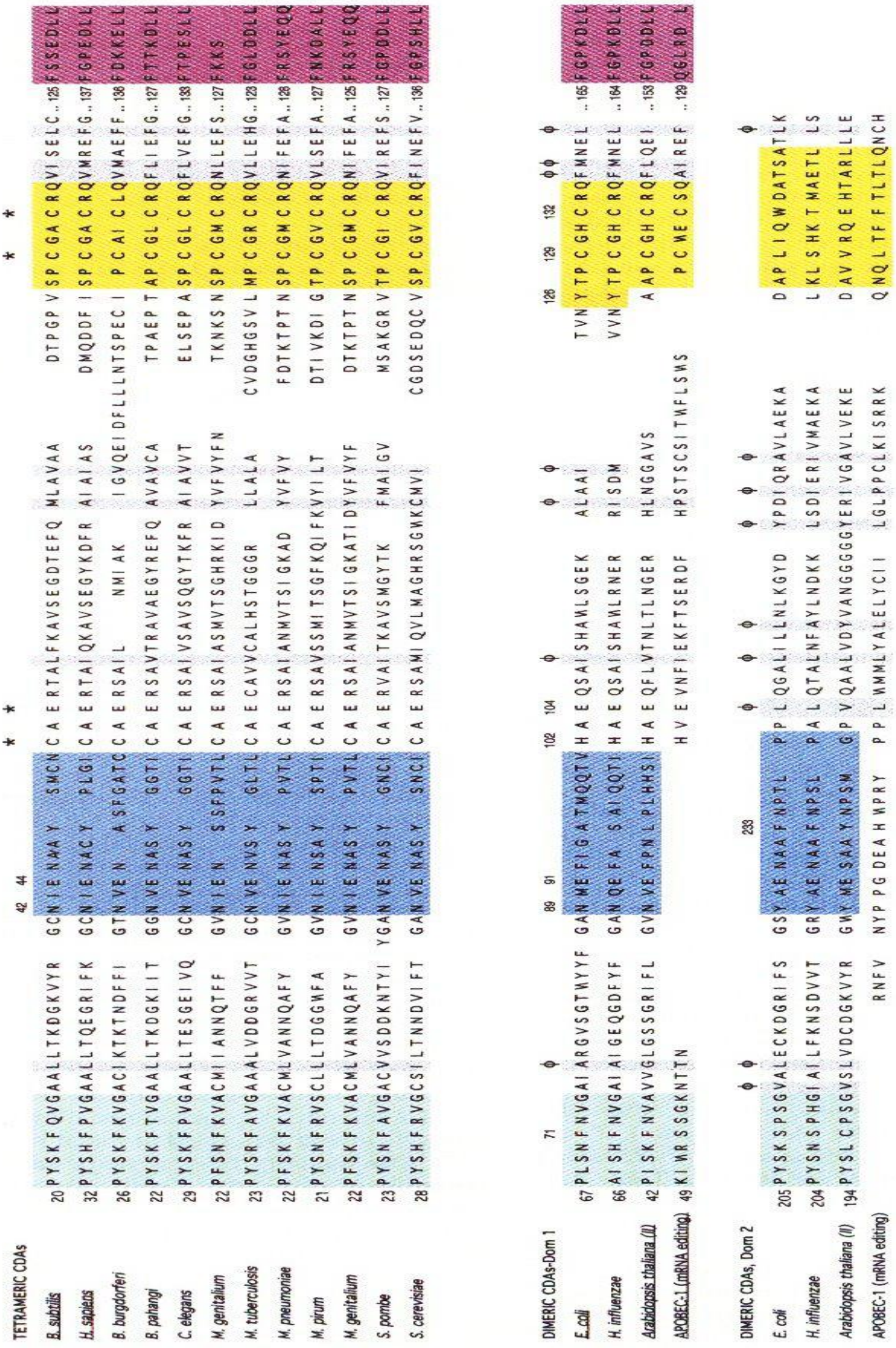


Figure1-4 Comparative alignment of amino acid sequences for tetrameric and dimeric CDA. Consensus motifs are in boldface. Asterisks indicate probable zinc ligands. Enzymes proven to contain zinc are underlined. (Figure taken from [51])

In this respect, the coordination motif of E. coli CDA appears to be unusual among hydrolytic enzymes, although it is also found in some other deaminases that act on cytosine derivatives, in deoxycytidylate deaminase and in recently discovered mRNA-editing enzyme from mammals, APOBEC-1. [92] In the small, tetrameric CDA of B. subtilis and human, [43] a cysteine residue is found at the position corresponding to the histidine residue in the first part of the zinc-binding motif of E. coli CDA [51] (Figure 1- 4).

The apparent involvement of three cysteine residues in zinc coordination, although common for "structural" zinc atom in proteins, appears to be unprecedented at the active site of a hydrolytic enzyme. Moreover coordinating zinc by three cysteine residues appears to be strictly correlated with a tetrameric quaternary structure of CDA, [51] (Figure1-5).

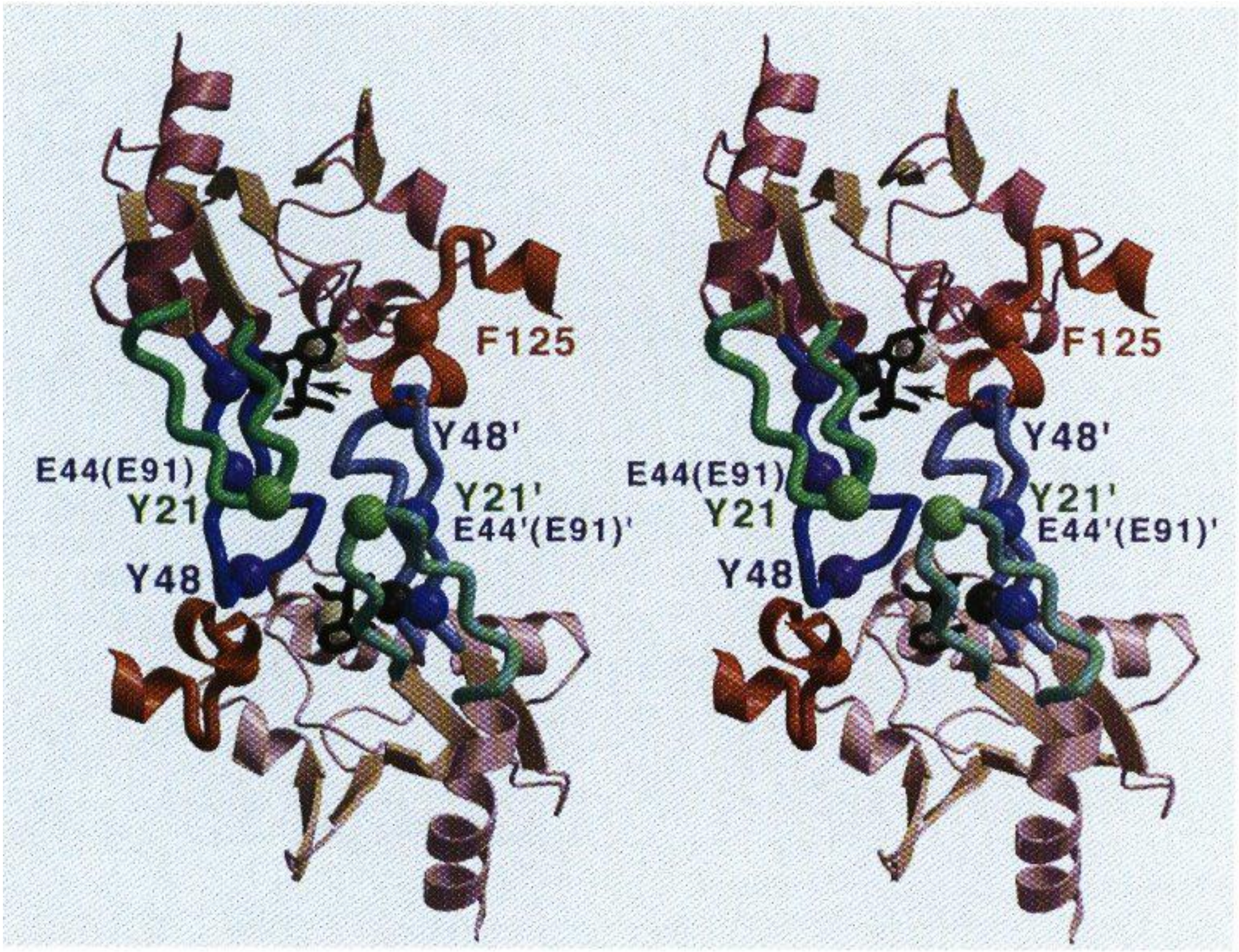


Figure1-5 Schematic drawing in stero of a novel interface in tetrameric CDA (Figure taken from [51])

Glutamic acid (Glu-104) in the active site of CDA can provide all of the necessary proton transfer functions involved in generating the zinc hydroxide nucleophile, and protonating the pyrimidine ring nitrogen atom and leaving amino group. [50] The hydrogen bond between the hydrogen group and Glu -104 carboxylate shortens in the transition-state, and may become a "low-barrier" hydrogen bond, since at the same time the bond between zinc and the Cys-132 thiolate ligand lengthens. [93] When Glu-104 is replaced by alanine, the affinities of mutant enzyme for substrate cytidine and product uridine are found to have increased, whereas free energy in kcat for deamination of cytidine has been reduced by 8 orders of magnitude. The carboxylmethyl group of Glu -104 appears to minimize the activation barrier for deamination, not only by stabilizing the altered substrate in the transition state, but also by destabilizing the enzyme-substrate and enzyme-product complexes.[94,95]

Several studies have been done to determine the role of amino acids in the active site of human CDA, and to show the role of cysteine residues in the active side, [96] and the role of glutamate-67 in the catalytic mechanism, [97] as well as the role of phenylalanine residues in a stabilizing interaction between ligand and enzyme. [98-99]

Crystal structures of the cytidine deaminase-uridine product complex prepared either by cocrystallizing enzyme with uridine or by diffusing cytidine into ligand-free crystal show that the product binds as a 4-ketopyrimidine (Figure1-6).

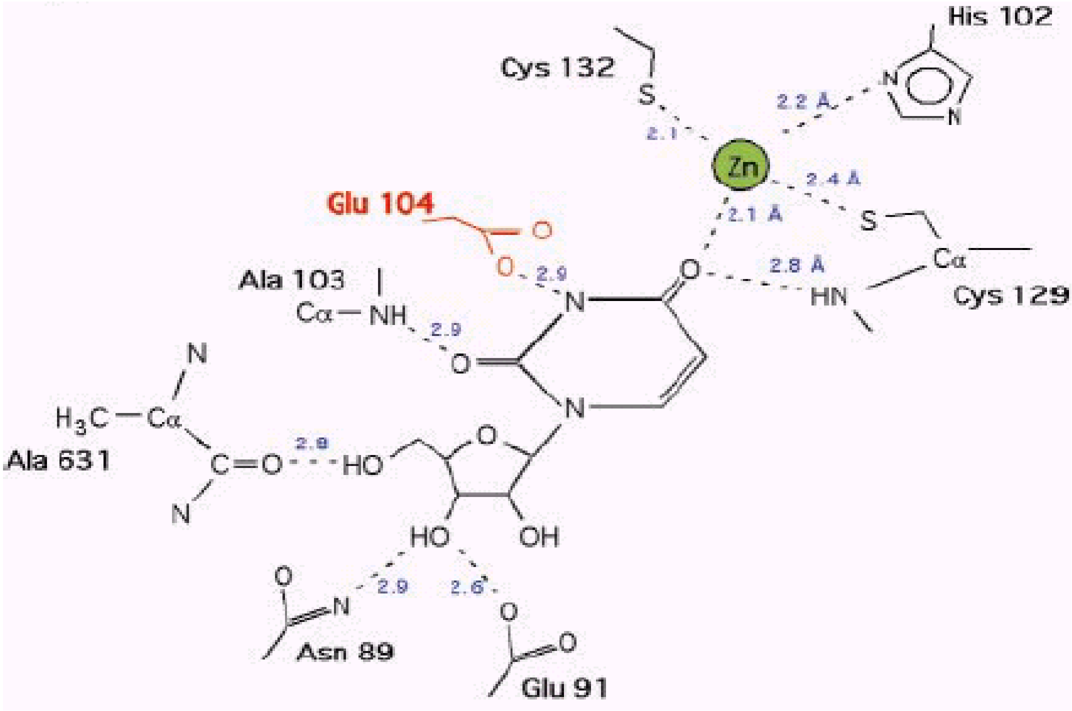


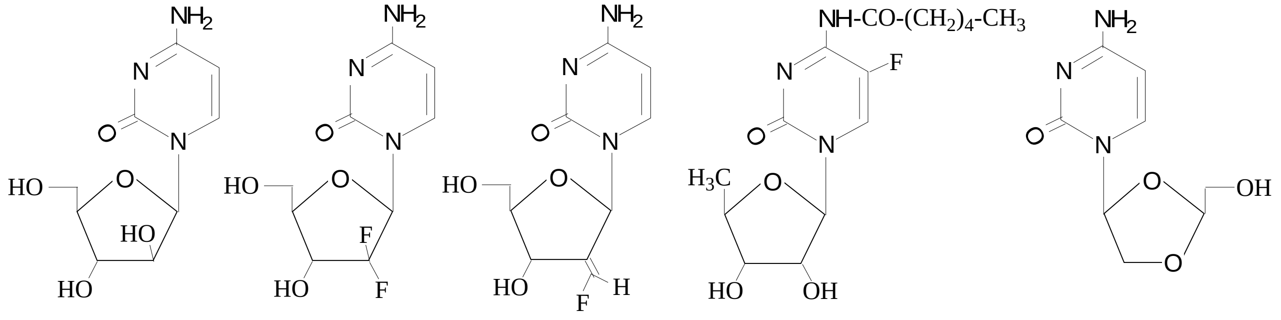
Figure 1-6 CDA-uridine complex, (Figure taken from [91])

They reveal four features of the catalytic process: (1) A water molecule bound to a site previously observed to bind the incoming 4-NH_2 group represents the site for the leaving ammonia molecule, (2) The Glu-104 carboxylate group rotates from its hydrogen bond to the oxygen atom of hydroxyl group in transition-state analog complexes, then Glu-104 transfers a proton from that group to forming a new hydrogen bond with the leaving group moiety. Thus, after stabilizing the hydroxyl group, amino group leaving, promoting enol to keto isomerization of the product. (3) Difference Fourier comparisons with transition-state complexes indicate that the pyrimidine rotates toward the zinc by approximately 10 degrees. The active site thus “pulls” the ring and 4-NH_2 group in opposite direction during catalysis. To preserve ring coplanarity of the 4-keto group with the pyrimidine ring, the N-C glycosidic bond (at position no.1) bends by approximately 19 degree out of the ring plane. This distortion may “spring load” the product complex and promote dissociation. (4) The Zn-S gamma Cys-132 bond, which lengthens in transition-state complexes, shortens as the oxygen atom (no.4) returns to state of negative charge in the planar product.[100]

CDA can also catalyze the deamination of cytosine nucleoside analogues used as anticancer drug. The deaminations of these analogues result in a loss of antineoplastic activity.[43] Cytidine analogues can be metabolized along two enzymatic pathways, by phosphorylation to mono, di and triphosphate derivatives of this analogues, a potent inhibitor of DNA synthesis, or through deamination to the inert metabolite uracil derivatives.[25,101] For this reason, it has been focusing on the synthesis of substituted cytidine analogues in either carbon atom (no.5) of pyrimidine ring or carbon atom (no.2) of ribose moiety, which are don’t involved in the active site of CDA as shown in Figure1-5, Figure1-6.

1.2.4 Role of CDA in Chemotherapy

CDA catalyses the hydrolytic deamination of various cytosine nucleoside into the corresponding uracil nucleosides.[24-26,30] This enzyme can also catalyze the deamination of cytosine nucleoside analogues used as anticancer drugs to the corresponding uracil nucleosides analogues, such as, cytosine arabinoside (ARA-C) or (cytosar), [24,25] 5-azacytidine (5-AZA C) ,[102,103] 5-aza-2`-deoxycytidine,[103-104] 5,6-dihydro-5-aza cytidine,[105-106] 2`-2`-difluorodeoxycytidine or (gemcitabine) ,[104] 5-fluorodeoxycytidine, [107] 2-deoxy-2`-fluoromethylendeoxy cytidine and 2`-flouro-2-deoxy-5-iodocytosine arabinoside [109] (Figure 1- 7).

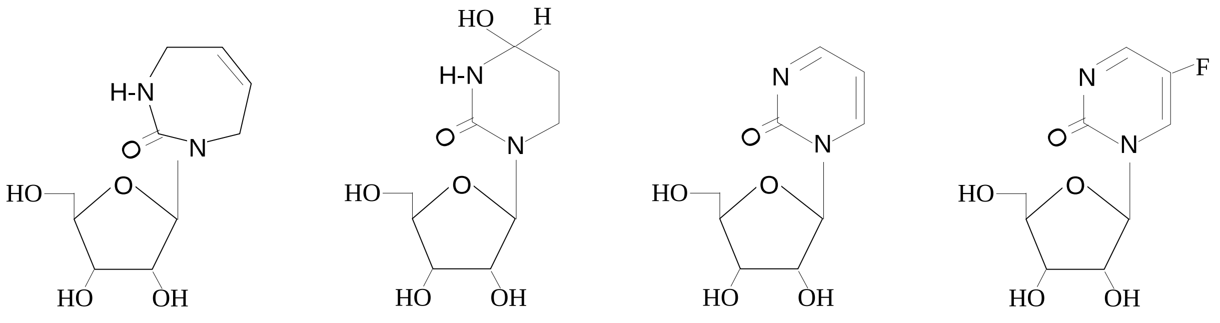


Cytarabine (ARA-C) **Gemcitabine** **FMdC** **Capecitabine (Xeloda)** **Troxacitabine**

Figure 1-7 Structures of cytidine and deoxycytidine analogues, (Figure taken from [118]) .

CDA can deaminate and thus inactivate these nucleoside analogues ,[104,110] and is also responsible for degradation of 2`, 3`dideoxy-cytidine, a selective inhibitor of human immunodeficiency virus (HIV) reverse transcriptase. [30]

To prevent the inactivation of these analogues, CDA inhibitors such as tetrahydrouridine (THU),[104,111-113] Zebularin, 5-flourozebularine, diazepinone riboside,[106-114-116] that could be an excellent candidate in combination chemotherapy with either ARA-C or AZA-CdR in patient with leukemia [106]or with other nucleoside analogues in different types of cancer chemotherapy, must be used. (Figure1-8).



Diazepinone Riboside **Tatrahydrouridine (THU)** **Zebularine** **5 -F-Zebularine**

Figure1-8 Structures of CDA inhibitors, (Figure taken from [197]).

Recently, many types of β-L-enatiomers of cytidine derivatives have been synthesized to overcome the inactivation of these analogues, such as β-L-enatiomer of 2`, 2`-dideoxycytidine or troxacitabine.[117] The chiral specificity of CDA does not allow L-enatiomer to be substrates and prevents the deamination of this analogue.[118]

Other types of drugs have been developed such as 1- (2-deoxy-2-methylene- β-D-erythro-pentofuranosyl) cytidine, and are considered to be CDA-resistant analogues.[119]

Three mechanisms have been suggested to account for the inhibition of nuclear DNA synthesis by cytosine nucleoside analogues in cancer chemotherapy. The relative importance of each mechanism may depend on the intracellular concentration of cytosine nucleoside analogue triphosphate (CNATP) derivative. The first mechanism is the inhibition of the initiation of new replication units in chromosome consequent to the incorporation of cytosine nucleoside analogues into the replicon-initiation primer.[120] The second mechanism is the retardation of DNA-chain elongation because of the incorporation of cytosine nucleoside analogues into DNA.[121,122] The third mechanism is that cytosine nucleoside analogues can inhibit the formation of the RNA oligomer required for the initiation of DNA synthesis.[123] (Figure1-9).

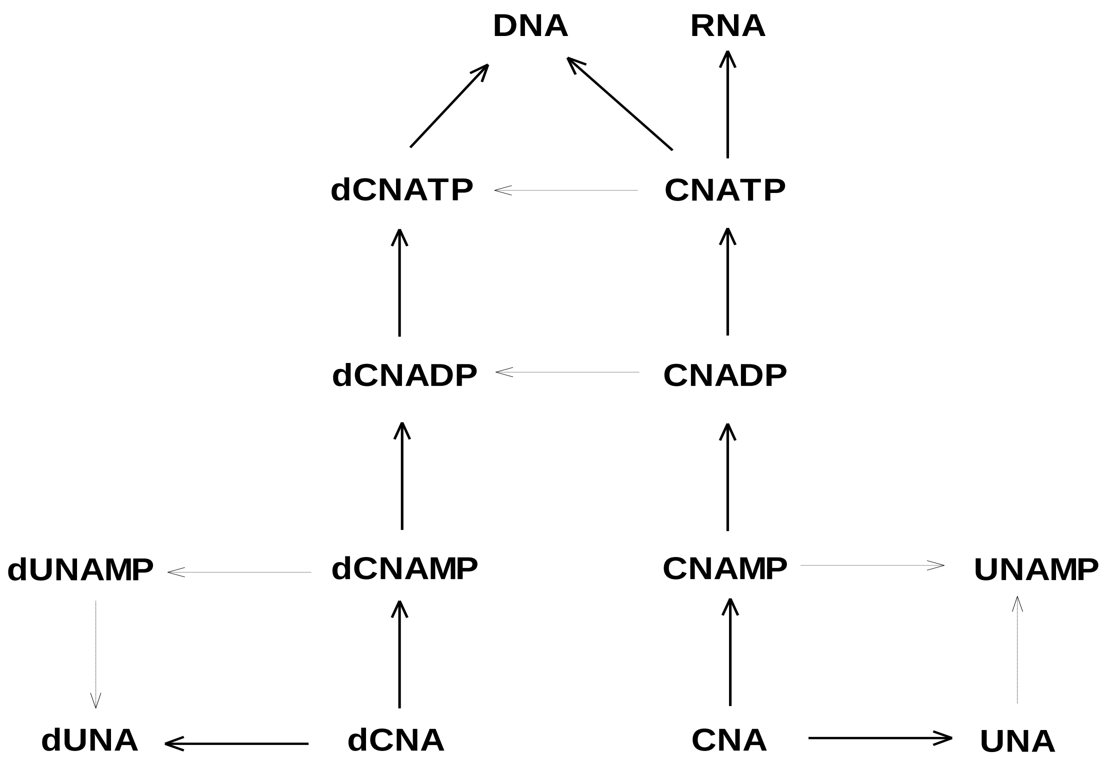


Figure 1-9 Metabolic Pathways of cytosine nucleoside analogues. C = cytosine, N = nucleoside, d= deoxy, A = analogues, U = uracil, P = phosphate, M= mono, D = di, T = tri, solid line indicate predominant pathway, Dashed lines indicate specific analogue pathway.

On the other hand, it is believed that the nucleoside analogue diphosphate of many drugs suppresses ribonucleotide reductase, the key enzyme in the biosynthesis of deoxyribonucleotides, whereas their nucleoside triphosphate suppresses DNA polymerase. [119]

Among several types of cytosine nucleoside analogue drugs, capecitabine or Xeloda® (Figure1-7) appears to be activated by the action of CDA in a mechanism of drug action far from that of other types of analogue. A liver carboxyesterase hydrolyses the N-pentyl carbomate chain to form 5`-deoxy-5-fluorocytidine, which is deaminated to 5`-deoxy-5-fluorouridine by CDA and finally thymidine phosphorylase hydrolyses 5`-deoxy-5-fluorouridine to produce 5-fluorouracil (5-FU), [124] a potent inhibitor of thymidylate synthase.[125]

1.2.5 CDA as a Biochemical Marker in Diseases

CDA has been identified in the human liver, [41] kidney, brain [126] placenta, [42,43] spleen [44] and granulocytes [26,45] and in a variety of biological sources. [126] The very high levels of CDA present in the liver and spleen are responsible for the short plasma half-life (15-20 minutes) of cytosine nucleoside analogues in man. In addition to the important role of this enzyme in the pharmacokinetics of nucleoside analogues, it has been reported that the leukemic cells from some patients treated with ARA-C or 5-AZA-CdR showed signs of drug resistance at relapse as indicated by increased levels of CDA.[110] CDA is present predominantly in polymorphonuclear cells (PMNC) in inflamed joints. Since CDA is probably released from PMNC by local cell death, CDA activity has been claimed to reflect local PMNC turnover in the inflamed joint,[127] and is thought to be released from granulocytes during phagocytosis and cell lysis, or both and its release might thus reflect granulocyte turnover or activity, rather than just their number.[123] CDA activity has been found to be very high in synovial fluids (SF) from rheumatoid arthritis (RA) and, crystal pyrophosphate disease (CPPD) followed by psoriatic arthropathy, reactive arthritis, spondyl arthropathy,[127] and in active systemic lupus erythematosus (SLE) rather than inactive SLE.[129] However, increase serum CDA activity in patients with gout and articular chondrocalcinosis has been reported, as well. [130] The concentration of CDA in joint fluid is 20 times more than in serum of the rheumatoid patients. [129]

Normally, CDA is present in serum, although at relatively low activity, [118] but an excessively elevated in serum CDA activity in abnormal pregnancies due to a damaged placenta under progressive deterioration, [126] therefore, it is reported to be a sensitive indicator of abnormal pregnancy. [126,131]

Also, massively high levels of CDA were observed in patients with fulminant meningococcal sepsis,[132] vasculitis of the small and mediumsized vessels, [133] gastric tissues from patients with gastric cancer [134] and various types of solid tumor cell line.[135,136]

1.2.6 The Methods of CDA Activity Determination

Several methods have been developed to determine the CDA activity, using different techniques. The ultraviolet (UV) spectorophotometric method is based on the loss of absorbance when cytidine is converted to uridine, at 282 nm, where $\Delta\varepsilon = -3600$.[20,33]

Paper chromatography can be used to determine CDA activity, using one-dimensional ascending chromatography on paper. This method based on the difference in retention factor (R_f) values in the n-Butanol-Water-Ammonia system, where R_f values of cytidine = 0.26 and R_f value of uridine = 0.34.[137]

A radio isotopic technique has been employed to determine the enzyme activity. The activity of CDA is assayed by measuring the amount of labeled uridine formed by the deamination of 2-C^{14} labeled cytidine.[138]

Also, high performance liquid chromatography (HPLC) techniques have been applied to the separation of a number of nucleosides. These techniques can be used to determine the CDA activity, based on the difference in the retention time (R_t) of cytidine and uridine in different volatile buffers. [139]

In addition, CDA activity can be estimated by a colorimetric procedure that determines the amount of ammonia liberated during the conversion of cytidine into uridine. [126,131]

1.3.1Cancer

Tumors are swellings or masses [55] and they are generally clonal, i.e. they develop from a single cell. This cell acquires a series of mutations or epigenetic changes that influence certain cellular pathways and processes. [140]

Tumors are divided into two types based upon the potential to spread: benign tumors and malignant tumors or cancer.[55] Cancer cells are characterized by three properties: (1) diminished or unrestrained control of growth, (2) invasion of local tissues, and (3) spread, or metastasis, to other part of body. Cell of benign tumors also show diminished control of growth but do not invade local tissue or metastasis to other parts of the body. [1,55]

Cancer is an ancient condition and was known to the early Egyptians. [141] The term cancer is derived from Greek *karkinos*, which means crab.[55] Cancers are classified according to the tissue and cell type from which they arise. Carcinomas are cancer that arise from the epithelial tissue or the ectoderm or endoderm. Sarcomas are cancers that arise from the mesoderm. Leukemias and lymphomas, which arise from mesoderm, are usually classified separately from sarcomas because of their distinctive clinical courses. [55]

Humans of all ages develop cancer, and a wide variety of organs are affected. Cancer has been a major cause of death in most countries of the world for the past few decades, second only to heart diseases. [1,5,16,142]

There are three major approaches to the cancer problem in most countries of the world: (1) prevention by identifying and eliminating the cancer-causing factor, (2) early discovery of the disease when it is localized in primary sites and is more responsive to therapy, and (3) development of effective therapeutic method. Of course, the best and most ideal way is to prevent the occurrence of cancer. [5,16,140]

A number of methods are currently available to detect cancer in its early stages. Biophysical methods play a very important role in early detection and treatment of cancer. Clinical laboratory testing for tumor markers can also represent a significant means for early cancer detection. Tumor markers tests measure either tumor-associated antigens or other substances present in cancer patients to aid the diagnosis, staging, monitoring, evaluation of response to therapy, and detection of recurrent disease.[1,5,16]

Tumor cells can be shed in body fluids and feces. Where tumor cell is characterized by specific mutation or chromosomal alterations, sensitive and specific detection of such changes should permit early diagnosis of cancer. For example, in the case of colorectal carcinoma, tumor cells are shed into the intestinal lumen at a very early stage. Only later tumor cell gain access to lymphatic and blood vessels, [140] (Figure1- 10).

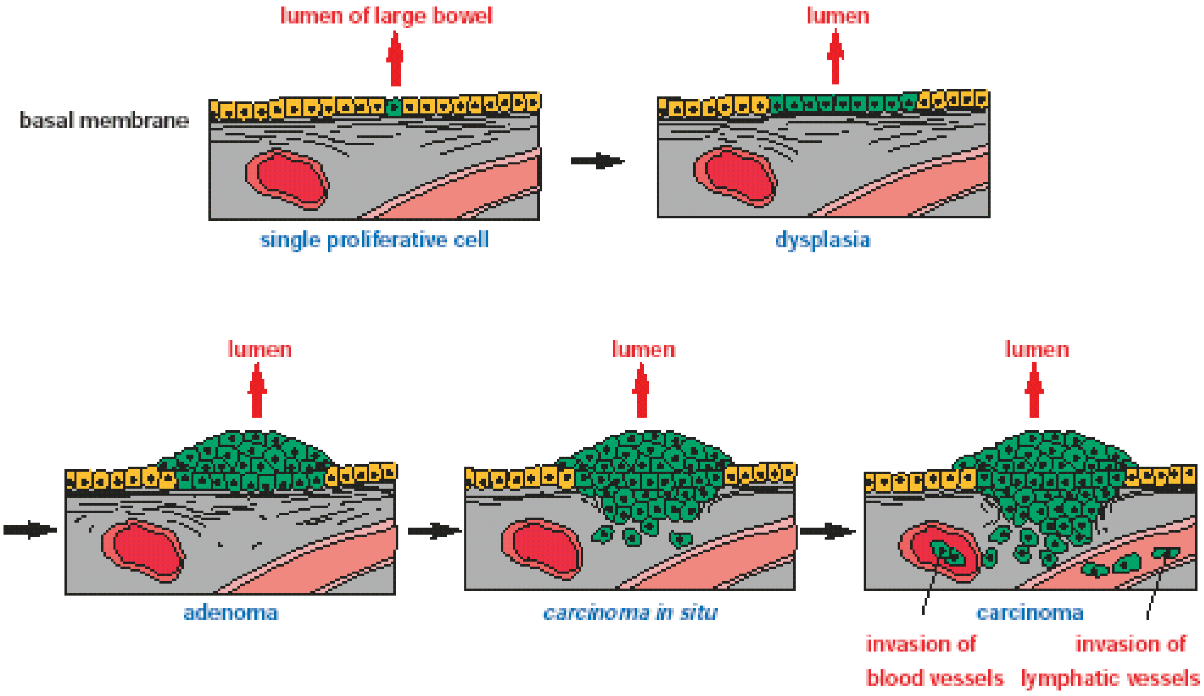


Figure1-10 Schematic representation of the development of the colorectal carcinoma (Figure taken from [140])

1.3.2 Tumor Markers

Tumor markers are substances, which in the presence of malignant tumors can be present in increased concentration in tumor tissues and / or body fluids. [140] Or it can be defined as biologic substances synthesized and released by cancer cell or substances produced by the host in response to concerous tissue [5,11]. They may be present in the circulation, in body cavity fluids, on cell membranes or in the cytoplasm. Tumor markers are also different from substances produced by normal cells in quantity and quality. [16,143]

Tumor marker may be classified into groups.[1,5,11,16,141,143]

(1) Oncofetal antigens such as carcinoembryonic antigen, alpha-fetoprotein, tissue polypeptide antigen, carbohydrate antigens, and prostate specific antigen.

(2) Placental proteins such as human chorionic gondotropin, human placental lactogen and placental alkaline phosphatase.

(3) Enzymes and isoenzymes as tumor markers, such as prostatic acid phosphatase (PACP), creatine kinase (CK)-BB isoenzyme, Alkaline phosphatase (ALP), neuron-specific enolase, lactate dehydrogenase (LD-1) isoenzyme and lysozyme.

(4) Hormones, such as eutopic hormones and ectopic hormones.

(5) Monoclonal immunoglobulins, such as IgG, A, M, D, E, and Kappa and lambda-light chains.

(6) Steroid receptors, such as estrogen and progesterone receptors.

(7) Immunophenotyping, such as lymphoid cells and myeloid cells.
(8) DNA analysis.
(9) Molecular diagnosis.

Ideally, tumor markers should provide the following potential clinical uses in cancer patient cure.[5,11,16,140]

(1) As a screening tool in the asymptomatic population.
(2) For diagnosis in patients with symptoms.
(3) As an adjunct in clinical staging of diseases.
(4) As an indicator of tumor volume.
(5) Aid for selecting appropriate therapy.
(6) For monitoring the response to therapy.
(7) As a prognostic indicator.
(8) For early detection of disease recurrence.

Unfortunately, almost all available tumor markers do not have sufficient sensitivity to be useful for screening the general population in a cost-effective manner. [16,140] Currently available tumor markers test can also not be used independently to establish a diagnosis of cancer. They may only be used as an aid and confirmatory method in diagnosis for symptomatic patients. [5,16]

1.3.3 Biochemistry of Cancer Cells

The essential changes that transform normal cells into cancer cells are due primary to somatic cell mutations. Several somatic mutations are required to produce cancer, and this concept is called the multistage theory of carcinogenesis. [55,140] These mutations involve the production of growth-promoting cellular oncogenes (cancer causing genes) and the inactivation of tumor suppressor genes (Figure 1-11).

Between two and seven somatic cell mutation are required for the initiation and progression of various malignant neoplasms. Cancer begins when a single cell begins to proliferate abnormally. This abnormal cell divides to form two abnormally proliferating cells, which in turn divide and so on. Additional somatic cell mutation in the progeny cells can produce more aggressive and rapidly proliferating cells (progression).[55,140]

It has been observed that nearly all type of cancer cell that forms a solid tumor produces more lactate than that of normal cell. Tumor cells contain a fetal form of phosphofructo kinase that is insensitive to inhibition by ATP and citrate, and this accounts for their increased production of lactate. [55]

Neoplastic cells secrete angiogenesis factors that stimulate the development of blood vessels to supply the tumor. Tumors produce metalloproteases and collagenases that facilitate the invasion of adjacent structures. The digestion of collagen and the basement membrane facilitates the invasion of lymphatic and capillaries and the metastasis of tumors to distant sites. [55]

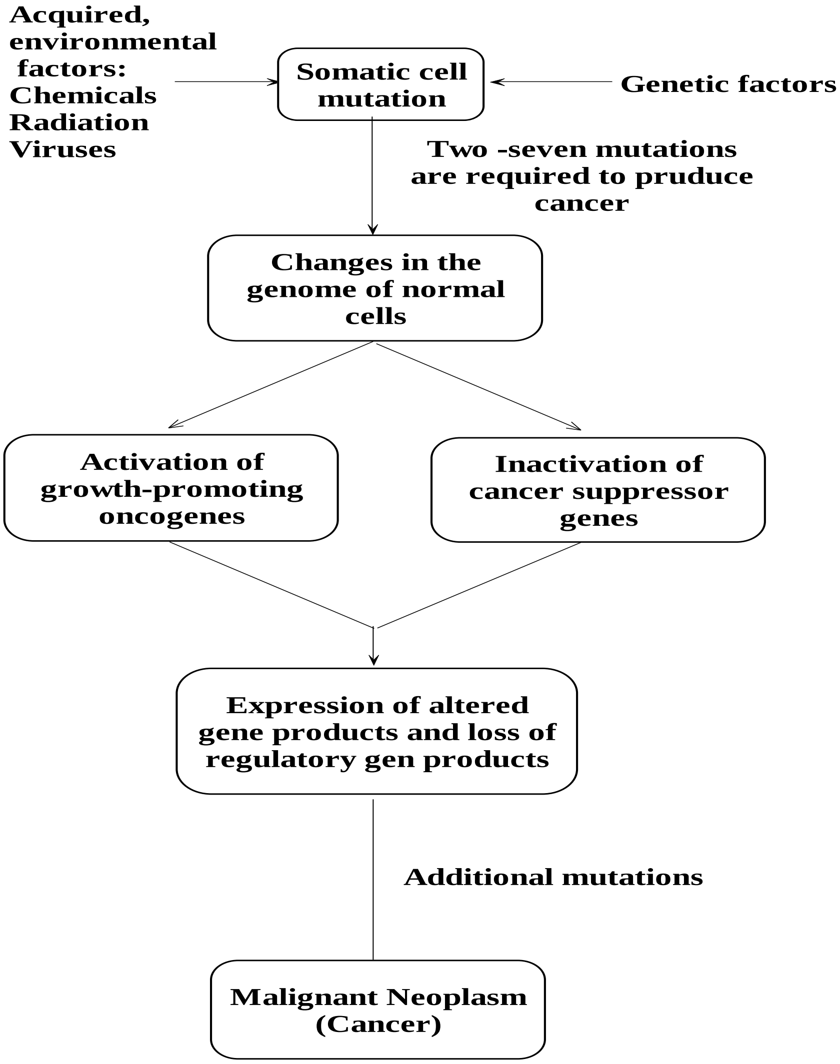


Figure 1-11 Simplified scheme of the pathogenesis of cancer (Figure taken from [55])

1.3.4 Causes of Cancer

1.3.4.1 Environmental Factors

It has been established that most human cancers are induced by environmental factors, including, chemicals, radiation, and biological agents.[144] There is great variation in the incidence of different cancers throughout the world. When people migrate to a new locale, they usually acquire the cancer incidence profile of the population at their new home within a generation. Thus, the environment can play a major role in the pathogenesis of malignant transformation.[55]

Polycyclic hydrocarbons are implicated in the causation of human lung and skin cancer. Tobacco smoke is the predominant cause of cancer mortality in many nations, and tobacco smoke contains several carcinogens, including polycyclic hydrocarbons, which form covalent adducts with bases of DNA.[1,5,141,144]

Ionizing radiation from radioactive chemicals and x-ray can produce cancer after exposure to high levels of radioactivity emanating from nuclear bombs or from major radioactive spills from nuclear power generators. High-energy x-ray and γ-ray produce point mutation in DNA nucleotide and chromosome breaks.

Ultraviolet (UV) irradiation from sunlight is the major cause of skin carcinomas and melanomas.[55] UV radiation has lower energy than ionizing radiation and can excite the pyrimidine molecules that are present in DNA to form a covalent link between adjacent pyrimidines. Thymine-thymine, cytosine-cytosine and cytosine-thymine dimers can be formed. [141]

Considerable numbers of reactive free radical or chemicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), (which will be discussed in detail later in this chapter), are generated by the action of ionizing radiation, or in the cells by normal metabolic pathways or under oxidative stress. [55,141,144] They can oxidize nucleic acid, proteins and lipids, and they have many of the characteristics of carcinogens. ROS and RNS generate structural alteration in DNA, decrease DNA repair by damaging essential protein and activate single transduction pathway. [141]

1.3.4.2 DNA and RNA Viruses

Both DNA and RNA viruses can produce tumor viruses that cause cell transformation by altering the types of protein made in the cell. Tumor viruses carry discrete genetic elements, called oncogenes that are responsible for the virus ability to transform cell. An “oncogene” (which is derived from Greek *onkos* which means “tumor”) is a gene whose product leads to neoplasia.[55,141]

Hepatitis B virus for example, which is responsible for serum hepatitis, is the most common virus linked to cancer. Hepatitis B virus, a DNA virus, is associated with liver cancer and responsible for 500,000 cancer deaths worldwide per year. [55]

Peyton Rous discovered the first tumorigenic virus early in the 20th century. Rous sarcoma virus produces sarcomas in chickens. The virus also transforms cell of other species in cell culture. This virus uses RNA as the genome, and it is a retrovirus with a DNA intermediate that integrates into the host genome.[55,141]

RNA tumor viruses are not involved in most neoplastic disorders that affect humans. Human T cell lymphotropic virus (HTCV), the only neoplastic disorder in humans that is caused by a retrovirus, is associated with a T lymphocytic neoplasia. [55]

1.3.4.3 Cellular Oncogenes

Oncogenes are genes capable of causing cancer.[1] Cellular oncogenes result from amplification, over expression, or mutation of normal cellular genes, and they are not the results of viral infection. Carcinogens or radiation can promote the mutation of protooncogenes (normal cellular genes into cellular oncogenes.[55] More than 60 oncogenes have been identified.[141]

Oncogenes are often named with a three-letter italic designtation, and the corresponding gene product or protein is designated by the same three leters in Roman type beginning with a capital letter.[55] A growth factor oncogene (*hst*) that is related to fibroblast growth factor is amplified (present in increased copy number) in some human breast carcinomas. Increased expression of this oncogene can stimulate cells that bear its receptor and contribute to malignant transformation. The genes from growth factor receptors become oncogenes when they are amplified and overexpressed or mutated so that the receptor remains active even without bound ligand.[55]

Approximately 30% of all human tumors contain mutated versions of Ras proteins. In some tumors, the incidence of *ras* (first described in a rat sarcoma) mutation is even higher; for example, 90% of pancreatic adenocarcinoma, about 50% of colon, endometrial thyroid cancer, and 30% of lung adenocarcinomas and myeloid leukemias show *ras* mutation. Indeed, mutation of the *ras* gene is the single most common abnormality of dominant oncogenes in human cancer.[145]

Changing in location of a gene in chromosomes (translocation) can alter its regulation. The basis of the translocation is that a piece of one chromosome is split off and then joined to another chromosome.[1] Human cells ordinarily have 23 pairs of chromosomes with well-defined structures, but tumor cells are usually aneuploid (i,e., they have an abnormal number of chromosomes, generally too many) and often contain translocation.[1,55]

Mutations or deletion of tumor suppressor genes can participate in neoplastic transformation. Tumor suppressor gene (a gene that inhibits tumor development) products block abnormal growth and malignant transformation. These genes are recessive, and both copies normal diploid suppressor genes must undergo mutation to allow for malignant transformation.[55]

1.4 Reactive Oxygen Species and Reactive Nitrogen Species: Overview

It is increasingly proposed that free radicals or reactive oxygen species (ROS) and reactive nitrogen species (RNS) play an important role in human cancer development, especially as evidence is growing that antioxidant may prevent or delay the onset of some types of cancer.[146] These species have been implicated in over 100 types of disease conditions, from arthritis and AIDS to cancer and diabetes mellitus.[147,148] A free radical is simply defined as any species capable of independent existence that contains one or more unpaired electrons, an unpaired electron being one that is alone in an orbital.[147] ROS is a collective term often used in the biomedical literature that includes oxygen-containing radicals such as super oxide ($O_2^{\cdot -}$), hydroxyl ($OH^{\cdot}$), peroxyl ($ROO^{\cdot}$), and alkoxyl ($RO^{\cdot}$), and non-radical species that can produce oxygen-containing radicals during their reaction; such as hypochlorous acid (HOCl), ozone (O_3), peroxynitrate ($ONOO^-$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2)[149]. RNS is a similar collective term that includes nitric oxide radical ($NO^{\cdot}$), $ONOO^-$, nitrogen dioxide radical ($NO^{\cdot}_2$), other oxides of nitrogen and products arising when $NO^{\cdot}$ react with $O_2^{\cdot -}$, $RO^{\cdot}$ and $ROO^{\cdot}$. Reactive is not always an appropriate term; H_2O_2 , $NO^{\cdot}$ and $O_2^{\cdot -}$ react quickly with very few molecules, whereas $OH^{\cdot}$ reacts quickly with almost any molecules. $ROO^{\cdot}$, $RO^{\cdot}$, HOCl, $NO_2^{\cdot}$, $ONOO^-$ and O_3 have intermediate reactivities.[146, 150]

There is increasing evidence that oxidative stress, defined as an imbalance between oxidants and antioxidant in favor of the former, or describes a condition in which cellular antioxidant defenses are insufficient to keep the levels of ROS/RNS below a toxic threshold, leads to many biochemical changes and is an important contributing factor in several human chronic disease, such as atherosclerosis and cardiovascular diseases, mutagenesis and cancer, and likely the aging process.[151, 152]

ROS and RNS have been shown to possess many characteristics of carcinogens.[153] Mutagenesis by ROS and RNS could contribute to the initiation of cancer, in addition to being important in the promoting and progression phases. For example, ROS and RNS can have the following effects:

(1) Cause structural alteration in DNA, such as base pair mutation, rearrangements, deletions, insertion, and sequence amplification. $OH^{\cdot}$ is especially damaging, but $^1O_2$, $ROO^{\cdot}$, $RO^{\cdot}$, HNO_2 , O_3 , $ONOO^-$ and the decomposition products of $ONOO^-$ are also effective.[146,149] ROS can produce gross chromosomal alterations in addition to point mutation and thus could be involved in the inactivation or loss of the second wild-type allele of a mutated proto-oncogene or tumor-suppressor gene that can occur during tumor promotion and progression, allowing expression of the mutated phenotype.[146, 149]

(2) Affect cytoplasmic and nuclear signal transduction pathways,[144,145] for example H_2O_2 (which crosses cell and organelle membranes easily) can lead to displacement of the inhibitory subunit from the cytoplasmic transcription factor, nuclear factor κB, allowing the activated factor to the nucleus. [141, 145]

(3) Modulate the activity of the proteins and genes that respond to stress and which act to regulate the genes that are related to cell proliferation, differentiation and apoptosis [10, 146] (Figure1-12).

1.4.1 Production of Free Radicals in Human Body

Free radical or ROS and RNS may be generated in vivo by endogenous and exogenous sources and cause DNA damage by a variety of mechanisms. [153] Free radical formation occurs continuously in the cells as a consequence of both enzymatic and non-enzymatic reaction in normal metabolic processes. Enzymatic reaction which serve as sources of free radicals include those involved in the respiratory chain, in prostaglandin synthesis and in the cytochrome P450 system. Free radicals also arise in non-enzymatic reactions of oxygen with organic compounds as well as those initiations by ionizing radiations. [145]

Some internally or endogenously generated sources of free radicals are: (1) Mitochondria, where mitochondrion consumes about 90% of the cell's oxygen, and the mitochondrial respiratory chain are the sources of a continuing flux of oxygen radicals. [152, 155, 156] (2) Phagocytes, where polymorphonuclear leukocytes produce $O_2^{\cdot -}$

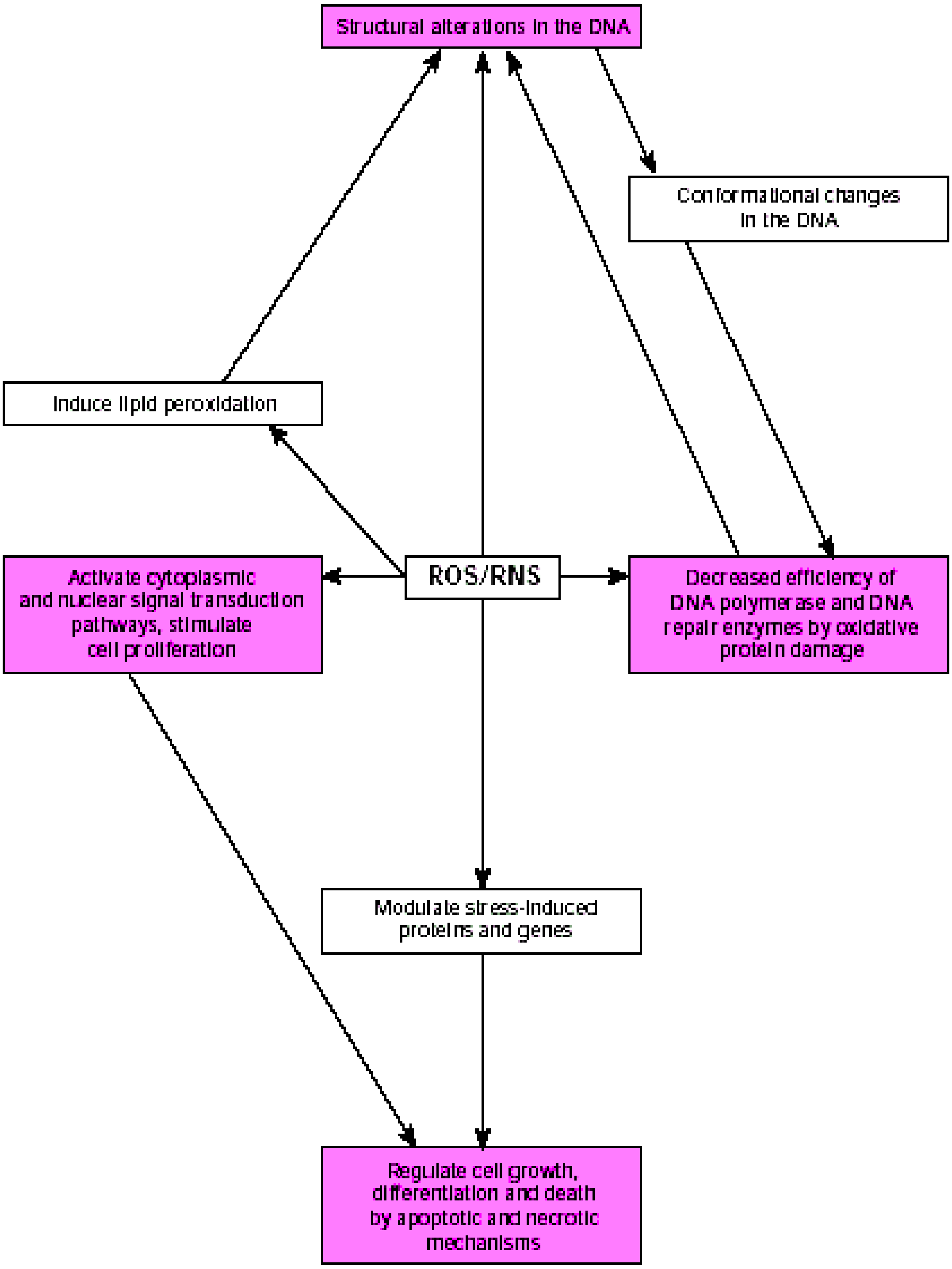


Figure 1-12 Potential carcinogenic characteristics of ROS and RNS (Figure taken from [146]).

during ingestion of bacteria.[157](3) Xanthine oxidase reaction. [152, 154, 157](4) Reaction involving iron or copper and other transition metals, such as Fenton reaction. [154, 156, 158, 159]

$$O_2 + e^- + H^+ \longrightarrow O_2^{\cdot\,-} + H^+ \xrightarrow{SOD} H_2O_2 \xrightarrow{Fe^{2+} \text{or } Cu^{1+}} 2OH^{\cdot} + Fe^{3+}$$

And Haber-Weiss reaction.[157,160]

$$\begin{array}{l}
H_2O_2 + Fe^{2+} \longrightarrow OH^{\cdot} + OH^{-} + Fe^{3+} \\
O_2^{\cdot -} + Fe^{3+} \longrightarrow O_2 + Fe^{2+} \\
\hline
H_2O_2 + O_2^{\cdot -} \longrightarrow OH^{\cdot} + OH^{-} + O_2
\end{array}$$

(5) Reactions do not involve metal ion. [160]

$$O_2^{\cdot -} + HOCl \longrightarrow OH^{\cdot} + Cl^{-} + O_2$$

(6) Arachidonate pathways [152, 161](7) Peroxisomes (8) Exercise (9) inflammation.[154]

Externally or exogenously generated sources of free radicals are (1) Cigarette smoke (2) Environmental pollutants (3) Radiation (4) UV light (5) Certain drugs, pesticides, anaesthetics and industrial solvents (6) ozone. [145]

$O_2^{\cdot -}$ and H_2O_2 , as well as other ROS and RNS, which are generated in all aerobic cells, do not appear to cause any DNA damage under physiological condition. The toxicity of these species has been attributed to the highly reactive $OH^{\cdot}$, which can be generated by different ways.[153]

1.4.2 Sources of $OH^{\cdot}$

The pattern of damaged to DNA bases in nuclear DNA suggests that $OH^{\cdot}$ attack occurs in vivo. If $OH^{\cdot}$ is attacking DNA, it must be produced very close to the DNA since it is so reactive that it cannot diffuse from its site of formation. [146] Exposure of living organisms to background levels of ionizing radiation lead to homolytic fission of oxygen-hydrogen bond in water to produce $OH^{\cdot}$ [149]. Other sources of $OH^{\cdot}$ include reaction of $O_2^{\cdot -}$ with HOCl [149] and reaction of $NO^{\cdot}$ with $O_2^{\cdot -}$.[146,162] $OH^{\cdot}$ can also be generated when H_2O_2 comes into contact with certain transition metal ion chelates, especially those of iron and copper, such as Fenton and Harber-Weiss reactions.[145, 149, 157-160] In general, the reduced forms (Fe^{2+}, Cu^{+}) and reducing agents, such as $O_2^{\cdot -}$ and ascorbic acid can often accelerate $OH^{\cdot}$ generation by metal ion / H_2O_2 mixtures.[149] Although iron and copper appear to be present in the nucleus and may be released from non-haem iron proteins in mitochondria, it remains to be established how they could reach the DNA. Iron and copper ions are normally and carefully sequestered by the body, into proteins such as ferritin, transferrin, ceruloplasmin and metallothionein. DNA is a powerful chelating agent for transition metal ion, and oxidative stress may however, cause the release of intracellular iron and / or copper ions into forms that could then bind to DNA. Thus $O_2^{\cdot -}$ can release some iron from ferritin, H_2O_2 can release iron from haem proteins and $ONOO^{-}$ can release copper from ceruloplasmin.[146]

1.4.3 Chemistry of DNA Damaged by ROS and RNS

The endogenous reactions that are likely to contribute ongoing DNA damage are oxidation, methylation, depurination, and deamination.[141, 159] Nitric oxide or more likely, reactive products derived from it, such as $NO_2^{\cdot}$, $ONOO^-$,N_2O_3 and HNO_2 , are mutagenic agents, with the potential to produce nitration, nitrosation and deamination reactions on DNA bases [141, 146] (Figure1- 13).

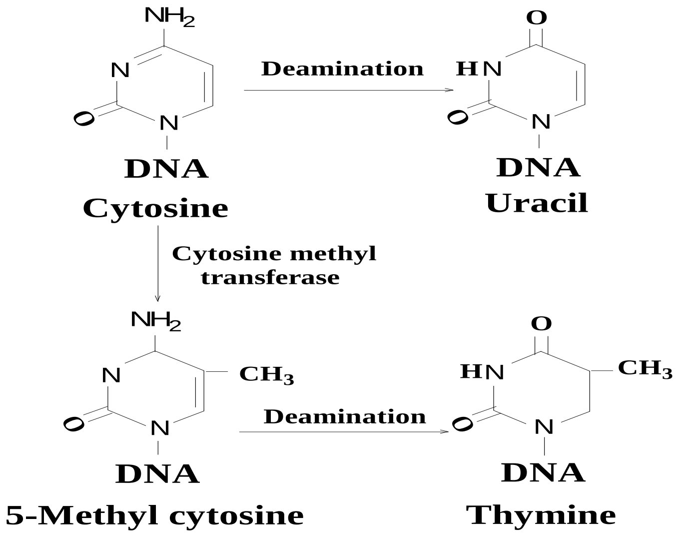


Figure1-13 Deamination and methylation of cytosine, and deamination of 5-methyl cytosine in DNA (Figure taken from [141])

Methylation of cytosines in DNA is important for the regulation of gene expression, and normal methylation patterns can be altered during carcinogenesis. Conversion of guanine to 8-hydroxyguanine (8-OHG), [141, 155] a frequent result of ROS attack, has been found to alter the enzyme catalyzed methylation of adjacent cytosines, thus providing a link between oxidative DNA damage and altered methylation patterns.[146]

Several models relate oxidative DNA damage to aging and cancer. For example, free radicals could react with nuclear DNA and produce somatic mutations including point or frame shift mutations, deletions, and strand breaks.[155]

DNA damage resulting from oxidative stress need not necessarily lead to cancer. Low level of damage may be efficiently repaired with a minimal risk of error. High levels of oxidative stress may lead to cell death, so that initiated cells do not remain in the organism. Thus, an intermediate level of damage is most likely to predispose to malignancy. [149]

Evidence exists that DNA damage by endogenous free radical occurs and accumulates in vivo, and that there is a steady state level of free radical-modified bases in cellular DNA. Continuous endogenous damage to cellular DNA by free radical and accumulation of such damage have been suggested to significantly contribute to carcinogenesis in human.[163] Different ROS affect DNA in different ways. $OH^{\cdot}$ generates a multiplicity of products from all four DNA bases and this pattern appears to be a diagnostic “finger-print” of $OH^{\cdot}$ protein attack.[149] By contrast $^1O_2$ selectively attacks guanine. The most commonly produced base lesion, and the one most often measured as an index of oxidative DNA damage, is (8-OHG).[146-145] Figure1-14 shows the structures of the products of ROS attack on DNA.[146]

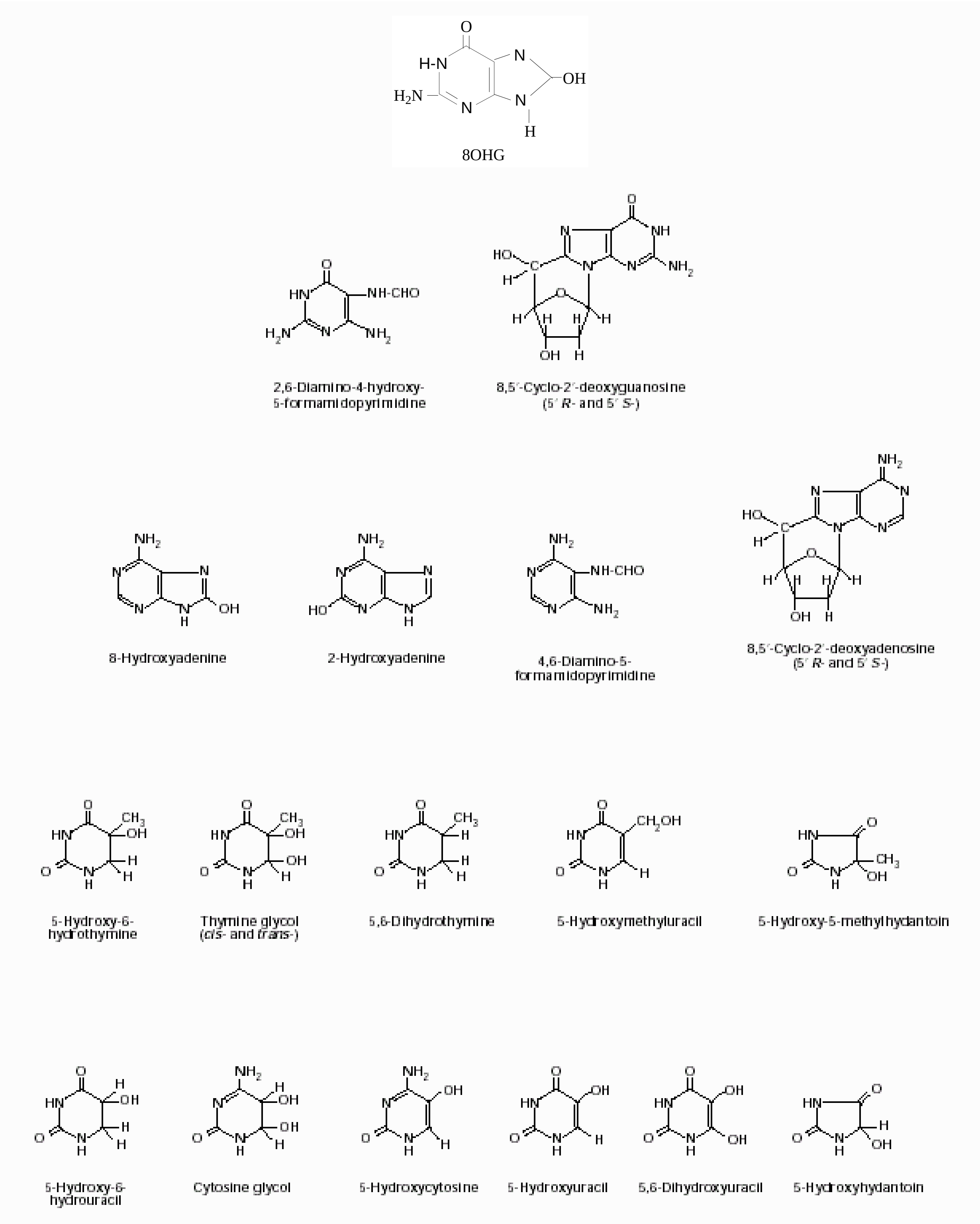


Figure 1- 14 Structure of Modified DNA Bases (Figure taken from [146,149, 163]).

Halliwell and Aruoma [149] have advanced two explanations of the DNA damage by ROS and RNS. First it is possible that the damage is due to $OH^{\bullet}$ radical formation. Thus, it is envisaged that H_2O_2 , which crosses biological membranes easily, can penetrate to the nucleus and react with ions of iron or copper to form $OH^{\cdot}$. Because of the high reactivity of $OH^{\cdot}$and its resultant inability to diffuse significant distances within the cell, this mechanism is only feasible if the $OH^{\cdot}$ is generated from H_2O_2 by reaction with metal ions bound upon, or very close to, the DNA. One possibility is that these metal ions might always be present bound to the DNA in vivo. A second possibility is that the metal ions might be released within the cell as a result of oxidative stress, and then binds to the DNA.

A second explanation of the ability of oxidative stress to cause DNA damage is that it triggers of a series of metabolic events within cell, that leads to the activation of nuclease enzymes, which cleave the DNA backbone. There has been much debate recently concerning the suggestion that oxidative stress causes rises in intracellular free Ca^{2+} -dependent endonucleases in a mechanism resembling that of apotosis (programmed cell death).

These two mechanisms (DNA damage by $OH^{\cdot}$ or by activation of nucleases) are not mutually exclusive, i,e. they could both take place [146, 149] (Figure 1-15).

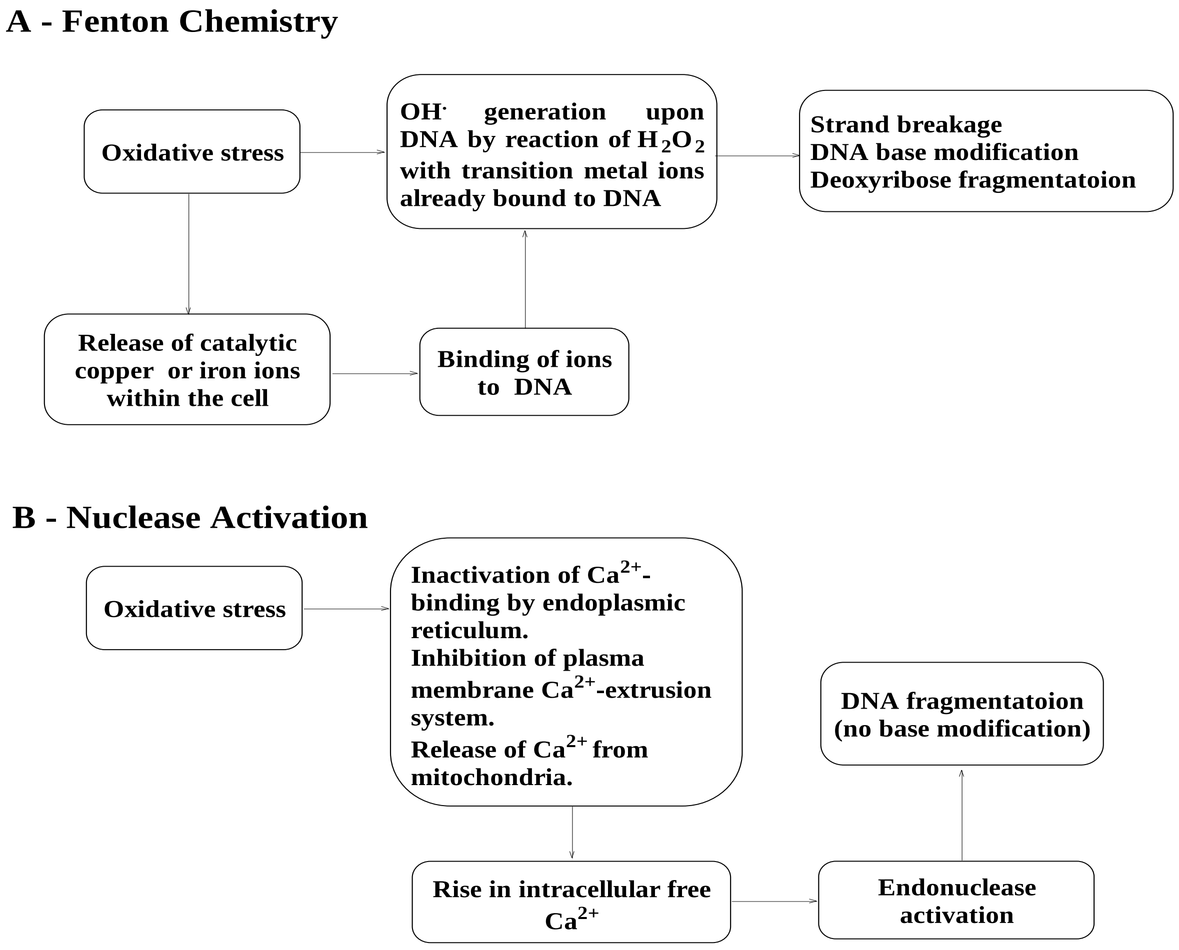


Figure 1-15 Hypotheses to explain DNA damage resulting form exposing cells to oxidative stress. (Figure taken from [149]).

1.4.4 DNA Damage by ROS/RNS Could Cause Mutation and Cancer

ROS and RNS can cause DNA base changes, strand breaks, damage of tumor-suppressor genes and enhanced expression of protooncogenes. Oxidative stress has been shown to induce malignant transformation of cells in culture.[55, 144, 146]

However, the development of human cancer depends on many other factors, including the extent of DNA damage, antioxidant defenses, DNA repair system, the efficiency of removal of oxidized nucleosides before they are incorporated into DNA, and the cytotoxic effects of ROS in large amount (a dead cell will not lead to cancer) as well as their growth-promoting effects in small amounts.[146]

Persistent excessive oxidative DNA damage may transmit signals to other cellular components (including the tumor-suppressor protein *p53*) and may trigger apoptosis.[141] In addition, DNA damage can result in *p53* accumulation in the nucleus, which may arrest cell growth at the G_1/S border in an attempt to allow repair of DNA lesions before replication.[146] The *bcl-2* gene is the prototype of a family of genes that inhibit apoptosis.[140, 141] Roles of ROS in apoptosis are frequently suggested; the antiapoptotic gene *bcl-2* decreases the over all cellular production of ROS and can trigger apoptosis. Unfortunately, the *bcl-2* gene is also an oncogene and the oncogenic properties of the *bcl-2* protein could relate to its ability to prevent useful apotptic cell death. The *bel-2* protein has a c-terminal membrane anchor and is localized to the nuclear, endoplasmic reticulum and mitochondrial outer membranes; it may protect cells by inhibiting ROS-induced lipids peroxidation, as well as down-regulation of the *bel-2* gene by the *p-53* tumor-suppresser protein in human breast cancer cells has been reported.[146]

Recent studies have attempted to identify mutations that are caused by ROS and RNS damage to DNA, with the aim of ascertaining their association with cancer.[146-148]

There are several different pathways leading from initial DNA base damage by ROS to subsequent mutation (Figure1-16). The first is the chemical modification of DNA bases causing a change in their hydrogen bonding specificity. The contributing of oxidative damage to polymerase-specific “hot spots” which is a likely major contributor to DNA polymerase-mediated mutagenesis, is a second possible mechanism. A third mechanism is linked to a conformational change in the DNA template that diminishes the accuracy of replication by DNA polymerases.[146]

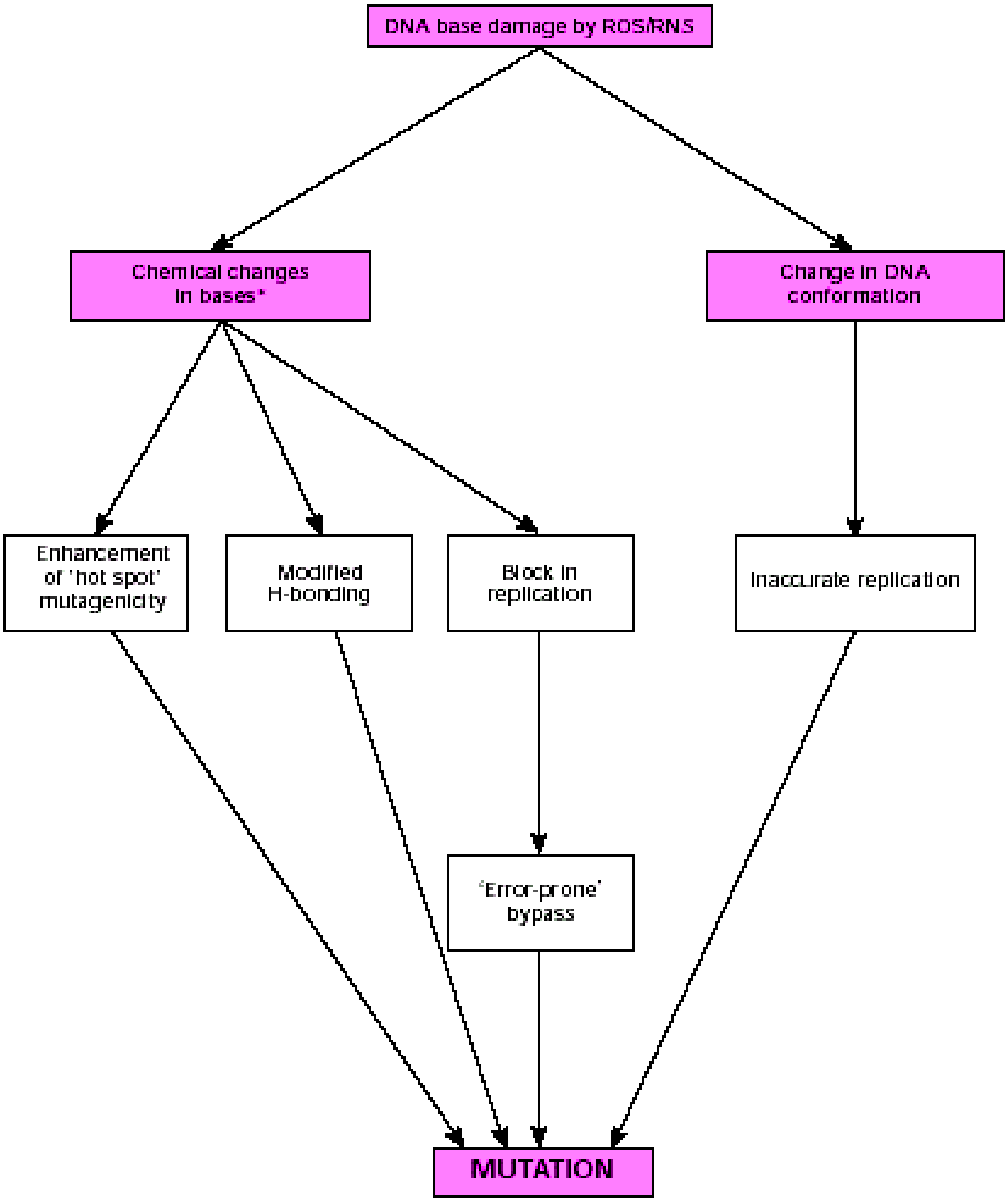


Figure 1-16 Pathways leading from initial base damage to subsequent mutation (Figure taken from [146])

1.4.5 Antioxidant

Free radicals have important regulatory functions in physiological processes and they constitute an important part of the defense against invading microorganisms. When free radicals are produced in excess, they may attack and injure several organs and may cause several diseases, including cancer. [164]

The human body has several mechanisms to counteract by free radicals and other ROS and RNS. These act on different oxidants as well as in different cellular compartments. One important line of defense is a system of enzymes, including glutathione (GSH) peroxidases, superoxide dismutase (SOD) and catalase, which decrease concentrations of most harmful oxidants in the tissues. Several essential minerals including selenium, copper manganese and zinc are necessary for the formation or activity of these enzymes. The second line of defense against free radical damage is the presence of antioxidants, such as GSH, vitamin C, vitamin E, [147, 151, 154, 164] (Figure 1-17).

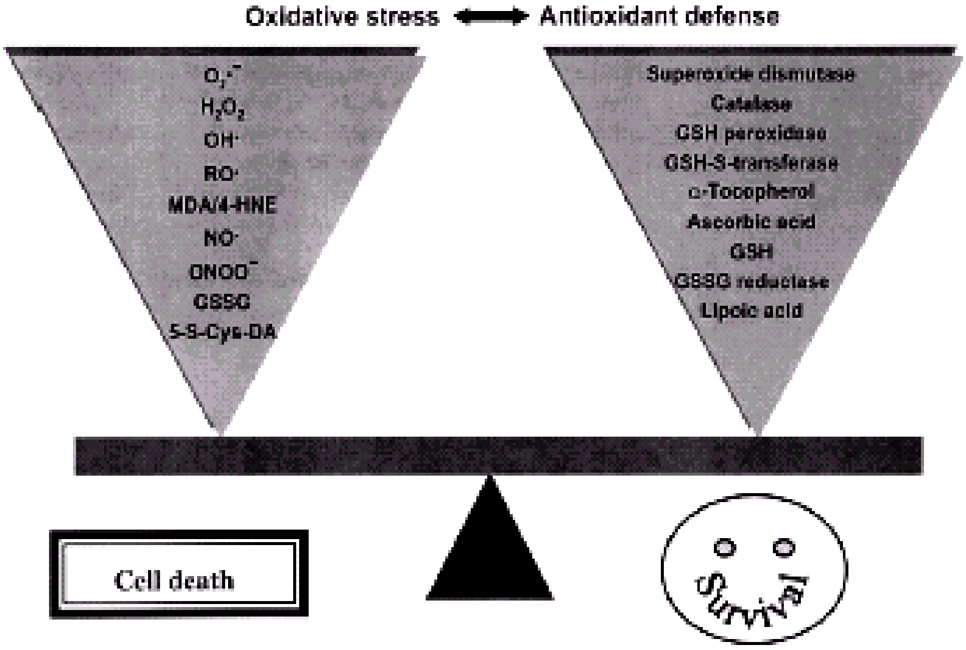


Figure 1-17 Balance of oxidants and antioxidant active systems (Figure taken from [152])

Halliwell[150] introduced a broader definition, an antioxidant is any substance that, when present at low concentration compared to those of an oxidizable substance, significantly delays or prevents the oxidation of that substrate. The term “oxidizable substrate” includes almost everything found in living cell, including proteins, lipids, carbohydrates, and DNA. [150]

Bagchi and Puri [154] introduced another definition, an antioxidant is a molecule stable enough to donate an electron to a rampaging free radical and to neutralize it, thus reducing its capacity to damage.[154]

The mechanism of antioxidants action can include removal of oxygen (e.g. the packing of food stuffs under N_2), scavenging ROS and RNS or their precursors, inhibition ROS and RNS formation, binding metal ion needed for catalysis of ROS, generation, and upregulation of endogenous antioxidant defenses. [150]

1.4.6 Measures of Oxidative Stress

It is difficult to directly measure free radicals in a biological sample due to their short half-life. The technique of choice is electron spin resonance (ESR), which often require previous “trapping” of the radicals in order to produce more stable molecules that can accumulate in larger amounts. However, estimation of radical damage is more easily performed. Radicals easily react with macromolecules of different classes, whereby more stable molecules are formed, which can subsequently be measured.[148]

Oxidatively modified groups on lipids, proteins and DNA can be used to indirectly measure the general ROS and RNS load in biological samples. [148]

The biological effects of free radicals are controlled in vivo by a wide range of antioxidants, such as vitamins E, C, and carotenoids, GSH, minerals zinc, selenium, copper, manganese, and iron, as well as proteins that either catalytically destroy oxidants, such as SOD, catalase, and GSH peroxidase, or scavenge oxidants in a sacrificial manner (i.e. replace a highly reactive free radical by a less reactive one), such as albumin or they sequester transition metals in away that prevents the metal ion from participating in free radical reactions, such as transferrin, ceruloplasmin, and albumin.[150, 154, 158, 164, 165]

The alteration in the levels of oxidants and antioxidants may be considered as a biochemical marker of free radical generation, which in turn, useful to measure the oxidative stress associated with diseases.[166]

One of most frequently used measures of lipid peroxidation is the thiobarbituric acid reactive substance method [148]. Protein carbonylation can be used as a measure of radical damage to protein.[148] Protein fragmentation can be measured by PAGE.[156] Also, radical damage to plasma proteins can be measured by quantitative determination of plasma protein profile. One of the most frequently used methods for estimating radical-modified DNA is to measure 8-OHG and (8-OHdG), because it can be assayed using HPLC technique. [146, 148] Many additional methods have been described e.g. gas chromatography-mass spectroscopy (GC-MS) has been used to analyze thymine glycol residues, uracil and malondialdehydequanine adducts. Analysis of uracil in DNA by GC-MS following its removal by uracil DNA glycosylase has been used to demonstrate that the inhibition of folic acid metabolism includes uracil accumulation in DNA,[141, 146] and by increasing the deamination of cytosine under oxidative stress. [141, 146]

1.4.7 Vitamin C (Ascorbic Acid)

Vitamin C or ascorbic acid (AA) in humans must be ingested for survival. Vitamin C is an electron donor, and this property accounts for all its known functions. As an electron donor, vitamin C is a potent water-soluble antioxidant. [167] The antioxidant properties of vitamin C may be involved in the prevention of cancer.[168]. Vitamin C, is a primary antioxidant in plasma and within cells, but it can also interact with the plasma membrane by donating electrons to α-tocopheroxyl radical and a trans-plasma membrane oxidoreductase activity. Ascorbate-derived reducing capacity is thus transmitted both into and across the plasma membrane. Recycling of α -tocopherol (vitamin E) by ascorbate helps to protect membrane lipids from peroxidation.[169]

Vitamin C generally functions as an antioxidant by directly reacting with ROS and has a vital role in defenses against oxidative stress. However, AA also has pro-oxidant properties and may cause apoptosis of lymphoid and myeloid cells. Also, dehydroascorbic acid (DHA), the oxidized from vitamin C, stimulates the antioxidant defenses of cells.[170]

Vitamin C readily scavenges ROS and RNS,[171] and may thereby prevent oxidative damage to important biological macromolecules such as DNA, lipids, and proteins. Vitamin C also reduces redox active transition metal ions in the active site of specific biosynthetic enzymes.[172, 173]

Humans and other primates luck gulonolactone oxidase, a key enzyme for ascorbic acid biosynthesis, and therefore it must be provided from external sources. Even in AA-synthesizing species, the majority of cells need AA from the outside. Vitamin C is absorbed from the gastrointestinal tract in the form of AA and circulates in the blood as ascorbate at pH of 7.4.[170] Within cell, DHA is regenerated into ascorbate at the expense of GSH.[174]

Vitamin C is an effective antioxidant for several reasons. First, both ascorbate and the ascorbyl radical (formed by one electron oxidative of ascorbate) have low reduction potentials and can react with most other biologically relevant radicals and oxidants. Second, the ascorbyl radical has a low reactivity due to resonance stabilization of the unpaired electron and readily dismutates to ascorbate and DHA (Figure 1-18). In addition, ascorbate can be regenerated from both the ascorbyl radical and DHA by enzyme-dependent and independent pathways.[172]

Another important biological function of vitamin C is its interaction with redox active transition metal ions, such as iron and copper.[172, 173] Vitamin C acts as a cosubstrate for hydroxylase and oxygenase enzymes involved in the biosynthesis of procollagen, carnitine, and neurotransmitters. A deficiency of vitamin C causes scurvy results from a decreased activity of these enzymes. [1, 7, 8, 172]

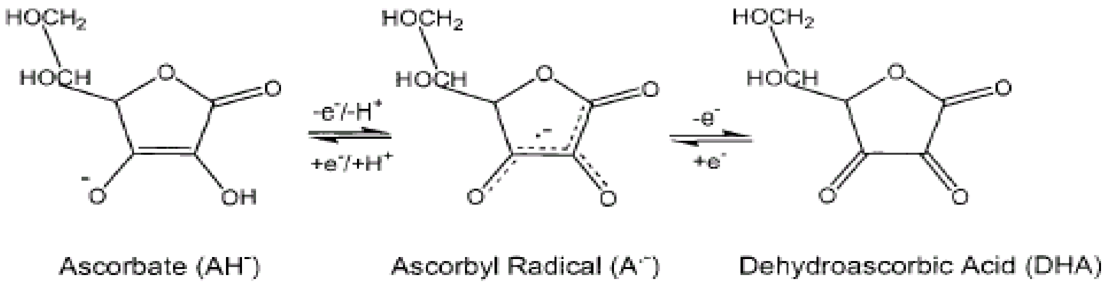


Figure 1-18 Oxidation of ascorbate ($AH^{\cdot}$) by two successive one electron oxidation steps to give the ascorbyl radical ($A^{\cdot -}$) and dehydroascorbic acid (DHA), respectively (Figure taken from [172])

The plasma concentration of vitamin C has been elevated by 60% after vitamin C supplementation. The levels of oxidized DNA bases, lipid peroxidation and protein oxidation have been reported to be significantly reduce, after vitamin C supplementation.[172]

A wide variety of methods involving different chromophores, detection systems, and enzymes have been developed for the determination of AA and DHA in biological samples.[175]

1.4.8 Glutathione (GSH)

Glutathione (GSH) is the most important non-protein thiol present in human and animal cells as well as in plant, and bacteria. It was discovered by F.G. Hopkins in 1921 and identified as the tripeptide γ-L-glutamyl-L-cysteinyl-glycine a few years later.[174, 176] GSH is synthesized in two steps catalyzed by γ -glutamyl-cysteine sythetase and GSH synthetase respectively.

$$\text{L-glutamate} + \text{L-cysteine} + \text{ATP} \rightleftharpoons \gamma\text{-L-glutamyl-L-cysteine} + \text{ADP} + \text{Pi}$$

$$\gamma\text{-L-glutamyl-L-cysteine} + \text{glycine} + \text{ATP} \rightleftharpoons \text{GSH} + \text{ADP} + \text{Pi}$$

The first reaction is considered rate limiting and inhibited by GSH, suggesting a physiologically significant feedback control of GSH synthesis. [177, 178] It is probable that the rate of GSH synthesis and, consequently, the GSH level are significantly influenced by the availability of intracellular cysteine. [177]

Cysteine molecule has a sulfer-containing portion, which gives the whole GSH molecule its biochemical activity, i.e. its ability to carry out the following vitally important functions:

Firstly, GSH is the major antioxidant produced by the cell, protecting it from free radicals, which, if left unchecked, will damage or destroy key cell components (e.g. membranes, DNA).[152, 174] In addition, GSH recycles other well-known antioxidants such as vitamin C and vitamin E, keeping them in their active state [1,178,179] as shown in Figure1-19.

Secondly, GSH is a very important detoxifying agent, enabling the body to get rid of undesirable toxins and pollutant. [179-181]

Thirdly, GSH plays a crucial role in maintaining a normal balance between oxidation and antioxidation. This, in turn, regulates many of the cell's vital function, such as the synthesis and repair of DNA, the synthesis of proteins and the activation and regulation of enzymes. [9,178]

Fourthly, GSH is required in many intricate steps needed to carry out an immune response. For example, it is needed for the lymphocytes to multiply in order to develop a strong immune response, and for killer lymphocytes to be able to kill undesirable cells such as cancer cells or virally infected cells.[179-181]

Low concentrations of GSH have been implicated in numerous pathological conditions, including diabetes, alcoholic liver disease, AIDS, acute hemorrhagic gastric crosions, cataracts, xenobiotic induced oxidative stress and toxicity, and aging.[182]

GSH concentrations in the blood may serve as a useful indicator of disease risk in humans.[182] Also, it has been reported that the level of oxidized GSH (GSSG) in serum may serve as index of several of diseases and aging. [183]

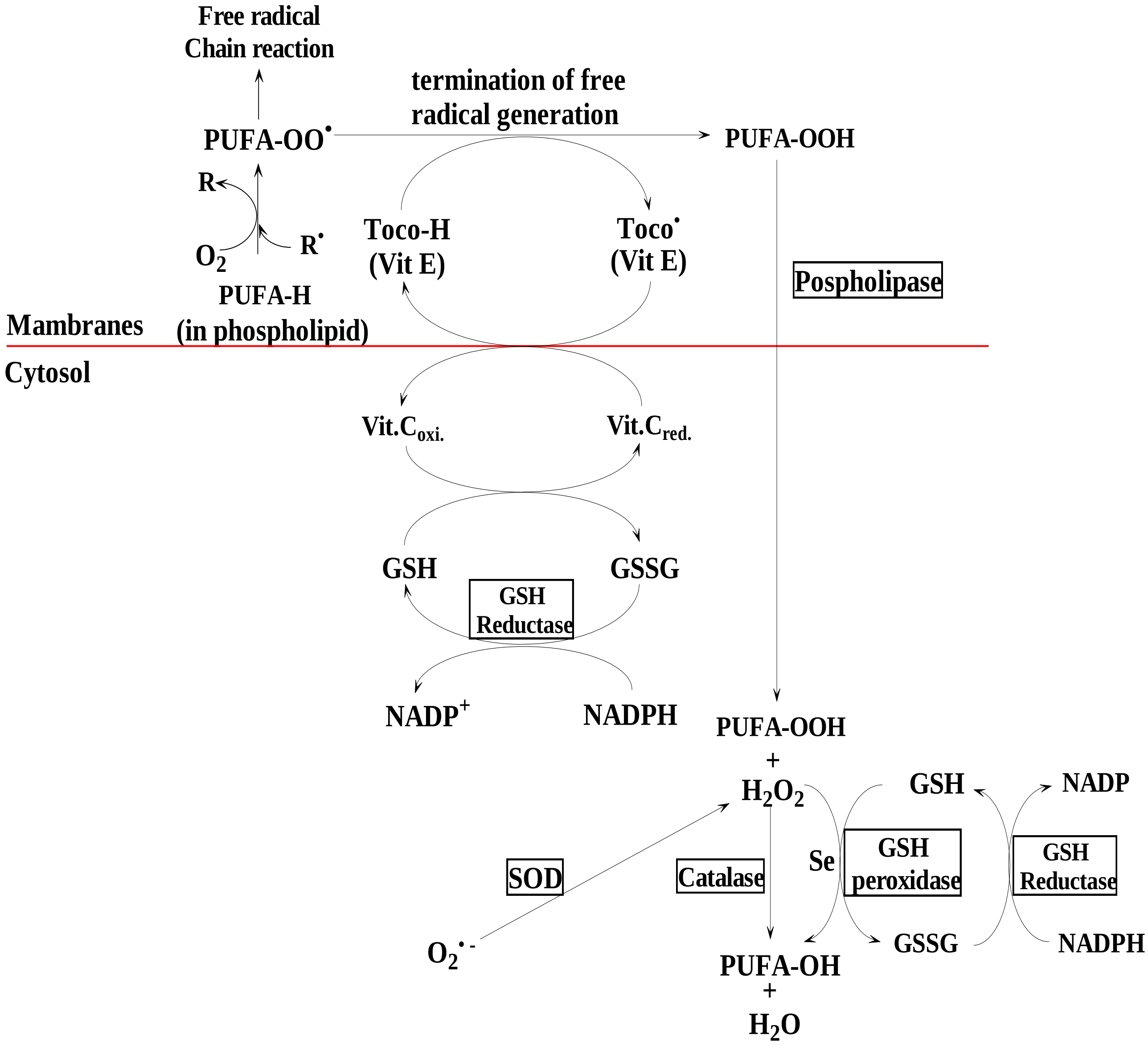


Figure 1-19 Interaction between antioxidant system operating in the membranes of the cell and cytosol. (Figure taken from [1])

1.4.9 Serum Proteins

The investigation of serum proteins in disease by the clinical laboratory can be considered in two ways; the first is the measurement of total protein and the relative distribution of the major proteins, and the second is the measurement of specific proteins in the serum. The first approach is not specific for pathological condition but can act as a screening test for many different conditions. The second approach, where a specific protein is measured, can give information about particular diseases. [2]

Although the protein in all fluid compartments plays a major physiological function, the plasma proteins are the most frequently analyzed. More than 500 plasma proteins have been identified.[5,16]

The word protein is based on the Greek word *proteios*, meaning first. It was introduced by the Dutch chemist Mulder in the late 1830s and implied that proteins were substances of primary importance in the body [2].

While plasma contains three major groups of proteins, albumin, fibrinogen, and globulins; serum contains only two major groups of proteins, albumin and globulins.

It was early recognized that plasma contains members of both the two major groups of proteins, the simple and conjugated, into which proteins had been divided. Simple proteins, composed only of amino acids linked by peptides bonds, include albumin and some globulins, while conjugated proteins such as fibrinogen and the rest of globulins containing other substance bound to the polypeptide protein such as glycoproteins, lipoproteins, and metalloproteins which containing carbohydrate, lipids, and metal ion, respectively. [13]

Serum protein plays a key role in varied functions ranging from nutrition and control of body water distribution, to transporting and protection, as well as their central role as a hormones and enzymes.[13] Also, some proteins act as antioxidants. [150]

Many plasma proteins, including albumin, fibrinogen and most globulins, are formed in the liver; whereas, reticuloendothelial system, lymph nodules and plasma cells produce immunoglobulines; the enzymes are released from various organs. [11,13]

1.4.9.1 Total Serum Protein

Total protein measurement is a fairly non-specific measurement as it can be influenced by many variables, and thus changes in one protein or group of proteins can be masked by opposite changes in other proteins. Total protein measurements can give an indication of gross changes brought about by a number of different of disease states. [2]

1.4.9.2 Albumin

Albumin is the most abundant protein found in the plasma, making up 55 - 65% of the total protein.[2,11] Albumin, with a molecular weight of about 66 kDa, is synthesized by the liver and is the most important protein in maintaining the osmotic pressure of plasma. Another important role of this protein is that of a non-specific transport protein, as it binds many non-polar compounds such as bilirubin, long-chain fatty acids, phospholipids, metallic ions, amino acids, and a number of drugs. Albumin also functions as a reservoir for a number of hormones, particularly thyroid hormones. [2,11,15]

Other important functions of albumin include:

(1) Albumin is an important component of plasma antioxidant activity, primary by binding free fatty acid, divalent cations, HOCl and bilirubin and scavenging of $O_2^{\cdot-}$ and $ONOO^-$.[11,150,156]

(2) Albumin acts as buffer in non-physiologic conditions.

(3) The binding of albumin to endothelial membrane-associated glycoproteins increases capillary permeability to small protein.[11]

1.4.9.3 Globulins

The globulin group of serum proteins consists of α_1, α_2, β_1, β_2, and γ fraction. Each fraction consists of a number of different proteins with different functions, as shown in the following: [2,5,15]

(1) α_1-Globulins fraction consists of α_1-antitrypsin, α_1-fetoprotein, α_1-acid glycoprotein (orosomucoid), α_1-lipoprotein (HDL), α_1-antichymotrypsin, inter- α -trypsin inhibitor, and Gc-globulin.

(2) α_2-Globulins fraction consists of heptoglobins which are divided into types 1,2, and 3, ceruloplasmin, and α_2-macroglobulin.

(3) β_1-Globulins fraction consists of pre β -lipoprotein (VLDL), transferrin, hemopexin, β -lipoproteins (LDL).

(4) β_2-Globulins fraction consists of β_2-microglobulin, and C_3, C_4, C_{19} complement, as well as C-reactive protein.

(5) γ -Globulin fractions consists of immunoglobulin G, A, M, D, and E. [5,16]

Globulins play a central role in the very varied function. Most globulins are formed in the liver; reticuloendothelial system and the lymph nodules, as well as plasma cells produce immunoglobulins.[11,13]

The serum globulins concentration is easily found by subtracting the serum albumin concentration from total serum protein concentration.[5]

Electrophoresis separates the serum globulins into five bands, which are mixtures of proteins that migrate together in the electric field. If special high resolution techniques are used, the serum proteins may separate into 15-20 separate bands. Thus electrophoretic pattern can be inspected visually to give much information about the patient. [2]

Serum protein changes; therefore, changes in the electrophoretic pattern are seen in liver disease, kidney disease, inflammatory conditions, infection, some cancers and conditions that affect specific proteins.[2]

1.4.9.4 Albumin / Globulins Ratio

In the assay of total serum proteins, useful diagnostic information can be obtained by determining the albumin fraction and total globulins. A reversal or significant change in the ratio of albumin and total globulin (A/G) was first noticed in diseases of the kidney and liver. [5,16]

To determine the (A/G) ratio, it is common to determine the total protein and albumin. Globulins are calculated by subtracting the albumin from the total protein.[184]

When an abnormality is found in the total protein or albumin, an electrophoretic analysis is usually made. If an abnormality is seen on the electrophoretic pattern, an analysis of individual proteins within the area of abnormality is made.[5,16]

1.4.9.5 Electrophoresis

Electrophoresis is a technique commonly used to separate the serum proteins into a number of bands, called an electrophoretogram or electrophoresis strip, using an electric current. The electrophoresis strip is visually inspected and can be used to screen for a number of diseases.[2,5] Changes in certain bands are clearly associated with particular disorders, making serum protein electrophoresis a valuable screening device. In the most electrophoretograms obtained from normal serum, usually only five bands (albumin, α_1, α_2, β, and γ) are seen, although a sixth band (β_2, C3 complement) may be visible if the serum is fresh.[11]

Electrophoresis is the movement of changed particles in an electric field. Proteins have a net charge derived from the ionization of the amino acid residues that make up the protein. The exact charge on the protein molecule will depend on the pH of the solution in which the protein is dissolved. At high pH values, where there is a lack of hydrogen ions, the protein will be negatively charged.[2,13]

Electrophoresis was discovered by the biochemist Arne Tiselius in Uppsala, Sweden. . Who was awarded a Nobel Prize in 1948 for his work in this area, he showed that blood proteins in serum could be separated into four major bands by applying an electric current.[2]

All types of electrophoresis are based on the same principles. The major difference among methods is the type of support medium, which can be either cellulose or thin gels. Cellulose is used as a support medium for low molecular-weight biochemicals, and polyacrylamide and agarose gels are widely used as support media for large molecules.[19]

Gel formed by polymerization of acrylamide has several positive features in electrophoresis:

(1) High resolving power for small and moderately sized proteins and nucleic acids.

(2) Acceptance of relatively large sample size.

(3) Minimal interactions of the migrating molecules with the matrix of gel.

(4) Physical stability of the matrix. [19]

1.4.10 Trace Elements

Many mineral elements occur in living tissues in such small amounts that the early workers were unable to measure their precise concentrations with the analytical methods then available. They were therefore frequently described as occurring in "traces" and the term trace elements arose to describe them. [185]

Concentrations of major elements in biological tissues are reported in gram per kilogram amounts. Trace elements are elements that occur in human and animal tissues in milligram per kilogram amounts or less. Correspondingly, human intake requirements of major element are expressed in grams or fractions of grams per day, whereas human requirements of trace elements are reported in milligrams per day.[5,11] Since the 1970s, the term ultratrace elements has been used to refer to elements present in tissues in microgram per kilogram amounts or less and with estimated dietary requirements indicated by micrograms per day. [11]

Trace elements appear to play a key role in most biological functions. The effect of these elements cannot be replaced by another element. It has been reported that action of a very small amount of trace and ultratrace elements are necessary for optimal performance of whole organism, and the lack of a small amount of them can result in clinical abnormalities.[2,11] On the other hand, deficient of these elements is associated with impairment of function and excess concentrations may be toxic.[5]

Trace elements are required in very small amounts to maintain health and they often form essential parts of enzymes and hormones, which regulate metabolism.[2]

Some of the trace and ultratrace elements have a scavenging property as antioxidant to free radical generation, thus the alteration in their levels may be considered a biochemical marker of free radical generation, such as zinc, selenium, copper, and iron.[166]

1.4.10.1 Zinc

The physiological functions of zinc are based largely on its present as an essential component of many metalloenzymes involved in virtually all aspects of metabolism. Zinc is an integral compound of nearly 300 enzymes in different species.[11]

The body contains an average of about 1.8 gm zinc. In men the prostate is the organ with the highest concentration of zinc with liver, kidney and muscle containing about half as much and heart about a third. Of the zinc in blood about 81% is present in the red cells almost wholly as carbonic anhydrase for which it is a cofactor, 16% is in the plasma and 3% in the leukocytes. About a third of the plasma zinc is bound loosely to albumin with the rest more firmly bound to globulin.[13] Zinc is transported in blood plasma mostly by albumin (60-70%) and by α_2-macroglobulin (30-40%), with a small associated with transferrin and free amino acids. [11]

Zinc is found in biological systems only in the (2+) valance state. This is the result of the extra stability associated with a filled d-orbital electron configuration .[2]

Zinc-containing enzymes are found in every enzyme class ALP, alcohol dehydrogenase, carbonic anhydrase, DNA polymerase, SOD contain zinc, and CDA (Figure1-3). Zinc-containing enzymes are essential to growth, wound healing, reproductive function, the immune system, and protection from free radical damage.[5]

Malabsorption chronic disease of liver and kidney, alcoholism, and diets high in phosphate or low in zinc are associated with zinc deficiency. Plasma zinc concentration does not change significantly until zinc deficiency is pronounced. Symptoms of deficiency include skin lesions, diarrhea, dwarfism, and susceptibility to infection. [5,11]

1.4.10.2 Selenium

Most of the selenium in tissues is present in two forms: selenocysteine and selenomethionine. Selenomethionine cannot be synthesized in the body and must be supplied by the diet, and is regarded as an unregulated strange compartment for selenium. Selenocyteine is a selenium-containing analogue of cysteine that is recognized as the 21st amino acid encoded by DNA.[11]

Selenium is incorporated into proteins, notably GSH peroxidase, which may be measured to reflect selenium states. Low selenium intake has been demonstrated in the cardiomyophaty of Keshan disease. Recent studies of effects of micronutrients on cancer have demonstrated that selenium supplementation is associated with a decreased risk of stomach carcinoma, lung, prostate, and total cancer.[5]

Selenocysteine is the biologically active form of selenium, is tightly regulated, and is found in the prokaryotic and eukaryotic kingdoms in active sites of enzymes involved in oxidation-reduction reactions. Selenium helps defend the organism against oxidant stress and is involving in the synthesis and metabolism of thyroid hormones.[11]

Selenium is present in selenoproteins, such as GSH peroxidase, iodothronine deiodinase, selenoprotein P, selenoprotein W, and thiorodoxin reductase.[9]

Selenomethionines are the most common selenium compounds taken as dietary antioxidant to reduce oxidative stress. Besides selenium, other dietary antioxidants, such as vitamin C or vitamin E are commonly supplemented and taken together with selenium to reduce the risk of cancer.[186,187]

Selenium has been shown to be essential for life and to be toxic at levels little above those required for health. Indeed, dietary levels of the desired amount of selenium are in a very narrow range; consumption of food containing less then 0.1 mg Kg^{-1} of this element will result in its deficiency whereas dietary levels above 1 mg Kg^{-1} will lead to toxic manifestations.[187]

1.4.10.3 Copper

Copper is a transition metal with three oxidation states. Cu^0,Cu^{1+}, and Cu^{+2} . [188] It may be present in biological systems in both (1+) and (2+) valance states,[11] but the cupric state is found most often in biological systems.[188] Thus the major functions of copper metalloproteins involved oxidation-reduction reactions; most known copper containing enzymes bind and react directly with molecular oxygen. Copper is an integral component of many metalloenzymes, including ceruloplasmin, cytochrom c oxidase, SOD contain Zn and Cu, dopamine-β-hydroxylase, ascorbate oxidase, lysyl oxidase, and tyrosinase.[11]

Cytochrom c oxidase catalyzes the reduction of oxygen to water in the last step of the electron transport chain. The enzyme contains two heme groups and two copper atoms that mediate the electron transfer. SOD is extremely important to antioxidant defense. SOD, containing copper and zinc, catalyzes the conversion of two $O_2^{\cdot-}$ to O_2 and H_2O_2 in the cytosol. The peroxide produced in this reaction is destroyed by catalase containing heme or GSH peroxidase containing selenium [5] (Figure 1-19).

Absorbed copper is rapidly transported as copper-albumin or copper-histidine complexes to the liver where it is stored, mostly as metallothionine-like cuproproteins. Copper is released from liver, mainly as ceruloplasmin, a multifunctional cuproprotein that accounts for over 95% of total copper in plasma.[11]

Copper deficiency is uncommon, but certain circumstances promote its occurrence. Example includes malnutrition, malabsorption, and conditions resulting in chronic copper loss. Zinc competes with copper for absorption from the intestine; therefore, increased intake of zinc could cause copper deficiency. Copper deficiency cause a microcytic, hypochromic anemia associated with low concentrations of ceruloplasmin.[5]

An early feature of copper deficiency is neutropenia, which has been observed in several conditions, including diet low copper, diarrheal loss, chelation therapy in Wilson's disease, peritoneal dialysis, and celiac disease.[9]

Accidental ingestion of copper compounds may cause acute copper toxicity and associated epigastric pain, nausea, and vomiting. Excess copper, as with excess iron, can cause free radical production and damage.[5]

1.4.10.4 Iron

Iron is the most important of essential trace elements.[11] The total iron content of the human body varies with age, sex, nutrition, and state of health. Normal adult man is estimated to contain 4-5 gm of iron.[155]

Most iron in the body is incorporated into heme of hemoglobin and a smaller proportion as myoglobin, heme-containing enzymes like catalase, and iron-sulfur proteins. A significant amount of iron is stored as ferritin and hemosiderin, primarily in the bone marrow, spleen, and liver. [5]

Iron deficiency is the most prevalent deficiency state affecting human population. In adult men and postmenopausal, the principle cause is chronic blood loss due to infections, malignancy and bleeding ulcers. However, anemia of dietary origin is not uncommon among the elderly, especially the institutionalized elderly.[185]

Iron is a transition element capable of reduction/oxidation (redox) activity, and potential harm to the body is circumvented by binding iron to transport or strong proteins. In recent years, the role of iron has been investigated as prooxidant, which contributes to lipid peroxidation, atherosclerosis. DNA damage, carcinogenesis, and neurodegenerative disease.[5]

Iron, as a transition metal with partially filled d-orbital tends to coordinate with a large number of electron donor atoms.[166] Fe^{3+}, released from binding proteins, can participate in the production of free radical by the Harber-Weiss or Fenton reaction and cause oxidative damage.[157-160]

CHAPTER TWO

2. MATERIAL AND METHOD

2.1 Materials

2.2.1Chemicals

All chemicals were used as supplied without further purification.

Chemicals	**Purity %**	**Supplied Company**
Meta-phosphoric acid (m-HPO_3)$_n$	60	Fluka
Thiourea	99.0	Fluka
Copper sulfate anhydrous	99.0	Fluka
Sulfuric acid	95	Fluka
Ethylenediaminetetracetic acid dihydrate (EDTA).2H_2O	99.5	Fluka
Bromophenol blue	99.5	Fluka
Triton X-100	99.5	Fluka
Methanol	99.8	Fluka
Coomassie brilliant blue R-250	99.5	Fluka
Ethanol	99.98	Fluka
Glycerol	87	Fluka
Nitric Acid	65	Fluka
Perchloric acid	70	Fluka
Hydrochloric acid	99.5	Merck
Tris (hydroxymethylene) aminomethane	99.5	Merck
Sulphosalicylic acid	99.5	Merck
Ascorbic acid	99.0	Biochemical

Chemicals	Purity %	Supplied Company
Glutathione	99.5	Biochemical
5,5`-Dithio bis (2-nitrobenzoic acid (DTNB)	99.5	Sigma chemical
Acrylamide	99.5	Sigma chemical
Methylene bis acrylamide (Bis)	99.5	Sigma chemical
TEMED	99.5	Sigma chemical
Glacial acetic acid	99.7	Chem. Supply
Ammonium persulphate	99.0	BDH
2,4-Dinitrophenyl hydrazine	99.0	BDH
Sodium azide	99.0	BDH
Glycine	99.5	Riedel-de Haën
Cytidine	99.5	Schuchart Munchen
Trichloroacetic acid	99.0	Hopkin and Williams
Standard solution of elements		Aldrich
Total protein kit		Randox
Albumin kit		Randox

2.1.2 Instrumental Analysis and Equipment

Instrument	Supplied Company
pH meter	Jenway (Germany)
Sensitive blance	Stanton 461 AN (Germany)
Vortex mixer	Karlkole (Germany)

Instrument	Supplied Company
Water Bath	Karlkole (Germany)
Shaker water bath	Tecam (England)
Oven	Hearson (England)
Magnetic stirrer	Gallin Kamp (England)
Spectrophotometer type 21	Molton Roy (Switzerland)
Electrophoresis	LKB 2117 Multiphor (Sweden)
Centrifuge	Heraeus (Germany)
Flame atomic absorption spectrophotometer and Flameless Graphic furnace.	Shimadzu 24AA-670/GU-7
Filter paper	Whatman No.1 (England)
Centrifuge tube	Mes (1.0 gm) Fisonsm.(England)
Plane tube	Afma-Dispo-(Jordan)
Micropiptte 20µl	Gilson (France)
Micropiptte 100-1000µl	Gilson (France)
Micropiptte 100µl	Oxford Sampler Micropipetting system (USA)
Micropiptte 40-200µl	Oxford Sampler Micropipetting system (USA)
Micropiptte 50µl	Nichiryo (Japan)
Micropiptte 500µl	Nichiryo (Japan)
Micropiptte 1000µl	Socorex (Swiss)
Disposable Syringe	Becton Dickinson (Spain)

2.1Methodologies

2.2.1 Collection of Blood and Serum preparation

The vein on the front of the elbow or forearm is almost employed. The arm should be warm to improve the circulation and distend the vein. The arm is extended and a tourniquet is firmly applied (10-15 cm) above the elbow. The skin over the vein will be sterilized with a small pad of cotton wool soaked with hapten. A disposable sterile needle fixed on to a disposable syringe is inserted into the vein. When the needle enters the vein the plunger of the syringe is slightly withdrawn. If blood appears, the tourniquet is released. When 5ml of blood has been drawn into the syringe, small pad of cotton wool soaked with hapten is placed on the arm where the needle was inserted and the needle is withdrawn. This pad is firmly pressed on until the bleeding stops. The needle is removed from the syringe and the blood slowly transferred to plain tube without anticoagulant. The blood is allowed to clot for 15 minutes, the clot shrinks and serum can be obtained by centrifuging for approximately 10 minutes at a relative centrifugal force (RCF) of 1000 x g to 2000 x g. [5,11,13]

2.2.2 Patients and Controls

Twenty one patients (13 males, 8 females) with Hodgkin's disease (HD), twenty three patients (13 males, 10 females) with non Hodgkin's lymphoma (NHL), thirty eight females with breast cancer, ten patients (5 males, 5 females) with acute lymphoblastic leukemia (ALL), eight males with bladder cancer, four females with acute myeloid leukemia (AML), three females with placenta cancer, three females with ovary cancer, three males with colon cancer, and two males with liver cancer admitted to Oncology Unit in Merjian Teaching hospital in Hilla city, have been subjected to present study, as well as fifty two apparently healthy individuals as a control (27 males, 25 females), after having been asked about their health.

2.2.3 Preliminary Experiments to Determine the Optimum CDA Activity

CDA activities in sera of healthy controls and patients were measured using direct spectrophotometric assay as described by (Cohen and Wolfenden, 1971),[20] based on the loss of absorbance when cytidine is converted to uridine, at 282 nm where $\Delta\varepsilon$ - 3600 for a 1cm light path with quartz cuvettes at pH 7.5 . The enzymatic reaction was terminated by the addition of 4 ml of 0.1M HCl.

The standard assay solution contained serum, cytidine (0.002M) and Tris-HCl buffer (0.05M, pH 7.5) in a final volume of 1 ml. One unit of activity was defined as the amount of enzyme that required to deaminate 1µmole of cytidine per minute in the stanedard assay solution at $25C^{\circ}$

2.2.3.1 Relationship Between Protein Content and Enzyme Activity

Sequent experiments have been done using different quantities of serum (20-200 µL) to determine the optimum amount of serum (which contains the enzyme), that gives the optimum enzyme activity. The reaction was carried out under standard conditions except for the amount of serum.

2.2.3.2 Relationship Between Incubation Period and Enzyme Activity

With each experiment described previously, the reaction mixture was stopped at different times (0.5-30 minutes) to determine the optimum period of incubation, which gives the maximum change in absorption at 282 nm as a function of enzyme activity.

2.2.3.3 Relationship Between pH and Enzyme Activity

A series of experiments has been done, using different buffer pH (6-9) to determine the optimum buffer pH, in which the enzymatic reaction gives the optimum enzyme activity.

2.2.3.4 Relationship Between Buffer Concentration and Enzyme Activity

Five various concentrations of Tris-HCl buffer pH 7.5 (0.01, 0.02, 0.05, 0.1, and 0.2 M) were examined to evaluate the optimum concentration of buffer solution, in which the enzyme gives the optimum activity.

2.2.3.5 Relationship Between Temperature and Enzyme Activity

Each enzyme functions optimally at a particular temperature, therefore, a sequence of experiments has been done to determine the optimum temperature of enzymatic reaction, by using various temperatures of incubation.

2.2.3.2 Relationship Between Substrate Concentration and Enzyme Activity

A sequent of experiments was conducted, using different substrate concentrations, to determine the optimum substrate concentration, which gives the maximum velocity of enzyme.

2.2.3.7 Preparation of Reagents

1- Tris-HCl buffer (0.05M, pH 7.5)

A-Hydrochloride acid (HCl) 0.2M

7.789 ml of concentrated HCl (sp.gr. 1.19, 32%) are added to 200 ml of double distilled water (DDW) and brought to a final volume of 250 ml.

B-Tris (hydroxymethyl) aminomethane (Tris) 0.2M

6.0572 gm of Tris are dissolved in a final volume of 250 ml of DDW.

207 ml of 0.2M HCl are added to 250 ml of 0.2M of Tris solution and diluted to 1 liter with DDW. pH is adjusted with 1 M HCl, then diluted to 0.05M with DDW.

2- HCl (0.1M)

9.57 ml of concentrated HCl (sp.gr. 1.19, 32%) are added to 900 ml of DDW and brought to a final volume of 1 liter.

3- Cytidine (0.002M)

0.0486 gm of cytidine is dissolved in a final volume of 100 ml of DDW.

2.2.3.8 Procedure

The procedure for determination of CDA activity is summarized as follows:
Serum is preincubated for 5 minutes at 25C°. Duplicates of each (sample) test tubes are prepared, then pipette into test tubes.

Reagents	Sample µL	Control µL	Sample Blank µL
Tris-HCl buffer	500	500	500
Cytidine	400	400	
DDW			400
Serum	100		100

Tubes are mixed, and incubated for 5 min at 25C° in shaker water bath.

Reagents	Sample µL	Control µL	Sample Blank µL
HCl	4000	4000	4000
Serum		100	

Spectrophotometer is adjusted zero at 282 nm with DDW. The absorption of sample (A_S), control (A_C), and sample blank (A_{SB}) is read.

2.2.3.9 Calculation of Enzyme Activity

There is a great variety of ways in which units of enzyme activity have been expressed. The recommendation of the Enzyme Commission of the International Union of Biochemistry and the International Federation of Clinical Chemistry was that all activities should be expressed in term of international units (IU) on such unit being the activity which transforms one micromole of substrate per minute under defined condition. The enzyme concentration was conveniently expressed as units per liter (U/L).[5,13,16]

$$\text{Enzyme activity U/L } (\mu \text{ mole/min/L}) = \frac{\Delta A/\text{min}}{\varepsilon \times b} \times \frac{V_t}{V_s} \times 10^6$$

where:

$\Delta A/\min$ = average absorbance change per minute.
V_t = total assay volume in ml.
V_s = volume of serum specimen in ml.
ε = molar absorptivity of substrate or product .
b = light bath.
10^6 = factor to convert mole to micromole.

$$\text{CDA activity (U/L)} = \frac{\Delta A/5}{6.0 \times 10^3 \times 1} \times \frac{5}{0.1} \times 10^6$$

$$= \Delta A \times 1666.6$$

where:

$\Delta A = (A_C - A_{SB}) - (A_S - A_{SB})$ = change in absorption at 282 nm
Incubation time = 5 minutes
Molar absorptivity of cytidine [33] = $6.0 \times 10^3\ cm^{-1}M^{-1}$.
Total assay volume = 5 ml.
Volume of serum specimen = 0.1 ml.
Light path = 1 cm.

2.2.4 Determination of Vitamin C (Ascorbic Acid)

Numerous analytical methods are available for assessment of ascorbic acid nutritional status. Chemical methods depend on either the reducing properties of the 1,2-enediol group that leads to absorbance changes in indicator dyes, or formation of hydrozones. Other methods, including fluorometric and HPLC techniques, have been developed. In most methods, protein is precipitated with metaphosphoric or perchloric acid before analysis.[11,19]

2.2.4.1 Determination of Serum Total Vitamin C (Ascorbic Acid)

2.2.4.1.1 Principle

In the 2,4-dinitrophenylhydrazine (DNPH) methods, AA is oxidized by Cu^{+2} to DHA and diketogulonic acid.[11] When treated with DNPH, the 2,4-dehydrophenylosazon product forms, which, in the presence of sulfuric acid, forms an orange-red complex that absorbs at 520 nm as shown in (figure 2-1).

2.2.4.1.2 Preparation of Reagents

1- Metaphosphoric acid (m-HPO_3) (0.75M)

30gm of m-HPO_3 are dissolved in a final volume of 500 ml of DDW. (Stable for 1 week).

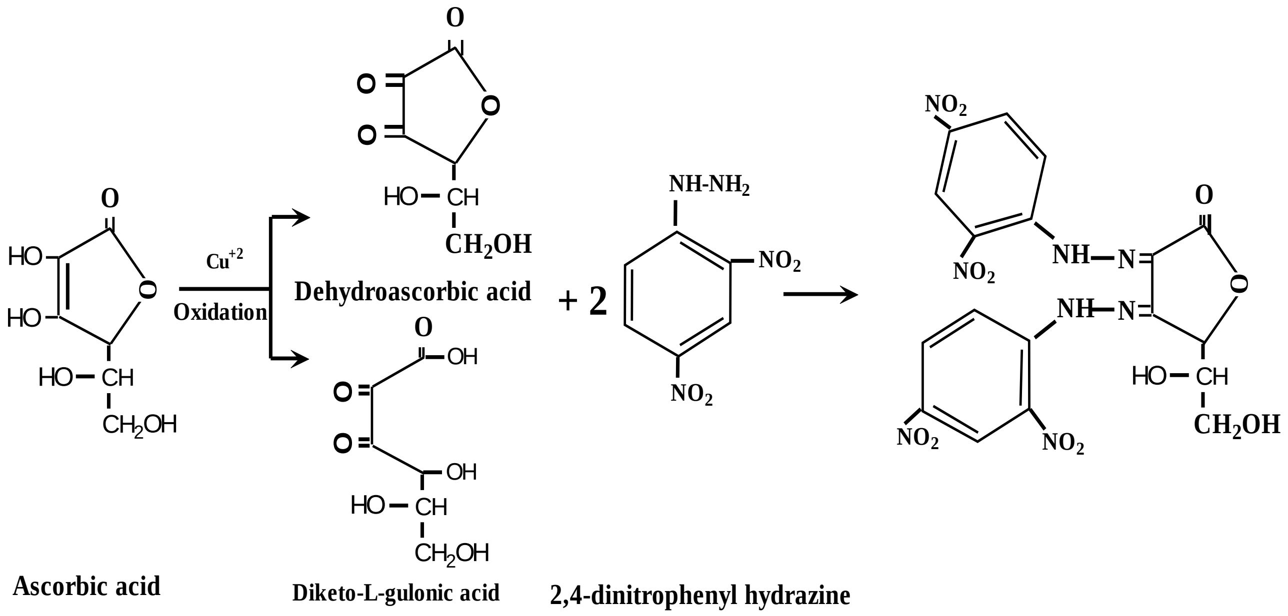


Figure 2-1 Reaction Between Ascorbic Acid and 2,4-DNPH (Figure taken from [189])

2- Sulfuric acid H_2SO_4 (4.5M)

Carefully 250 ml of concentrated H_2SO_4 are added to 500 ml of cold DDW. When the solution has cooled to room temperature, DDW is added to 1 liter, with mixing. (Stable for 2 years).

3- Sulfuric acid H_2SO_4 (12M)

Carefully 650 ml of concentrated H_2SO_4 are added to 300 ml of cold DDW and brings to a final volume of 1 liter (Stable for 2 years).

4- 2,4-DNPH reagent (0.01M)

10 gm of 2,4-DNPH are dissolved in 400 ml of 4.5M H_2SO_4 and bring to a final volume of 500 ml with 4.5M H_2SO_4, then refrigerated overnight, and filtered. (Stable for at least 1 week at refrigerated temperature).

5- Thiourea (0.66M)

5 gm of thiourea are dissolved in a final volume of 100 ml of DDW. (Stable for 1 month at 4C°).

6- Copper sulfate (0.027M)

0.6 gm of anhydrous copper sulfate is dissolved in a final volume of 100 ml of DDW. (Stable for 1 year at room temperature).

7- DTCS reagent

5 ml of the thiourea, 5 ml of the copper sulfate, and 100 ml of the 2,4-DNPH reagent are combined. (Store in bottle at 4C° for a maximum of 1 week).

8- Ascorbic acid standards

Stock standard solution (2.8 mM) is prepared by dissolving 50 mg of ascorbic acid in a final volume of 100 ml of m-HPO_3. Dilutions are made in m-HPO_3 to 2.5, 5 and 20 mg/L (0.014, 0.028, and 0.11 mM) respectively. There are the working standards (All working standards should be prepared daily).

2.2.4.1.3 Procedure

The procedure for the determination of total vitamin C in serum by 2,4-DNPH method is summarized as follows:
Duplicates of each standard and sample test tubes are prepared, then pipetted into test tubes.

Reagents	Sample(µL)	Reagent Blank(µL)	Standard(µL)
m-HPO_3	800		
Serum	200		

Tubes are mixed in vortex mixture, then centrifuged at 2500 x g for 10 minutes

Reagents	Sample(µL)	Reagent Blank(µL)	Standard(µL)
Supernatant	600		
Standards			600
m-HPO_3		600	
DTCS reagent	200	200	200

Tubes are capped and mixed in vortex mixture, then incubated in a water bath at 37C° for 3 hours.
The tubes are removed from the water bath and chilled for 10 minutes in an ice bath, with slowly mixed.

Reagents	Sample(µL)	Reagent Blank(µL)	Standard(µL)
Cold H_2SO_4 (12M)	1000	1000	1000

Tubes are mixed in vortex mixture and returned immediate to the ice bath. The spectrophotometer is adjusted with blank to read zero absorbance (A) at 520 nm, and the absorbance of standards and sample is read.

2.2.4.1.4 Calculation of Serum Total Vitamin C

The concentration of the samples is obtained from the calibration curve (Figure 2-2) and is multiplied by 5 (to correct for dilution of the serum by m- HPO_3) to give the concentration of vitamin C (ascorbic acid) per liter of serum.

The concentration of ascorbic acid can be determined directly from a standard as fellows:

$$\frac{A_{\text{sample}}}{C_{\text{sample}}} = \frac{A_{\text{std.}}}{C_{\text{std.}}}$$

$$C_{\text{sample}} = \frac{C\ \text{std.} \times A_{\text{sample}} \times 5}{A_{\text{std.}}}$$

where:

C = concentration (mg/L) or (µM) of AA in sample and standard.
A = absorbance at 520 nm for sample and standard.
5 = factor is added to correct for the dilution of sample.

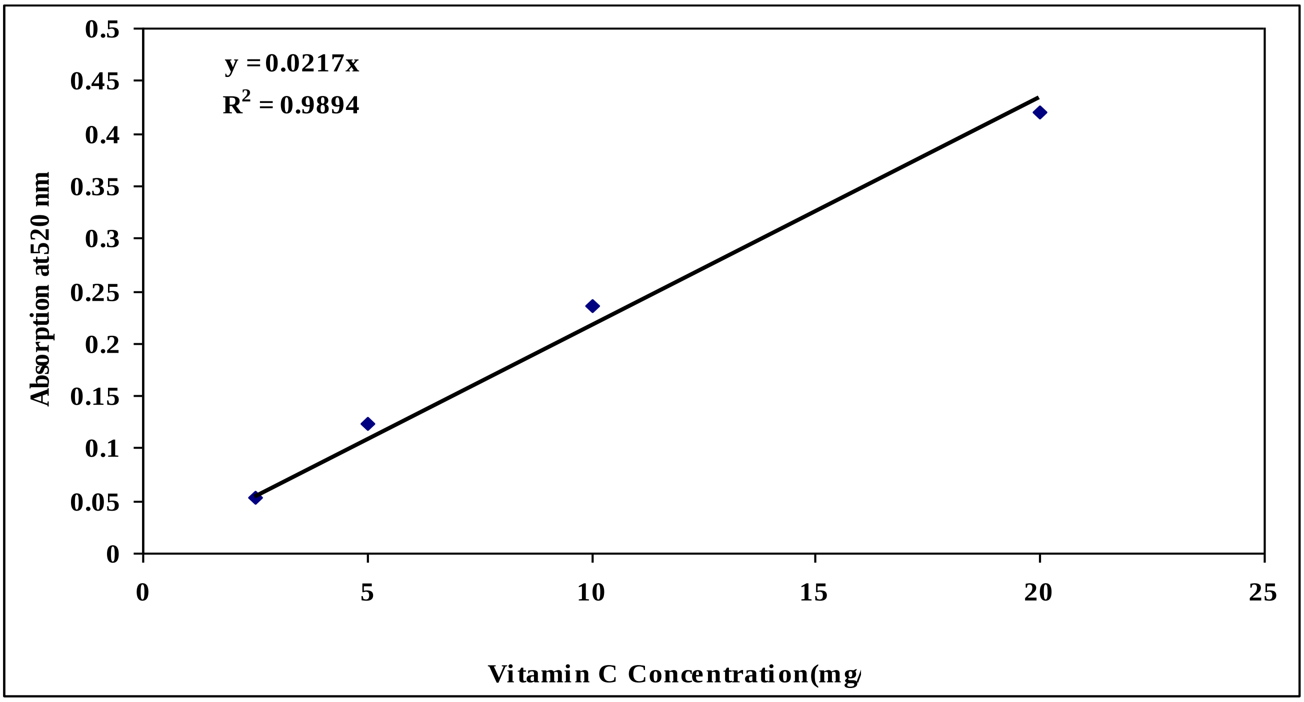


Figure 2-2 Standard Curve for 2,4-DNPH method for ascorbic acid.

2.2.4.2 Determination of Serum Reduced Vitamin C (Ascorbic Acid)

2.2.4.2.1 Principle

The reducing properties of ascorbic acid are utilized in the 2,6-dichlorophenolindophenol (2,6-DCIP) method.[19] AA causes a reduction in the dye from the oxidized to the colorless leuco form. The change in color can be monitored photometrically at 505 or 520 nm or titrametrically. 2,6-DCIP is blue compound, being red in acid solution and on titration with a solution of ascorbic acid is reduced to the colorless leuco base, the AA being oxidized to DHA. It is thus seen that if a mixture of AA and DHA is present, only the former reacts with the dye,[13] as shown in (Figure 2-3).

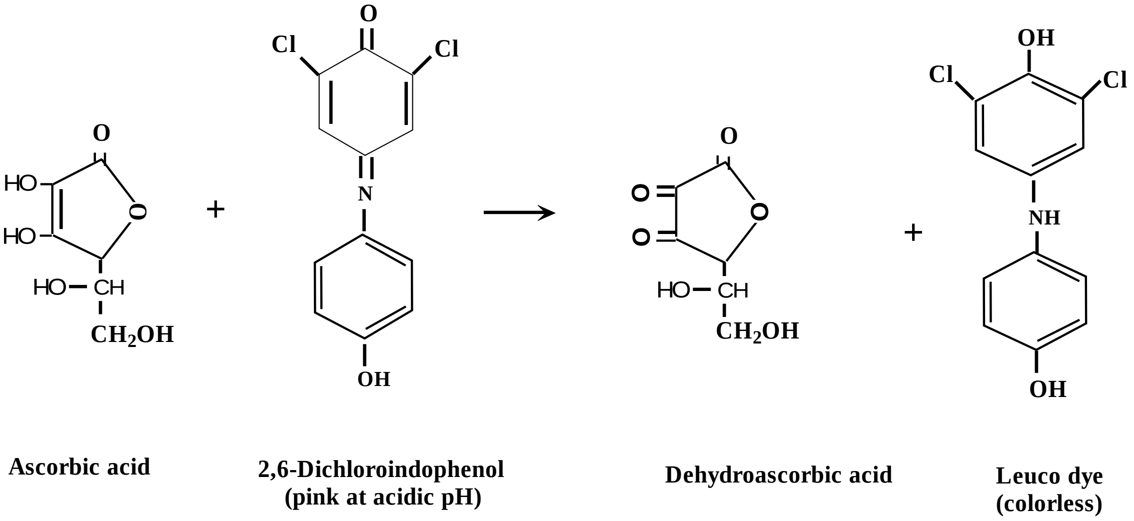


Figure 2-3 Reaction between Ascorbic Acid and 2,6-DCIP (Figure taken from [189])

2.2.4.2.2 Calculation of Serum Reduced Vitamin C (Ascorbic Acid)

In the present study, reduced AA was calculated from a correlation study between the DNPH and DCIP methods conducted on 611 patient sample, and showed good agreement.[189]

DNPH = 1.088 (DCIP) + 0.12 (r = 0.95)

Therefore,

$$\text{Reduced Ascorbic Acid} = \frac{\text{Total ascorbic acid (DNPH method)} - 0.12}{1.088}$$

2.2.5 Determination of Serum Reduced Glutathione (GSH)

All analytical methods such as, photometric enzymatic,[11,190,191] flourometric, and HPLC methods,[183] that used to determine tissues homogenate, erythrocyte, and serum glutathione (GSH) depend on the action of sulfhydryl groups.

2.2.5.1 Principle

5,5´-Dithiobis (2-nitrobenzoic acid) (DTNB) is a disulfide chromogen that is readily reduced by sulfhydryl group of GSH to an intensely yellow compound. The absorbance of the reduced chromogen is measured at 412nm and is directly proportional to the GSH concentration[11](Figure2-4).

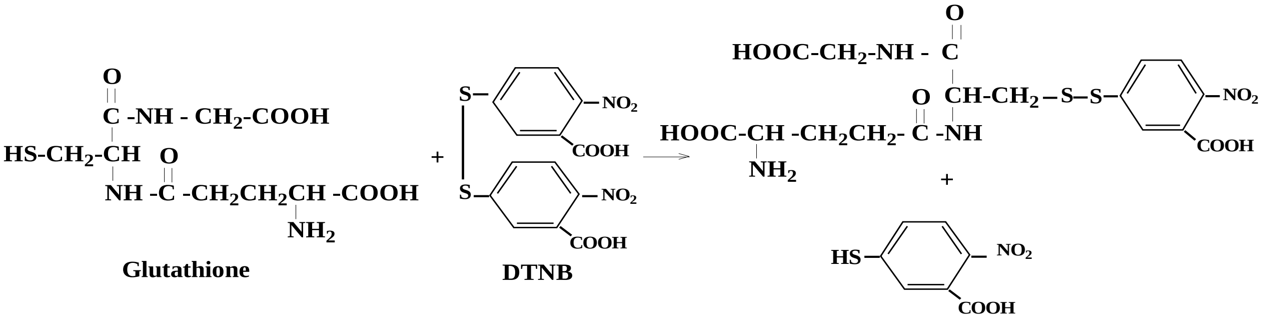


Figure 2-4 Reaction between GSH and DTNB (Figure taken from [190,191])

2.2.5.2 Preparation of reagents

1- Precipitating solution. Trichloroacetic acid (TCA) 50%

50gm of TCA are dissolved in a final volume of 100ml of DDW.

2- Ethylenediamine tetracetic acid-di sodium (EDTA Na_2) (0.4M)

148.9gm of EDTA are dissolved in a final volume of 1liter of DDW.

3- Tris-EDTA buffer (0.4M) pH 8.9

48.458gm of Tris are dissolved in 800ml of DDW. 100ml of (0.4M) EDTA solution are added and bring to a final volume of 1liter with DDW. The pH was adjusted to 8.9 by the addition of 1M of HCl. (Stable for at least 10 days).

4- DTNB reagent (0.01M)

0.099gm of DTNB is dissolved in absolute methanol, and bring to a final volume of 25ml. (This reagent is stable for at least 13 weeks at 4C°).

5- GSH standards

Stock standard solution (0.001M) is prepared by dissolving 0.0307gm of GSH in a final volume of 100ml of (0.4M) EDTA solution. Dilutions are made in EDTA solution to 2, 5, 10, 15, 20, 30, 40 and 50μM. (This working standard solution should be prepared daily).

2.2.5.3 Procedure

Serum GSH was determined by using a modified procedure utilizing Ellman's reagent (DTNB), which is summarized as follows:

Duplicates of each standard and sample test tubes are prepared then pipetted into test tubes.

Reagents	Sample µL	Reagent Blank µL	Standard µL
Serum	100		
Standard			100
DDW	800	900	800
TCA	100	100	100

Tubes are mixed in vortex mixture intermittently for 10-15 minutes, and centrifuged for 15 minutes at 3000 x g, then pipetted into test tubes.

Reagents	Sample µL	Reagent Blank µL	Standard µL
Supernatant	400	400	400
Tris-EDTA buffer	800	800	800
DTNB reagent	20	20	20

Tubes are mixed in vortex mixture. The spectrophotometer is adjusted with reagent blank to read zero absorbance (A) at 412nm, and the absorbance of standards and sample is read within 5 minutes of the addition of DTNB.

2.2.5.4 Calculation of Serum GSH

The concentration of serum GSH is obtained from the calibration curve in µM. (Figure 2-5).

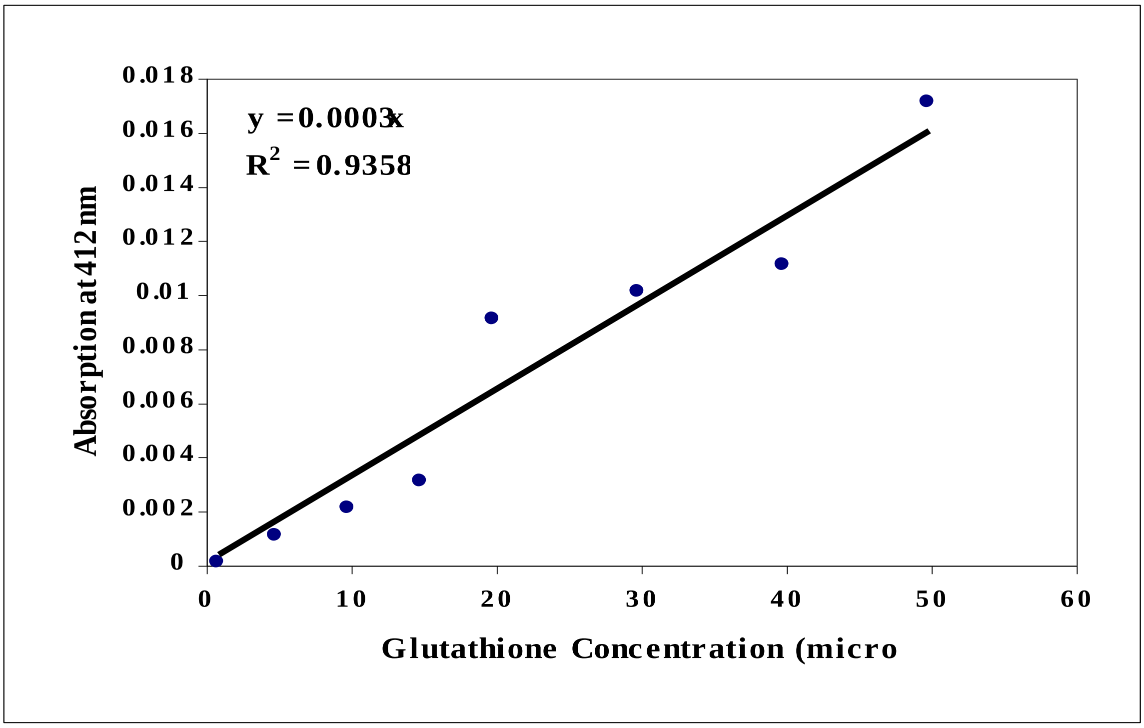


Figure 2-5 Standard Curve of Glutathione Concentration.

2.2.6 Determination of Total Serum Protein

When the total protein concentration of biological fluids such as serum and urine is measured, two assumptions are arbitrarily made: (1) all protein molecules are pure polypeptide chains, containing on the average 16 % by weight of nitrogen; and (2) each of the several hundred individual proteins present in serum reacts chemically like every other protein. Clearly, the first assumption is not true at all, and the second cannot always be true. Nevertheless, these simplifying assumptions make measurement of total protein a practical, although empirical, procedure.[5,11]

Many methods have been developed to measure the total protein content of biological fluids, such as, biuret, ultraviolet absorption, dye binding, Folin-Ciocalteu (Lowry), Kjeldahl, salt precipitation, electrophoresis, refractometry, turbidimetric, and nephelometric methods.[5,11]

In the present study, the total serum protein was measured by using commercially available kits (Randox Laboratories Ltd., UK) based on biuret method. [5,11,13,16]

2.2.6.1 Principle

Peptide bonds of protein react with cupric ion (Cu^{2+}) in alkaline solution to form a colored product whose absorbance is measured spectrophotometrically at 540 nm. In an alkaline medium and in the presence of at least two peptide bonds, a violet-colored chelate is formed. The intensity of color produced is proportional to the number of peptide bonds. The reagent also contains sodium potassium tartrate to complex cupric ions to prevent their precipitation in the alkaline solution, and potassium iodide, which acts as an antioxidant.[5,11,13]

In addition to the (NHCO) group that occurs in the peptide bond, cupric ions will react with any compound that has two or more of the following groups: (NH_2CH_2), (NH_2-C-), and (NHCS). The method was given its name because a substance called biuret ($NH_2CONHCONH_2$) reacted with cupric ions in the same manner[5].

One Cu^{2+} is probably linked to six nearby peptide linkages by coordinate bond,[11] or four molecules of biuret, the linkage being to the central nitrogen atom,[13] as shown in (Figure 2-6).

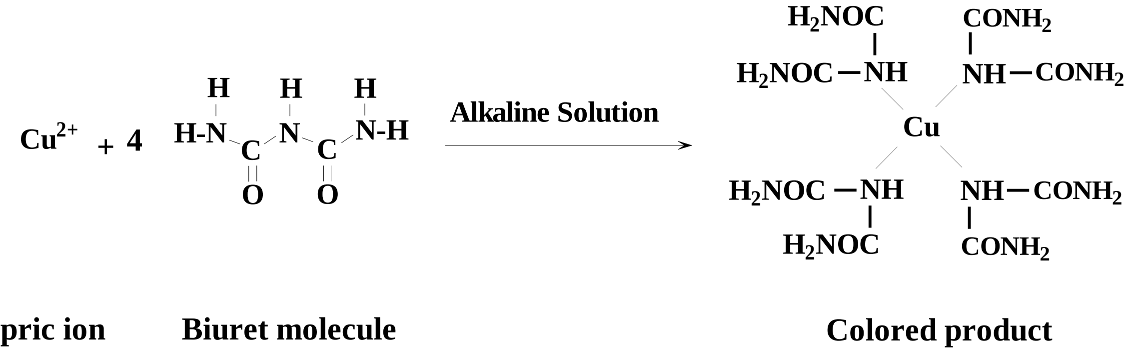


Figure 2-6 Reaction between Biuret Molecules (Figure taken from [13])

2.2.6.2 Reagents

The commercially (Randox Laboratories Ltd., UK) kit consists of the following:

1- Biuret reagent

A- Sodium hydroxide NaOH (100mM)
B- Na-K-tartrate (16mM)
C- Potassium iodide KI (15mM)
D- Cupric sulfate $CuSO_4$ (6mM)

2- Blank reagent

A- Sodium hydroxide NaOH (100mM)
B- Na-K-tartrate (16mM)

3- Standard

Human serum albumin 60g/L (6.0g/dL)

2.2.6.3 Preparation of Solutions

1- Biuret Reagent

The contents of bottle 1 are diluted with 400ml of DDW. (Stable for one year when sealed and stored at +15-25C°).

2-Blank Reagent

The contents of bottle 2 are diluted with 400ml of DDW.(Stable for one year when sealed and stored at +15-25C°).

3-Standard

Ready for use. (Stable up to expiry date when stored at 15-25C°).

2.2.6.4 Procedure

Total serum protein was determined by using biuret method, which is summarized as follows:

A duplicate of each standard and sample test tubes is prepared, then pipetted into test tubes.

Reagents	Sample µL	Standard µL	Reagent Blank µL
Serum	20		
Standard		20	
Blank reagent			20
Biuret reagent	1000	1000	1000

Tubes are mixed and incubated for 30 minutes at 20-25C°. The spectrophotometer is adjusted with reagent blank to read zero A at 540nm, then the absorbance of standard and sample is read.

2.2.6.5 Calculation of Total Serum Protein

Total serum protein in the sample was calculated from the following formula:

$$\text{Total Serum Protein Concentration (gm/dL)} = \frac{A_{\text{sample}} \times 6}{A_{\text{std.}}}$$

where:

A_{sample} = Absorbance of serum sample.
A_{std}. = absorbance of standard.
6 = Concentration of standard in (gm/dL)

2.2.7 Determination of Serum Albumin

The determination of albumin in serum or plasma is usually based on the binding behavior of the protein with anionic dyes such as, bromocresol green (BCG), 2,4-hydroxy-azobenzene-benzoic acid (HABA), and bromocresol purple (BCP) in a manual or automated procedure. Electrophoretic and immunochemical methods are also available for the analysis of albumin.[11]

In the present study, serum albumin was measured by the use of commercially available kits (Randox Laboratories Ltd., UK), based on bromocresol green method.[192]

2.2.7.1 Principle

The measurement of serum albumin is based on its quantitative binding to the indicator 3,3`, 5, 5`-tetrabromo-m-cresol sulphonphthalin (bromocresol green, BCG)(Figure 2-7). The absorption of BCG-albumin complex is determined spectrophotometrically at 628nm. At pH 4.2, albumin acts as a cation to bind the anionic dye.[11]

The binding of BCG to albumin is not wholly specific; some dye binds also to α_1-and α_2-glubulins, although more slowly than with albumin.[11,13]

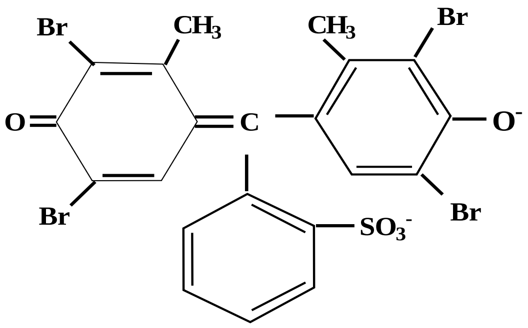


Figure 2-7 Structure of Bromocresol Green (Figure taken from [13])

2.2.7.2 Reagents

The commercially (Randox Laboratories Ltd., UK) kit consist of the following:

1- BCG concentrate

A- Succinate buffer (75mM; pH 4.2)
B- Bromocresol green (0.15mM)
C- Brij 35
D-Preservative

2-Standard

Human serum albumin 45gm/L (4.5gm/dL)

2.2.7.3 Preparation of Solution

1-BCG concentrate

The contents of one bottle are diluted, which contain 35ml of concentrate with 215ml of DDW. (Stable for 3 months at 15-25C°).

2-Standard

Contents ready for use. (Stable up to expiry date when stared at 15-25C°).

2.2.7.4 Procedure

Serum albumin was determined by using BCG method that is summarized as follows.

Duplicates of each standard and sample test tubes is prepared, then pipetted into test tubes.

Reagents	Sample µL	Standard µL	Reagent Blank µL
Serum	10		
Standard		10	
DDW			10
BCG reagent	3000	3000	3000

Tubes are mixed and incubated for 5 minutes at 20–25C°. The spectrophotometer is adjusted with reagent blank to read zero A at 630 nm, then the absorbance of standard and sample is read.

2.2.7.5 Calculation of Serum Albumin

The albumin concentration in the sample was calculated from the following formula:

$$\text{Albumin Concentration (gm/dL)} = \frac{A_{\text{sample}} \times 4.5}{A_{\text{std.}}}$$

where:

A_{sample} = Absorbance of sample.
$A_{\text{std.}}$ = absorbance of standard.
4.5 = Concentration of standard in (gm/dL)

2.2.8 Determination of Serum Albumin/Globulins (A/G) ratio.

To determine the albumin/globulins (A/G) ratio, it is common to determine total protein and albumin.

Globulins are calculated by subtracting the albumin from the total protein.
Total serum protein - serum albumin = serum globulins.[5,11,13,16]

2.2.9 Determination of abnormal Serum Protein by Use Polyacrylamide Gel Electrophoresis (PAGE)

The separation of serum proteins on polyacrylamide, cellulose acetate, and agrose gel is almost always performed on serum to avoid the complication of a fibrinogen band.[11]

In the present study, the separation of serum proteins was conducted by using conventional 3.5% and 5ml slots PAGE with (LKB 2117 multiphor, Sweden), to avoid the precipitation of some proteins of the gel surface in the slot. [193]

2.2.9.1 Principle

Electrophoresis is the name given to the movement of charged particles through an electrolyte subject to an electric field. If these are differently charged, they will move in the opposite direction.[13]

The rate of migration of particles of like charge will depend among other things on the number of charges each carries.[13] Polyacrylamide gel (PAG) can be prepared with different pore sizes by changing the concentration of cross-linking agents. Electrophoresis through PAG leads to enhanced resolution of sample component because the separation is based on both molecular sieving and electrophoretic mobility.[11,13,19]

PAG are prepared by the free radical polymerization of acrylamide and the cross-linking agent N,N`-methyline-bis acrylamide (Bis). Chemical polymerization is controlled by an initiator-catalyst system, ammonium persulfate-N,N,N,N`-tetramethylethylene diamine (TEMED). Figure (2-8)

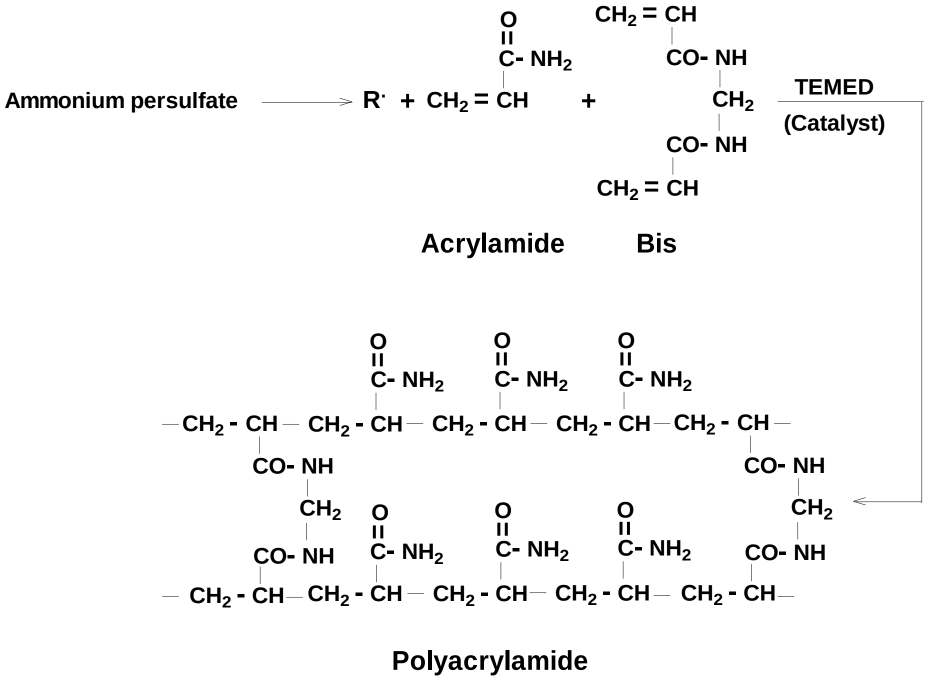


Figure 2-8 Chemical Reaction Illustrating the Copolymerization of Acrylamide and N, N`-methylene - bis – acrylamide (Figure taken from [19])

2.2.9.2 Preparation of Solutions

1- Tris-glycine buffer stock solution 0.2M (pH 8.9)

75.1gm of glycine and 2.5gm of sodium azide are dissolved in approximately 3L of DDW, and titrated with Tris to adjust pH at 8.9. The volume is made up to 5L with DDW and the pH is checked after making up.

2- Electrode buffer (0.1M)

One buffer stock solution is diluted with one part of DDW.

3- Sample buffer (0.01M)

5ml of buffer stock solution are pipetted and made up to 100ml with DDW.

4- Acrylamide solution

22.2gm of acrylamide, specially purified for electrophoresis and 0.6gm of Bis are dissolved in a final volume of 100ml of DDW. The solution is filtered through a Whatman No.1 filter paper if it appears cloudy. (Store in a dark bottle at 4C°).

5- Ammonium persulphate solution

0.15gm ammonium persulphate is dissolved in a final volume of 10ml of DDW. (Store in a dark bottle at 4C°).

6- Bromophenol blue 0.25% (w/v)

0.025gm of bromophenol blue is dissolved in a final volume of 10ml of sample buffer.

7- Detergent solution

0.1ml of Triton X-100 is diluted to a final volume of 100ml of DDW.

8- Fixing solution

57gm of trichloroacetic acid (TCA) and 17gm of sulphosalicylic acid are dissolved in a mixture of 150ml of methanol and 350ml of DDW.

9- Staining solution

1.25gm of Coomassie brilliant blue R-250 is dissolved in the solution of 227ml of methanol and 227ml of DDW. 46ml of glacial acetic acid are added to the mixture. Insoluble material is removed by filtration through a Whatman No.1 filter paper. (Store in a dark bottle).

10- Destaining solution

500ml of acetic acid are added to 1500ml of ethanol, and bring to a final volume of 5 L with DDW.

11- Preserving solution

300ml of ethanol, 100ml of acetic acid, and 100ml of glycerol are mixed and bring to a final volume of 1 L with DDW.

The solutions should be used within one month, except for the ammonium persulphate solution, which is made up freshly every week. Those solutions with a specific pH value should be checked with a pH-meter.[193]

2.2.9. 3 Procedure

2.2.9. 3.1 Preparation of the Gel

The set for gel moulding is mounted as follows: Slot former, rubber gasket, glass plate 1mm and glass plate 3mm respectively. The rubber gasket should be straight on the long sides and the clamps should be putted.

19.3ml of DDW with 33ml of buffer stock solution and 10.4ml of acrylamide solution are mixed in a vacant flask, and deaerated for 5 minutes. 3.2ml of ammonium persulphate solution and 0.1ml of TEMED are added, and mixed carefully without introducing too much air. Immediately the solution is poured into the moulding set with the help of a plastic funnel with a pressed tip. The last few milliliters are added carefully, using a Pasteur pipette. Air bubbles are removed by taping on the gel moulding. The rubber gasket is closed without trapping air. The polymerization reaction will be complete in 40 minutes. After polymerization, the cassette is putted without clamps in a refrigerator for 15 minutes, to facilitate the removal of the slot former. The gasket is removed very carefully, especially when loosening the edge of the gel from the slot former. The gel is stored at room temperature. It is recommended that the gel be used not before 12 hours.[193]

2.2.9.3.2 Preparation of sample solution

Serum is diluted 1: 3 with 0.01M of Tris-glycine sample buffer. For detection of the front, 10µL of 0.25% bromophenol blue is added to each 250 µL of sample solution. [193]

2.2.9.3.3 Samples

Two electrophoresis assays have been done by using PAGE.

1- The sequence of sera sample on the gel slots of the first assay is shown in Table 2-1.

Table 2-1 The Slots Number and the Diagnosis of Patient and Their Sex for the First Assay.

No. of slot	Diagnosis of patient	Sex
1,2	NHL	M
3,4	HD	F
5,6	Breast cancer	F
7,8	ALL	M
9,10	Placenta cancer	F
11,12	Bladder cancer	M
13,14	Ovary cancer	F
15,16	Liver cancer	M
17,18	Healthy control	M

2- The sequence of sera samples on the gel slots of the second assay is shown in (Table 2-2).

Table 2-2 The Slots Number and the Diagnosis of Patient and Their Sex for the Second Assay.

No. of slot	Diagnosis of patient	Sex
1	HD	M
2	HD	M
3	HD	F
4	HD	F
5	NHL	M
6	NHL	M
7	NHL	F
8	NHL	F
9	Breast cancer	F
10	Breast cancer	F
11	Breast cancer	F
12	Breast cancer	F
13	ALL	M
14	ALL	M
15	ALL	F
16	ALL	F
17	Placenta cancer	F
18	Placenta cancer	F
19	Healthy control	M

2.2.9.3.4 Pre-electrophoresis

To make it possible to detect the marker dye front during electrophoresis, a white paper is placed under the cooling plate. A line is drawn on the cooling plate with water proof ink 16 mm from the edge of each long side. Each of the buffer tanks of Multiphor are filled with 1.2 L of electrode buffer.

A few milliliters of detergent solution are applied to the cooling plate to give a good thermal contact with the gel plate. The gel plate is placed on the cooling plate without trapping air. 8-10 layers of electrode wicks are soaked with buffer. The wicks should be exactly parallel. The edge of the wet wicks is applied along the lines on the cooling plate. They should overlap the gel by 10-12 mm, then the cooling water bath is switch on at 10°C. Pre-electrophoresis should be performed with a preset constant current 50 mA for 30 minutes. [193]

2.2.9.3.5 Sample Application

The slots are filled up using micro syringe with a short needle, with 5 µL of each sample. Filling should be done as quickly as possible to prevent the diffusing of bromophenol blue dye.[193]

2.2.9.3.6 Electrophoresis

The power supply immediately switched on after the samples have been applied. The current is adjusted to 20mA for 5-10 minutes, to concentrate the samples at the anode side of the slot. The field strength is increased after the concentration step to be 15 Vcm^{-1} and the current is 45mA for 2 hours.

The run should be performed with constant current; therefore, it is interesting to measure the field strength during the run with LKB voltage probe connected to D.C. voltage meter. The value read should be divided by 4 to obtain Vcm^{-1}. If the slot dry out during the run, they should be filled up with dilute stock solution (1: 1).[193]

2.2.9.3.7 Fixation

To avoid the loss of small amount of protein and to minimize diffusion, the gel should be fixed in fixing solution immediately after electrophoresis, for 30 minutes at room temperature. [193]

2.2.9.3.8 Staining

The gel is left in the staining solution for approximately one hour. The adhesiveness of the gel to the glass plate reduces during staining. [193]

2.2.9.3.9 Destaining

Excess dye is removed by diffusion in destaining solution. The more frequent the changes of destainer, the faster will destaining take place. The gels show no dye back round after destaining for one day. [193]

2.2.9.3.10 Preserving

To make it possible to dry the gel without cracking, it must be soaked for one hour in preserving solution. Gel storing should be on glass plate or on a foil. Drying should be performed at room temperature. [193]

2.2.10 Determination of Serum Zinc, Selenium, Copper, and Iron

Zinc has been determined colorimetrically using dithiozone and flourimetrically.[13] The method of choice now is atomic absorption spectrophotometer (AAS).[11]

Selenium has been determined spectroflourometically and by using neutron activation analysis (NAA) and AAS. Recommended methods for determining selenium in biological specimens are flameless ASS.[11]

A variety of color reagents have been used to determine serum copper, such as sodium diethyl dithiocarbamate, dithizone,[13] bis-cyclohexanone, oxaldihydrazon (cuprizone) and bathocuprione as chromogens.[11] The method of choice for determining serum copper is AAS after direct dilution.[13]

Early colorimetric methods for determining serum iron used thiocyanate or ferricynide, then were replaced by organic reagents which contained the “ferroin” group –N=C–C=N–, such as 2,2`-dipyridyl, 2,2`,2``-tripyridyl, 1,10-phenanthroline, Ferrozin, and triazines which form complexes with greater molar absorbtivity.[13] Other method for determination of serum iron has been developed such as AAS and auto analyzer.[11,13]

In the present study, the determination of the trace elements zinc, selenium, copper, and iron were done using flame AAS for zinc, copper and iron, and flameless graphite furance AAS with deuterium background correction for selenium.

2.2.10.1 Principle

In AAS, the element is merely dissociated form its chemical bonds and placed in an unexcited or ground state (neutral atom). Thus, the neutral atom is at a low energy level in which it is capable of absorbing radiation at a very narrow band width corresponding to its own line spectrum.[11,13]

2.2.10.2 Reagents

1- Deionized water is used to dilute sample and standard.
2- Concentrated nitric acid HNO_3.
3- Concentrated perchloric acid $HClO_4$.
4- Concentrated sulfuric acid H_2SO_4.
5- Standard solution of elements from Aldrich Chemical Co.

2.2.10.3 Instrumentation

Instrument setting for determination of zinc, selenium, copper, and iron is listed in Table 2-3.

Table 2-3 Instrument Setting for Zn, Se, Cu, and Fe Determination

Element	Wave length	Slit width nm	Current mA	Injection volume
Zinc	213.9	0.5	4.0	20
Selenium	196.0	0.3	12	20
Copper	324.8	0.5	3.0	20
Iron	213.0	0.5	4.0	20

2.2.10.4 Procedure

Zinc, copper, and iron were determined by direct digestion of serum with nitric acid, then injected into auto sample cup of instrument.

Selenium was determined by pre-treatment of the serum with HNO_3 and $HClO_4$ or H_2SO_4, then injected into auto sample cup of instrument.

2-3 Statistical Analysis

All values were expressed as mean ± standard deviation (SD). Student’s t-test was used to estimate differences between the groups, and the differences were considered significant when the probability (P) was $P< 0.05$.

CHAPTER THREE

3 RESULT AND DISSCUTION

In the present study, the control values of different variants were determined in sera of apparently healthy individuals, to compare with different variants in patient groups. Then, the determination of different variants was done in sera of patients with different types of cancer.

The ages of healthy controls and patients subject to present study are shown in Table 3-1.

Table 3-1 Age of Healthy Controls and Patients

Groups	Sex	No.	Age (years)	±SD	Upper value	Lower value
Healthy Control	M	27	34.32	9.48	55	21
Healthy Control	F	25	31.32	11.55	56	20
NHL	M	13	48.84	17.64	60	20
NHL	F	10	47.5	16.84	79	30
HD	M	13	26.15	16.18	51	4
HD	F	8	41.5	15.02	65	52
Breast Cancer	F	38	46.15	11.08	72	25
Bladder Cancer	M	8	61.12	20.36	70	54
ALL	M	5	29.8	17.72	58	13
ALL	F	5	27.8	17.78	60	10
AML	F	4	29.75	14.41	45	21
Placenta Cancer	F	3	42	19.15	47	32
Ovary Cancer	F	3	52.33	32.79	46	42
Colon Cancer	M	3	52	22.52	53	51
Liver Cancer	M	2	34	29.95	65	3

3.1Primary Experiments for Determination the Optimum CDA Activity

The rate at which an enzymatic reaction proceeds depends on several reaction conditions [5]. Primarily, CDA activity was measured by the use of Cohen and Wolfenden (1971) method previously described. [20] Since CDA is usually determined indirectly by measuring the decrease in its substrate (cytidine), several factors must be carefully controlled if reliable results are required.[13]

3.1.1 Effect Protein Content on CDA Activity

A sequence of experiments has been done using different quantities of serum, as described previously. The results indicate that 100µl is the optimum amount of serum which gives the optimum enzyme activity, Figure 3-1.

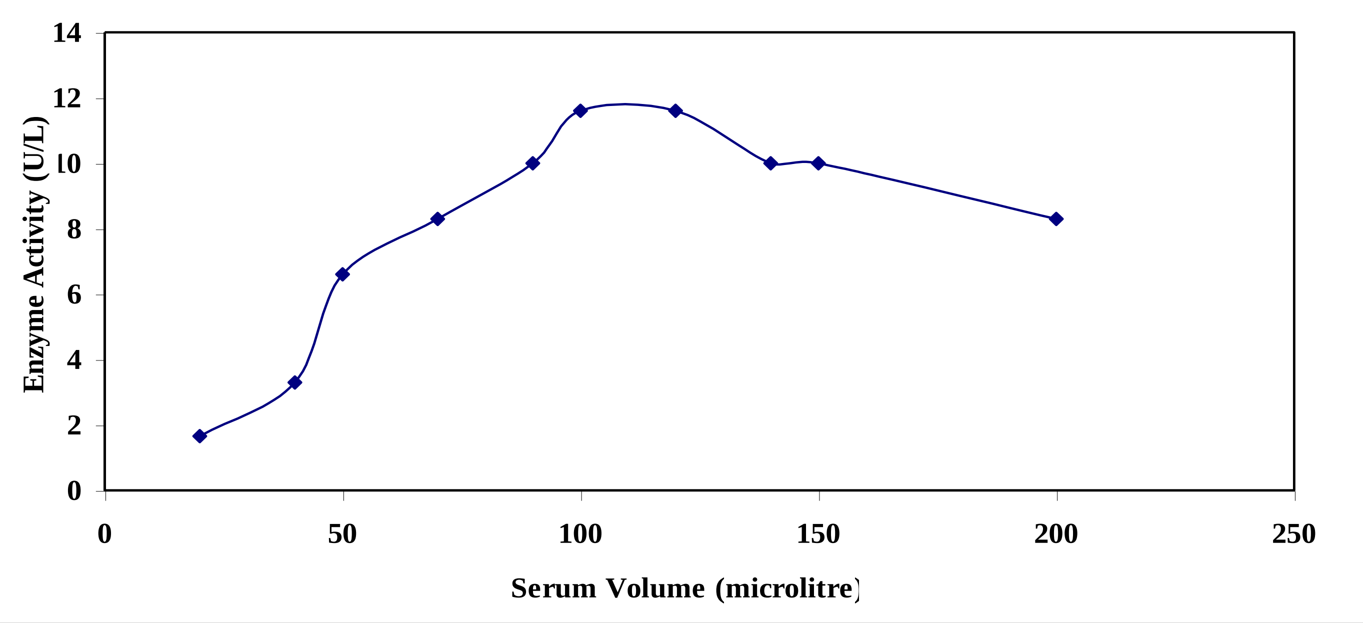


Figure 3-1 Effect of Protein Content and CDA Activity.

The rate of reaction and uridine formation is proportional to the concentration of enzyme present in serum. The results indicate that CDA activity increased and was proportional with the increase of serum. This result supports that the cytidine disappearance proceeds across enzymatic reaction.

This result agrees with those result obtained by Hammodat [194] in human serum, and disagrees with those obtained by Al-Mashhadani in *Leishmania.*[38]

3.1.2 Effect of Incubation Period on CDA Activity

The time course of the reaction catalyzed by CDA was tested by terminating the enzymatic reaction at altered time, as described previously.

All results showed maximum change in absorption at 282nm at 5 minutes. Therefore, all experiments done later based on this period of incubation, Figure 3-2.

The disappearance of cytidine is proportional to reaction time at the optimum conditions, until optimum time is reached.

The optimum period of incubation of CDA reaction was found to be 8 minutes in *Leishmania,*[38] 2 minutes in human serum,[194] 10 minutes in *S. typhimurium*, 5-10 minutes in yeast,[38] 30 minutes in E. coli,[33] 10 minutes in mouse spleen,[138] and 8 minutes in cow liver (unpublished date).

Apparently, there are wide varieties in the incubation period of the CDA reaction from different sources. These results may reflect the presence of different types of CDA.

When the concentration of the product of CDA action increases and cytidine is used up, the rate of the reverse reaction increases and is proportional to uridine concentration. This phenomenon has been termed "product inhibition" or feed back inhibition.[13] This issue may explain the decrease in the latter part of the curve in the Figure 3-2.

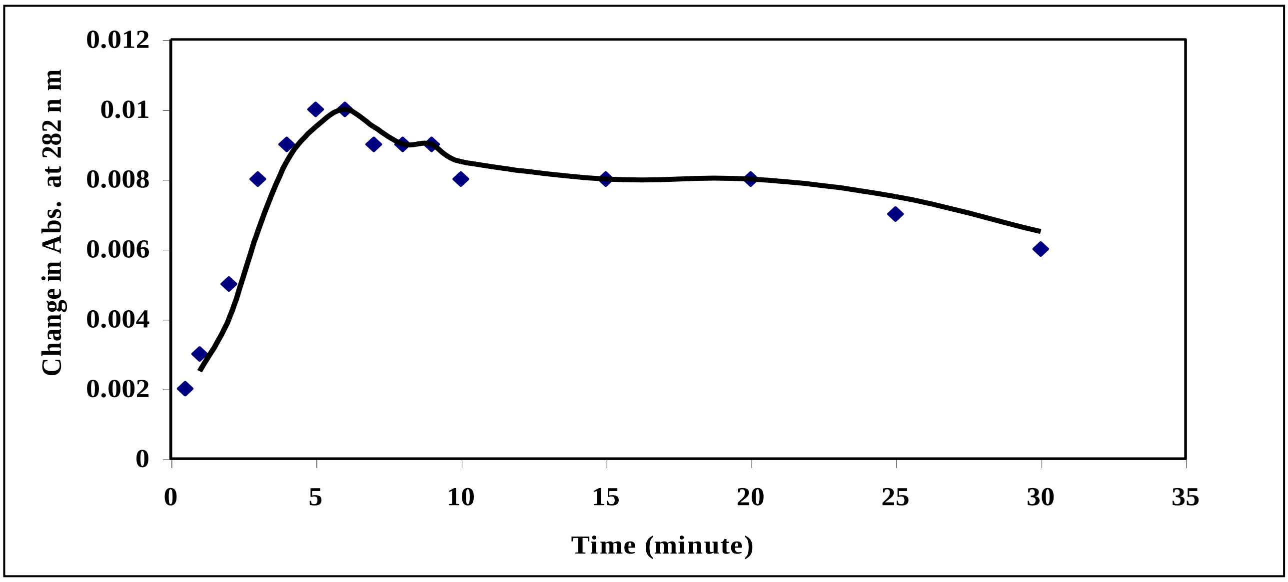


Figure 3-2 Effect of Incubation period on CDA activity.

3.1.3 Effect of pH on CDA Activity

Enzymes are proteins that carry net molecular charge. Extreme pH levels may denature an enzyme or influence its ionic states, resulting in structural changes or a change in the charge on amino acid residues in the active site. Hence, each enzyme operates within a specific pH range and maximally at a specific pH.[5]

The relationship between pH and CDA activity was studied, and the results indicated that the optimum buffer pH was found to be at 7.5 in Tris-HCl buffer, in which the enzyme gives optimum activity. Figure 3-3.

This result agrees with several previous studies, such as optimum buffer pH in E. coli, [20-22,30,32,33] human serum,[131,194] human placenta,[42,43]whereas, optimum buffer pH was found to be 7.4 in mouse kidney and *Leishmania*,[38] 8 in human liver, [41] and leukemic mouse spleen,[138] and 7.9 in HeLa cell culture.[195]

These results may indicate that CDA from different sources operates within imminent range of pH.

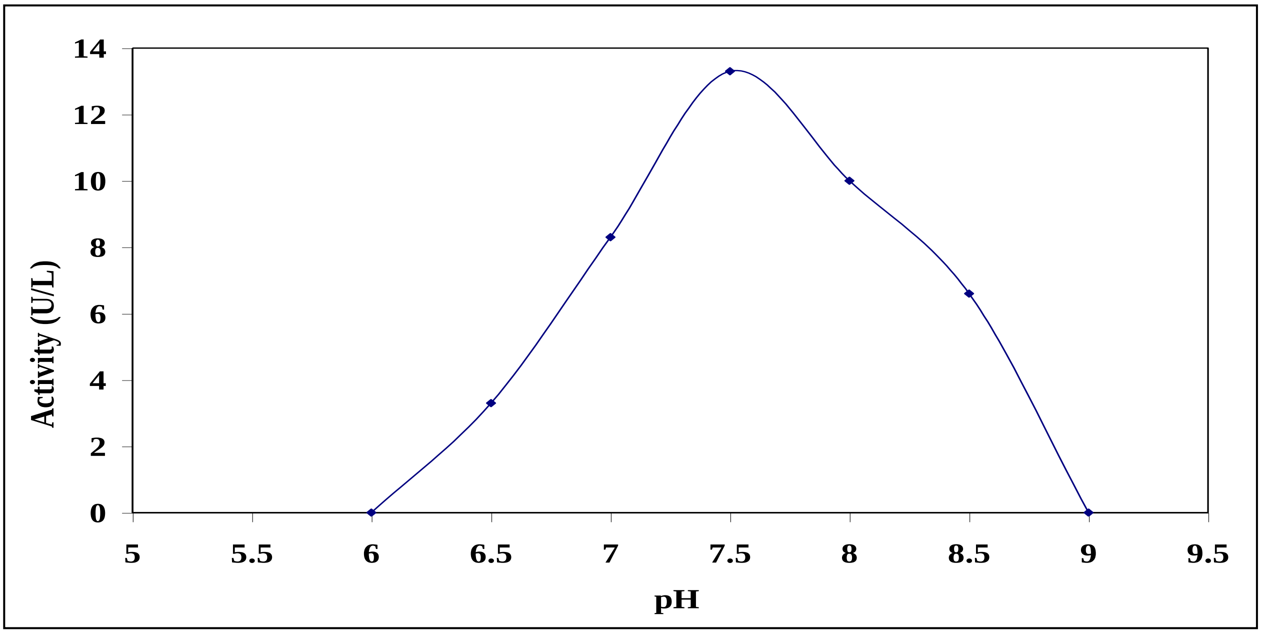


Figure 3-3 **Effect of pH on CDA activity**

3.1.4 Effect of Buffer Concentration on CDA Activity

The relationship between buffer concentration and enzyme activity was study, as shown previously.

The result indicate that (0.05M, pH = 7.5 Tris-HCl buffer is the optimum concentration of buffer solution which given the optimum enzyme activity. (Figure 3-4).

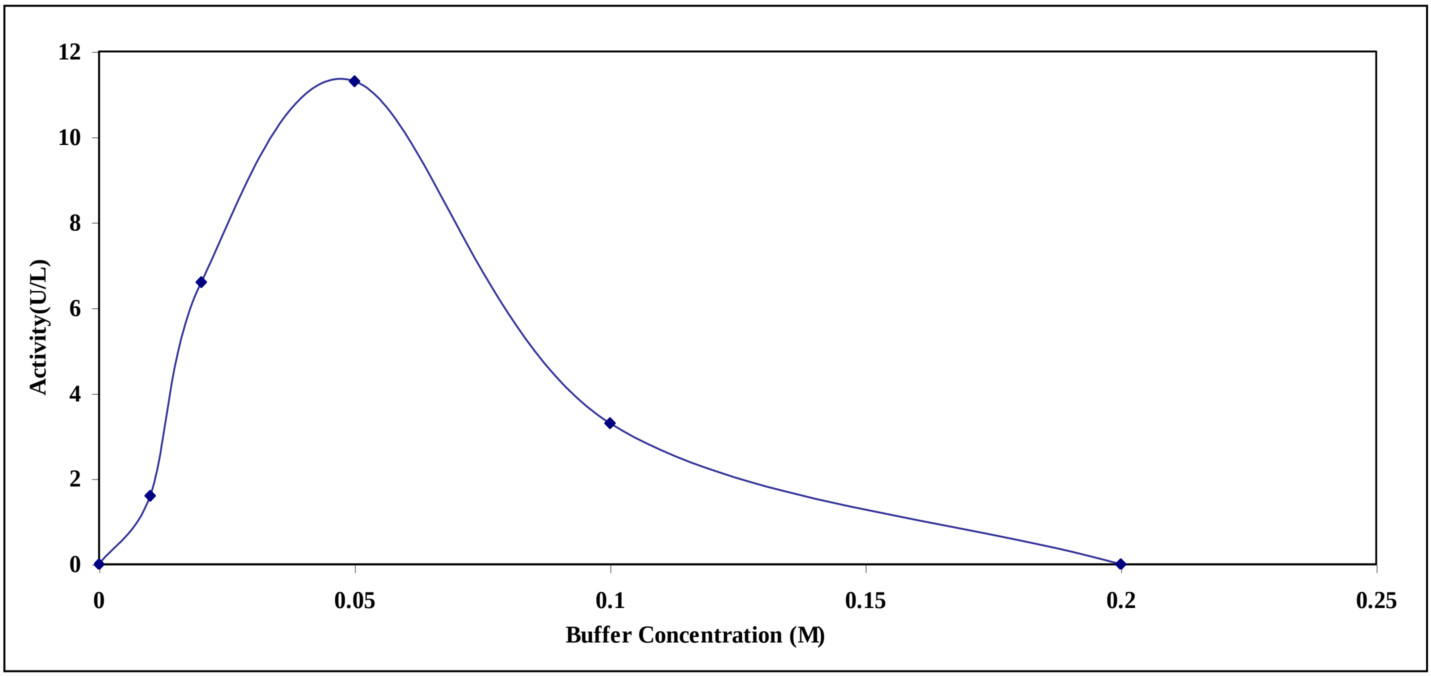


Figure 3-4 Effect of Buffer Concentration on CDA Activity.

This result agrees with those obtained in similar studies such as, the concentration of Tris-HCl buffer of CDA reaction in E. coli [20-22,32] and human placenta.[43] Other studies show disagreement with this result, where, Tris buffer concentration was found to be 0.1M in the CDA activity measurement in E. coli,[33] human placenta,[42] 0.01M in human liver,[41] 150 and 250µM in the human red blood cell (RBC) heamolysate and serum respectively,[194] and 400µM in *Leishmania.*[38]

The optimum pH for a CDA activity measurement may differ with its sources and ionic strength.

3.1.5 Effect of Temperature on CDA Activity

Increasing temperature usually increases the rate of a chemical reaction by increasing the movement of molecules, the rate at which intermolecular collisions occur, and the energy available for the reaction. Such is the case with enzymatic reaction, until the temperature is high enough to denature the protein composition of the enzyme.[5]

The relationship between temperature and CDA activity was studied as described previously, to determine the optimum temperature, in which CDA functions optimally. The results show optimum activity of CDA round about 25°C. Figure 3-5

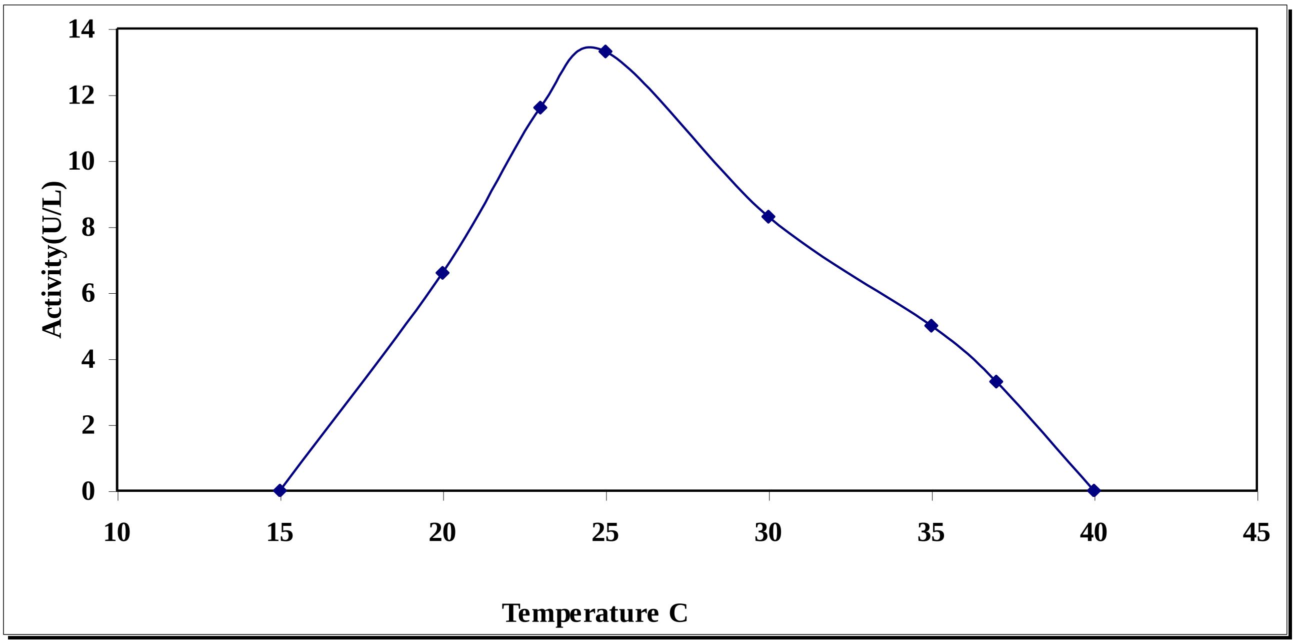


Figure 3-5 Effect of Temperature on CDA Activity .

Although, this result may not be at a physiological temperature, CDA reaction proceeds optimally at 25°C, in several previous studies, such as CDA reaction in E.coli[20-22] and human liver.[41] Other studies show that CDA reaction proceeds at physiological temperature (i.e. 37°C), such as, CDA reaction in human placenta,[42,43] mouse spleen,[46,138] mouse kidney, human serum, HeLa cell culture,[195] and in *Leishmania*.[38]

Hammodat found the optimum temperature of CDA reaction to be 30°C in human serum.[194] The International Union of Biochemistry (1961) in agreement with the International Federation of Clinical Chemistry recommended 25°C for enzyme assays then replaced this by 30°C in 1965. However, it appears that a majority of workers still use 37°C for most determination, because it is the body temperature.[13]

3.1.6 Effect of Substrate Concentration on CDA Activity

At low substrate concentration, the rate of an enzyme reaction is dependent on the concentration of substrate. The rate of reaction changes as substrate concentration changes until a maximum rate is reached called the maximum velocity (V_{max}). At this point the enzyme is saturated by its substrate and the enzyme reaction is independent of substrate concentration.[2]

The effect of substrate concentration on CDA activity was studied under standard condition, and the results indicate that 0.8µM is the optimum concentration of cytidine as a substrate which gives the optimum enzyme activity, Figure 3-6.

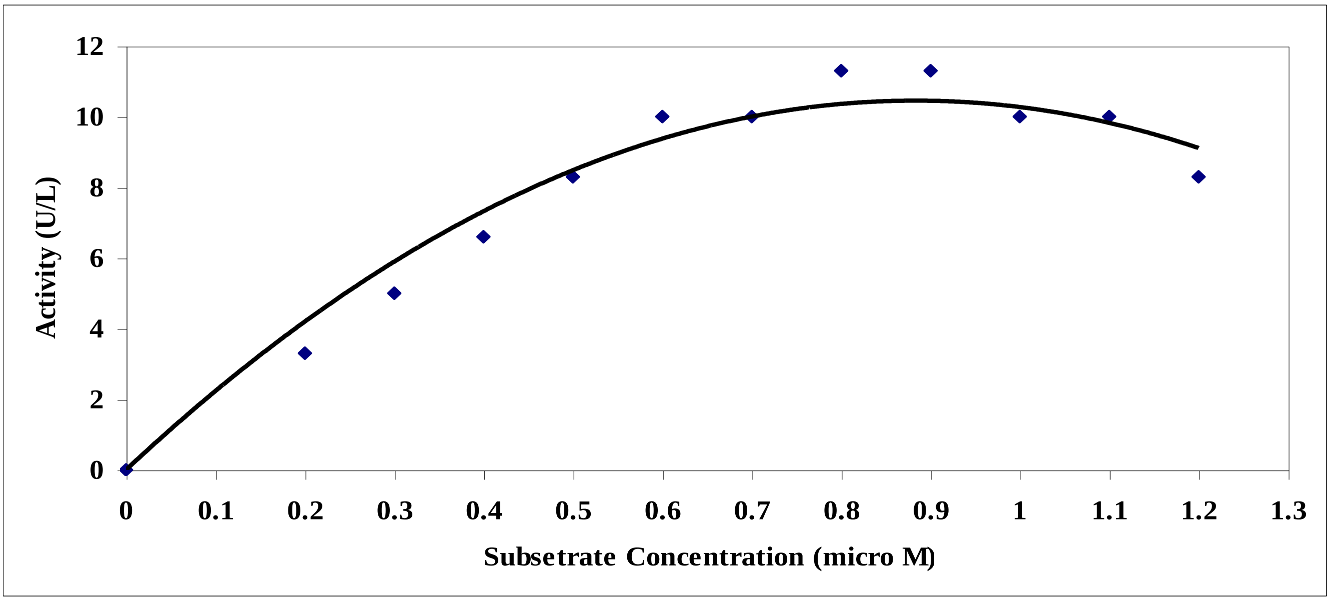


Figure 3-6 Effect of Substrate Concentration on CDA Activity.

This result disagrees with the previous study done on human serum and RBC haemolysate which was found to be 3 µM,[194] and on *Leishmania* [38] which was found to be 400 µM.

Where the reaction is proportional to the substrate concentration, that is when the enzyme is not saturated, the enzyme reaction is said to be of first order kinetics. In a situation where there is excess substrate and the enzyme is saturated, the reaction rate is constant, and at this point the enzyme reaction is said to be of zero order kinetics, i.e. independent of the substrate concentration.[2]

The early part of the curve in Figure 3-6 rises until it reaches the maximum velocity (V_{max}) or the maximum rate of reaction, then the curve decreases less than the optimum concentration, due to inhibition of reaction by the substrate concentration.[11] It will be necessary to use a concentration less than the optimum.[13]

3.1.7 Michaelis Menton Constant (K_m) and Maximum Velocity (V_{max})

The initial rate of reaction catalyzed by CDA was plotted against substrate concentration by using Lineweaver-Burk plot to obtain Michaelis Menton constant (K_m) and maximum velocity (V_{max})

$$\frac{1}{v} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}}$$

where

v = Measured velocity of reaction

[S] = Substrate concentration

The result indicates that the apparent K_m value is 1.12 µM and V_{max} is 24.75 µmole per minutes, (Figure 3-7).

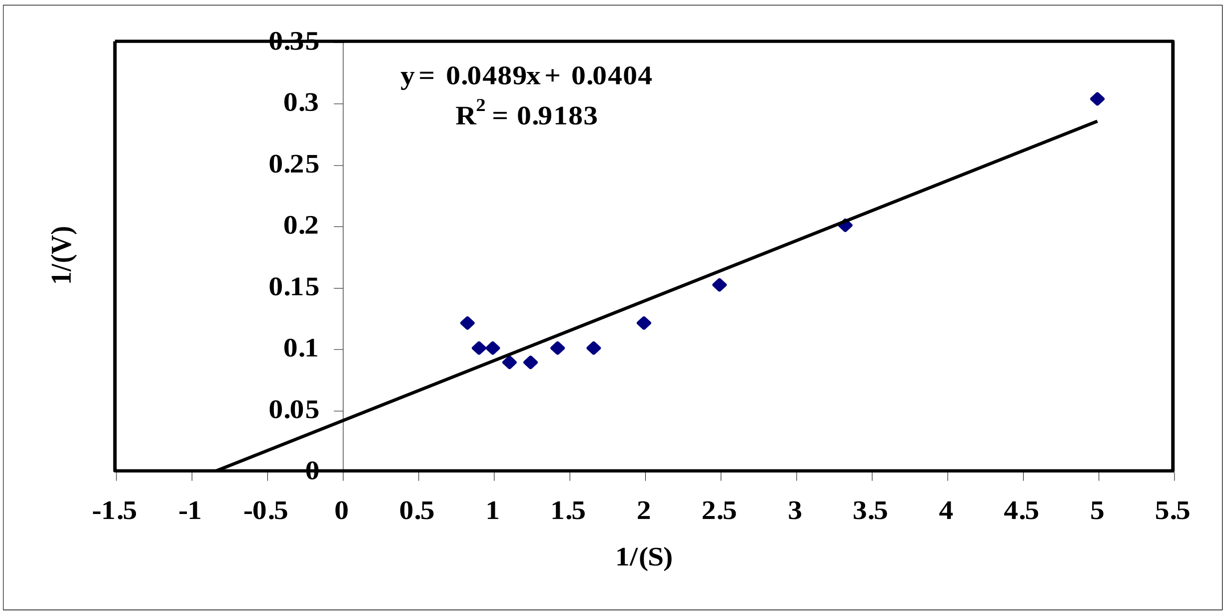


Figure 3-7 Linewever-Burk Plot of CDA in Serum.

K_m values of CDA vary according to their sources. It is found to be 2.7×10^{-5}M in *Leishmania*,[38] 2.2×10^{-4}M in E. coli,[20-22] 9.2×10^{-6} in human liver, [41] 5×10^{-5} in HeLa cell cultures,[195] 1.4×10^{-5} in human serum,[131] and 6.3×10^{-5} in mouse spleen.[46,138]

K_m is equal to the substrate concentration at which the rate of reaction is half maximum[13]. K_m is important as an indicator of enzyme-substrate affinity. The enzyme, which has lower K_m value to its substrate, is said to be of high affinity to its substrate.[13]

K_m values of CDA may reflect the difference in affinities of CDA to its substrate from different sources.

3.1.8 Optimum Condition for Measurement of CDA Activity

The optimum condition for the measurement of CDA activity in serum was conducted by using of 100 μL of serum, after preincubation period of serum for 5 minutes at 25°C, 400 μL of 0.002M cytidine and 500 μL of 0.05M Tris-HCl buffer, pH 7.5, in a final volume of 1.0ml.

The reaction was initiated by the addition of serum, (which contained the enzyme), and the reaction mixture was incubated for 5 minutes at 25°C. The enzymatic reaction was terminated by the addition of 4ml of 0.1M HCl. The decrease in absorbance was read at 282 nm for 1cm light path with quartz cuvettes against sample blank.

3.2 CDA Activity in Patient and Healthy Controls

Compared with healthy controls, CDA activities was found to significantly increase in sera of patients with NHL, breast cancer, placenta cancer, and liver cancer. Also, CDA activities increased in sera of males with ALL, females with AML, and females with ovary cancer whereas, CDA was found to decrease in patients with HD, males with bladder cancer, females with ALL, and males with colon cancer as shown in Table 3-2 and Figure 3-8.

Table 3-2 CDA Activity (U/L) in Sera of Patients and Healthy Controls. (M = males, F = females, S = significant (when P < 0.05), NS =not significant).

Groups	Sex	CDA(U/L)	± SD	Upper value	Lower value	P	Sig.
Healthy Control	M	6.94	4.07	13.3	0.0		
Healthy Control	F	4.96	2.29	11.6	0.0		
NHL	M	15.46	5.44	26.6	6.6	0.000	S
NHL	F	15.13	1.89	18.3	11.6	0.000	S
HD	M	4.83	3.03	10.0	0.0	0.085	NS
HD	F	3.71	2.11	8.3	1.6	0.21	NS
Breast Cancer	F	13.3	3.67	21.6	6.6	0.000	S
Bladder Cancer	M	5.21	2.8	8.3	1.6	0.18	NS
ALL	M	9.28	1.99	11.6	6.6	0.097	NS
ALL	F	4.3	1.7	6.6	1.6	0.519	NS
AML	F	7.07	2.98	10	3.3	0.321	NS
Placenta Cancer	F	22.8	2.09	25	20	0.007	S
Ovary Cancer	F	8.32	2.71	11.6	5	0.23	NS
Colon Cancer	M	6.63	3.57	11.6	3.3	0.917	NS
Liver Cancer	M	19.15	0.85	20	18.3	0.001	S

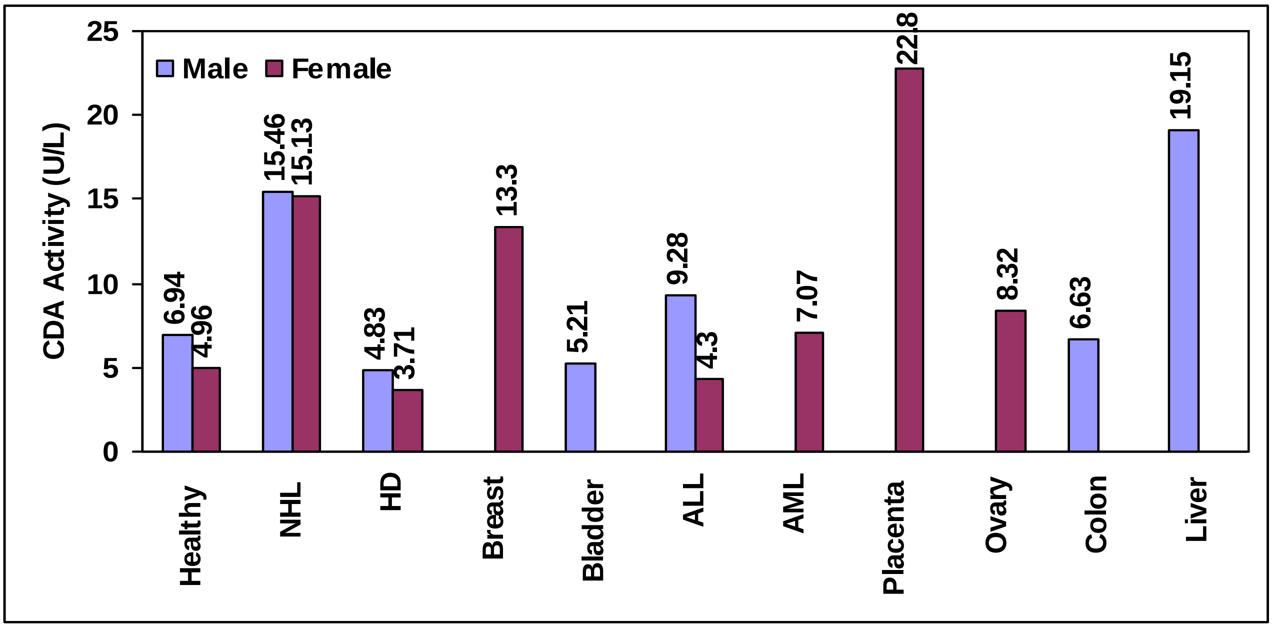


Figure 3-8 CDA Activity in Sera of Patients and Healthy Controls

Previous studies have shown higher levels of CDA in SF compared to blood in arthritic condition, favoring the view of intra-articular release of this molecule.[127] CDA activities in SF seem to be a marker of local gronulocyte turnover or activity, or both.[128]

CDA is present in several types of cell, such as liver, lung, kidney cells, and also in minute amounts in lymphocytes.[128] It is claimed that CDA may leak out of damaged cells and reflect the turnover rate of this cells.[129]

An exaggerated increase of CDA activity was found in patients with abruptio placenta (46.3 U) and amnionitis associated with intrauterine fetal death (55.9U).[131] The cells of the infected amnion and the damaged placenta, both of which contained high CDA levels, appeared to leak CDA into the maternal circulation. High levels of CDA activity were observed in abnormal pregnancy such as pre-eclampsia, intrauterine death and cholestasis of pregnancy when compared with normal pregnancy.[126]

Massively high levels of CDA were reported in gastric tissues from patients with gastric cancer and various types of solid tumors cell line.[135,136]

An increase in spontaneous release of CDA was noted in active SLE PMN (10.003 ± 2.637 U/5×10^6 PMN) that equal to 10.003 ± 2.637 U/L of PMN, compared with inactive SLEPMN (5.358 ±1.624 U/5×10^6 PMN) or normal group (5.449 ± 1.358 U/5×10^6 PMN).[129]

YU C.L. et al.[129] attributed their findings to several possibilities (1) serum factor such as autoantibodies or immune complexes which may activate or injure PMN in vivo; (2) intrinsic defect of SLE PMN; (3) the influence of drug therapy; (4) the combination of (1), (2) and or (3).

Several observations were noted in the present study:

(1) Increase in CDA activity in NHL may be attributed to the damaged B or T cell which mainly increase in this disease.[142]

(2) Liver and placenta cells have high levels of CDA,[41-43] and when such cells are involved in the uncontrolled cell proliferation (cancer), they appear to leak out CDA into the circulation.

(3) Increase in CDA in sera of patients with breast cancer may be attributed to the existence of CDA in mammary glands in high level, and the leak out of CDA from damaged cells may be reflect to the turnover rate of these cells.

(4) The decrease of CDA activity in sera of patients with HD may be attributed to the presence of Reed -Sternberg cell regarded as the neoplastic component of this disease[143] that may suppress CDA release from lymphocyte, or the presence of some immune complex or specific antigens that suppress its formation or its release.

(5) Although leukemic blast cell contained very low levels of CDA,[110] fluctuation of CDA activity in sera of patients with ALL and AML was observed. These results may reflect the induction of ARA-C to expression of CDA gene in patients treated with this drug where elevated levels of this enzyme, were shown,[119,196,197] and in patients treated with other drug.

(6) In addition to the above suggestions, it may be the serum factors such as autoantibodies or immune complexes or specific antigens, or the influence of drug therapy that stimulate the release of CDA, or suppress its release from cells that contain it.

(7) Oxidative stress associated with cancer may be due to CDA release from cell in which plasma membrane is damaged by ROS or RNS, and the cells which are exposed to more oxidative stress may reveal high CDA activity.

3.3 Effect of Oxidative Stress on Vitamin C Concentration

Ascorbic acid is an important vitamin which participates in a great variety of biological events concerning electron-transport reaction, the oxidative catabolism of aromatic amino acid, and scavenging ROS and RNS and may thereby prevent oxidative damage to important biological macromolecules such as DNA, lipid, and protein.[172,198]

Oxidative DNA damage in humans has been estimated to occur at a rate of 10^4 to 10^5 hit per cell per day. The repair of these oxidative lesions by specific DNA glycosylases and other repair mechanisms is not 100 % efficient, therefore lesions accumulate with age and may result in mutations and cancer. Numerous studies have shown an inverse association between vitamin C intake or plasma status, and the risk from different types of cancers.[172]

Vitamin C functions as an antioxidant by reacting directly with ROS or regenerating vitamin E from α- tocopheroxyl radical (Figure 1-21). Thus, it protects cell membranes from external oxidants and has a GSH-sparing effect. However, vitamin C cannot be efficiently transported into blood cells. The existence of DHA has long been considered evidence of ascorbate oxidation.[170]

Compared with healthy controls, total (ascorbic acid plus DHA) and reduced form (ascorbic acid) of vitamin C was found to significantly decrease in sera of most patients with different types of cancers subjected to the present study. Also, total and reduced vitamin C was found to decrease in sera of females with ALL, AML, and ovary cancer, but not significantly, as shown in Table 3-3, Table 3- 4, Figure 3-9, and Figure 3-10.

Table 3-3 Vitamin C Concentrations (mg/L) in Sera of Patients and Healthy Controls. (M = males, F = females, S = significant (when P < 0.05), NS =not significant).

Groups	Sex	Total Vit. C mg/L	± SD	Upper value	Lower value	P	Sig.	Reference Range
Healthy Control	M	11.28	2.94	18.0	6.36			4-15
Healthy Control	F	9.61	1.62	11.9	6.78			4-15
NHL	M	5.528	2.55	10.19	1.2	0.000	S	
NHL	F	6.16	2.37	9.4	2.91	0.0016	S	
HD	M	8.44	1.87	11.98	5.54	0.001	S	
HD	F	7.31	1.92	9.93	5.42	0.0016	S	
Breast Cancer	F	7.5	2.75	12.82	3.42	0.0003	S	
Bladder Cancer	M	5.99	2.06	9.41	3.53	0.000	S	
ALL	M	3.1	1.89	4.48	0.15	0.000	S	
ALL	F	7.18	2.1	10.82	4.41	0.078	NS	
AML	F	8.75	2.9	11.81	4.33	0.64	NS	
Placenta Cancer	F	5.47	1.24	7.12	4.1	0.021	S	
Ovary Cancer	F	8.67	1.17	9.5	7.01	0.366	NS	
Colon Cancer	M	4.313	2.78	8.04	1.36	0.007	S	
Liver Cancer	M	3.49	3.19	6.68	0.3	0.000	S	

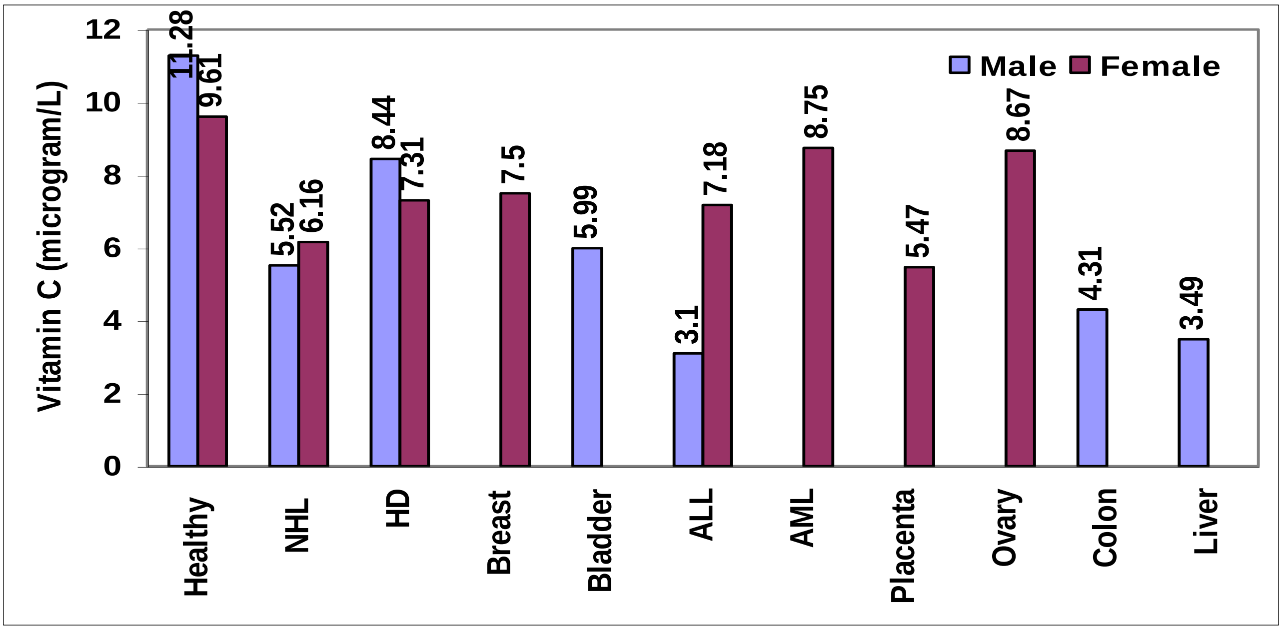


Figure 3-9 Vitamin C Concentrations in Sera of Patients and Healthy Controls.

Table 3-4 Reduced Vitamin C Concentrations (mg/L) in Sera of Patients and Healthy Controls. (M = males, F = females, S = significant (when P < 0.05), NS =not significant).

Groups	Sex	Reduced Vit. C mg/L	± SD	Upper value	Lower value	P	Sig.	Reference Range
Healthy Control	M	10.25	2.7	16.43	5.73			3.5 - 13.5
Healthy Control	F	8.729	1.49	10.82	6.12			3.5 - 13.5
NHL	M	4.97	2.34	9.25	0.99	0.000	S	
NHL	F	5.55	2.17	8.53	2.56	0.0016	S	
HD	M	7.65	1.71	10.9	4.98	0.001	S	
HD	F	6.61	1.18	9.01	4.87	0.0016	S	
Breast Cancer	F	6.78	2.52	11.67	3.03	0.0003	S	
Bladder Cancer	M	5.39	1.89	8.53	3.13	0.000	S	
ALL	M	2.74	1.73	4.0	0.02	0.000	S	
ALL	F	6.49	1.93	9.83	3.94	0.078	NS	
AML	F	7.93	2.66	10.74	3.87	0.64	NS	
Placenta Cancer	F	4.92	1.14	6.43	3.65	0.021	S	
Ovary Cancer	F	7.85	1.07	8.62	6.33	0.36	NS	
Colon Cancer	M	3.85	2.85	7.28	1.14	0.007	S	
Liver Cancer	M	3.09	2.93	6.03	0.16	0.000	S	

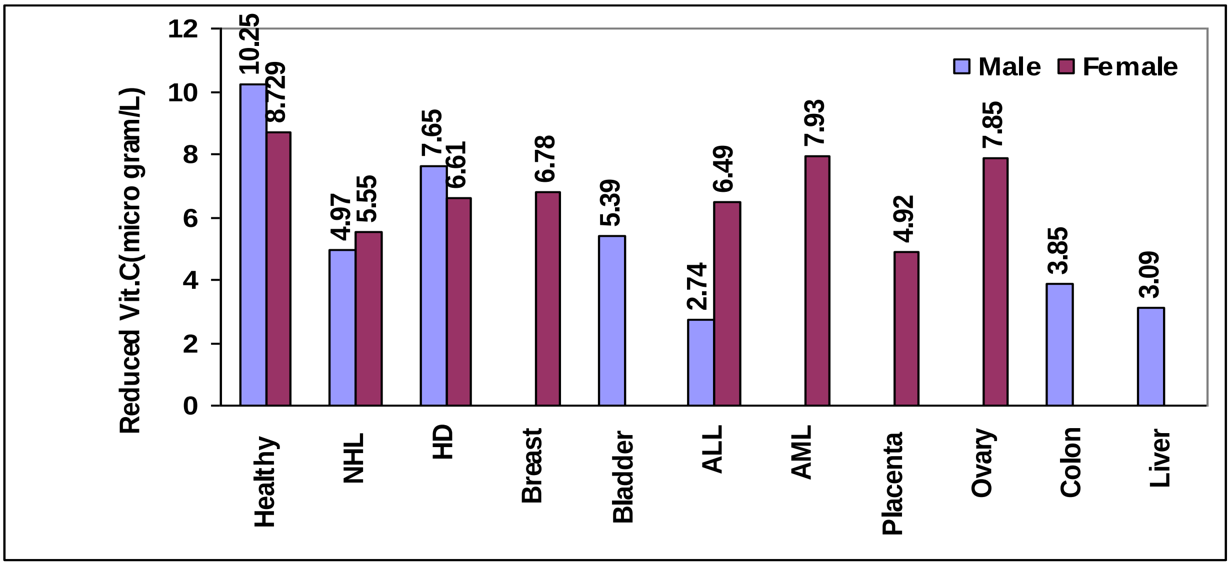


Figure 3-10 Reduced Vitamin C Concentrations in Sera of Patients and Healthy Controls.

Although all values of serum vitamin C decreased in the present study, most of them were found to be within reference interval except those results of male with ALL and liver cancer, which decrease in comparison with the reference interval.

Free radicals formed in the body fluids in patients with cancer are detoxified by antioxidant including vitamin C.[165] This may explain the depletion in vitamin C concentration in the present study.

In people with adequate intake of vitamin C, plasma or serum concentrations of total vitamin C are between 4-15 mg/L (23-85 μmole/L). The lower limit value may be seen in some cases with subclinical vitamin C deficiency in older individuals.[11]

Serum levels of 3-5.9 mg/L are considered acceptable (low risk of developing scurvy), whereas concentrations of 2-2.9 mg/L are considered medium risk. Serum values less than 2 mg/L are clearly deficient and are associated with a high risk of scurvy.[1,5,11]

Depleted serum Vitamin C levels were shown in several conditions related to oxidative stress, such as diabetes mellitus,[165] cardiovascular disease and cancer.[167]

Adequate serum concentrations of ascorbic acid do not always reflect tissue pools. Clinical symptoms of scurvy have found in some persons with serum AA concentration above 2.0 mg/L but with depleted body pools of this vitamin.[11]

In addition, serum reduced vitamin C concentration in the present study do not reflect the exact values, because they are estimated by mathematic manner. Therefore, we must rely on the comparison between healthy controls and patients values.

3.4 Effect of Oxidative Stress on GSH Concentration

GSH, a ubiquitous cellular antioxidant, play a central role in defense against a variety of disease and both exogenous and endogenous insults. Its functions include the detoxification of xenobiotics, carcinogens, free radicals, and peroxides; regulation of immune function; and maintenance of protein structure, function, and turnover[182]. GSH is not required in the diet of animals and humans but is synthesized in virtually all animal and human cells.[174]

Vitamin C and GSH are strongly correlated. Within the cell, DHA is regenerated into ascorbate at the expense of GSH. However, ascorbate cannot regenerate GSH from its oxidized from (GSSG). GSSG is reduced to GSH at the expense of NADPH, which is produced by pentose phosphate pathway by the action of GSH redactase.[1,170]

Numerous laboratory studies have suggested that GSH is a critical factor in protecting organisms against toxicity and disease. Blood and serum GSH concentrations may serve as an indicator of GSH status and, thus disease risk in human subjects.[182,183]

Compared with healthy controls, GSH concentrations were found to significantly decrease in sera of patients with different types of cancers, in the present study, except those of females with NHL, placenta cancer, ovary cancer, and males with colon cancer, in which GSH levels decreased but not significantly, and GSH levels were found to be within, or higher than, reference interval.[199] Table 3-5. Figure 3-11.

Previous studies have shown low levels of GSH in patients with hemolysis from primaquine, diabetes mellitus, alcoholic liver disease, AIDS, acute hemorrhagic gastric crosions, cataracts, xenobiotic induced oxidative stress and toxicity and aging.[11,182]

Table 3-5 Glutathione Concentrations (µM) in Sera of Patients and Healthy Controls. (M = males, F = females, S = significant (when P < 0.05), NS =not significant).

Groups	Sex	GSH µM	± SD	Upper value	Lower value	P	Sig.	Reference Range
Healthy Control	M	28.05	10.18	47	7.5			4-20
Healthy Control	F	19.22	4.84	30	15			4-20
NHL	M	15.61	6.46	30	5	0.000	S	
NHL	F	16.65	3.34	27	12.5	0.098	NS	
HD	M	15.57	4.87	40	5	0.000	S	
HD	F	11.87	3.47	24	10	0.000	S	
Breast Cancer	F	15.68	6.18	44	5	0.015	S	
Bladder Cancer	M	12.43	7.75	30	5	0.000	S	
ALL	M	7	1.87	10	5	0.000	S	
ALL	F	13.3	3.529	17	7.5	0.02	S	
AML	F	11.87	1,08	12.5	10	0.000	S	
Placenta Cancer	F	14.66	3.86	19	10	0.20	NS	
Ovary Cancer	F	15.33	3.77	18	10	0.26	NS	
Colon Cancer	M	13.38	7.51	24	5	0.09	NS	
Liver Cancer	M	10.5	5.5	16	5	0.000	S	

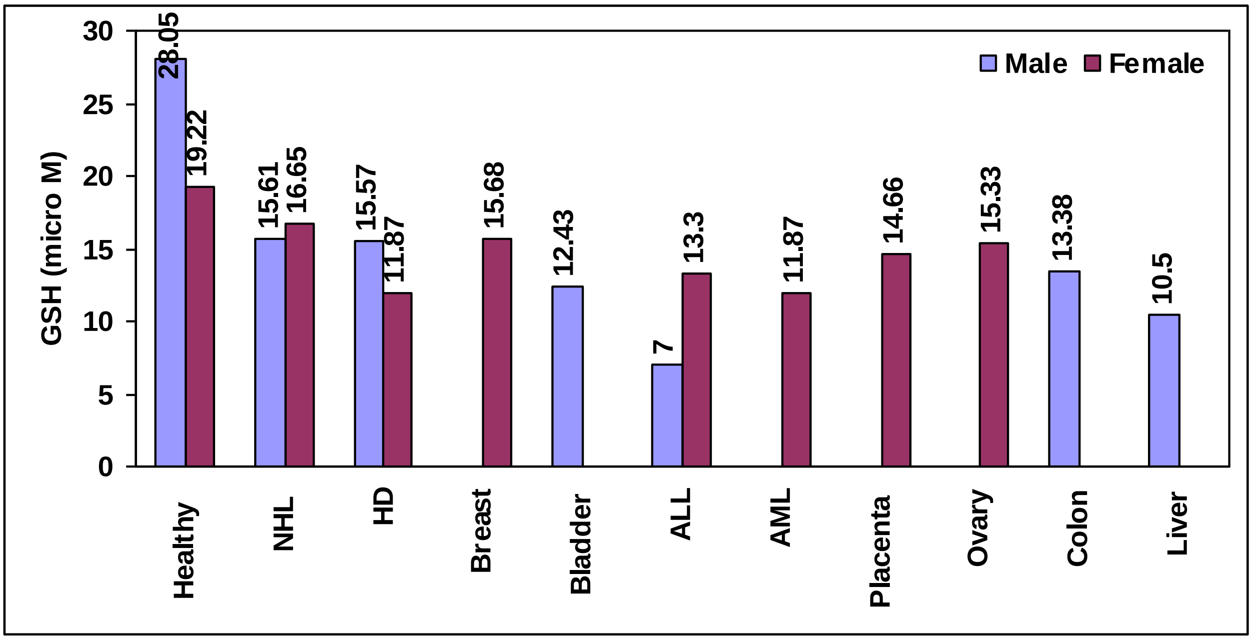


Figure 3-11 Glutathione Concentrations in Sera of Patients and Healthy Controls.

Another studies has reported that GSH concentrations were 8 –10 % greater in smokers than in nonsmokers, and no sex differences.[182]

Intracellular GSH, which is normally present at millimole per liter concentration, has a protective role against oxidative damage by neutralizing vitamin

C or by the action of GSH peroxidase. GSH peroxidase is one of the main enzyme neutralizing H_2O_2, it catalyses the reaction:

$H_2O_2 + 2GSH \longrightarrow GSSG + 2H_2O$

There is a family of GSH peroxidases rather than one enzyme all members of which contain for subunits with one selenium atom at the active site.[145,148]

Oxidative stress may be important in the development of coronary artery disease because the heart, in contrast to the liver or lung, is relatively poorly defended against oxidative stress. The levels of oxidized GSH in serum serves as an index of myocardial oxidative stress during and after reperfusion.[183]

The depletion of GSH levels in the present study, when compared with healthy control supports the hypothesis that considers GSH a protective factor against the development of deferent types of cancers.

3.5 Effect of Oxidative Stress on Total Serum Protein Concentration

Measurement of total serum protein concentration provides general information reflecting disease states in many organ systems.[5] Serum proteins play several roles in the human body. Some of them, such as albumin may be considered an important component of plasma antioxidant activity, primarily, binding free radicals, free fatty acid, divalent cations, HOCl and bilirubin.[11,150,158] Thus, total serum proteins concentration may change under oxidative stress associated with cancer.

Compared with healthy controls, total serum proteins, in the present study, in patients with different types of cancers, were found to significantly decrease, with few exception such as in sera of females with NHL, AML, ovary cancer, and in males with colon cancer, where it is found to decrease but not significantly. The results of present study show slight excess of total serum proteins in males over females, Table 3-6, Figure 3-12.

An increased concentration of total serum protein can be due to either increase in an individual protein or group of proteins, or increase in all proteins. More commonly, an elevated total serum protein is caused by dehydration of patient, where fluid has been lost.[15]

Low total serum protein levels can be found in cases of severe disease such as liver disease or severe malnutrition where the synthesis of protein is reduced. More commonly, low total protein levels are found in situation where there is dilution of the blood (haemodilution) caused by over-hydration. Total serum protein levels can also be affected by the absence of one of the major blood proteins, for example hypoalbuminaemia or hypogammaglobulinaemia.[2]

The results of the present study suggest that the decrease in total serum protein may be due to the involvement of albumin in the defense against oxidative stress associated with cancer. Globulins were elevated, but not sufficient to substitute the significant decrease in albumin.

Table 3-6 Total protein Concentrations (gm/dl) in Sera of Patients and Healthy Controls. (M = males, F = females, S = significant (when $P < 0.05$), NS =not significant).

Groups	Sex	Total Protein gm/dl	± SD	Upper value	Lower value	P	Sig.	Reference Range
Healthy Control	M	7.64	0.54	8.71	6.49			6.6-8.7
Healthy Control	F	7.49	0.5	8.4	6.4			6.6-8.7
NHL	M	6.22	0.974	7.9	3.87	0.000	S	
NHL	F	6.61	1.22	8.9	3.72	0.063	NS	
HD	M	7.09	0.6	8.6	6.25	0.015	S	
HD	F	6.38	0.78	7.09	4.54	0.006	S	
Breast Cancer	F	6.81	0.76	7.93	4.63	0.000	S	
Bladder Cancer	M	6.53	0.24	6.87	5.97	0.000	S	
ALL	M	6.26	0.615	7.32	5.48	0.008	S	
ALL	F	6.64	0.63	7.58	5.68	0.003	S	
AML	F	7.42	0.19	7.59	7.1	0.000	NS	
Placenta Cancer	F	6.63	0.67	7.28	5.71	0.021	S	
Ovary Cancer	F	7.12	0.7	7.62	6.13	0.544	NS	
Colon Cancer	M	6.58	0.941	7.9	5.75	0.25	NS	
Liver Cancer	M	6.04	0.04	6.08	6.0	0.000	S	

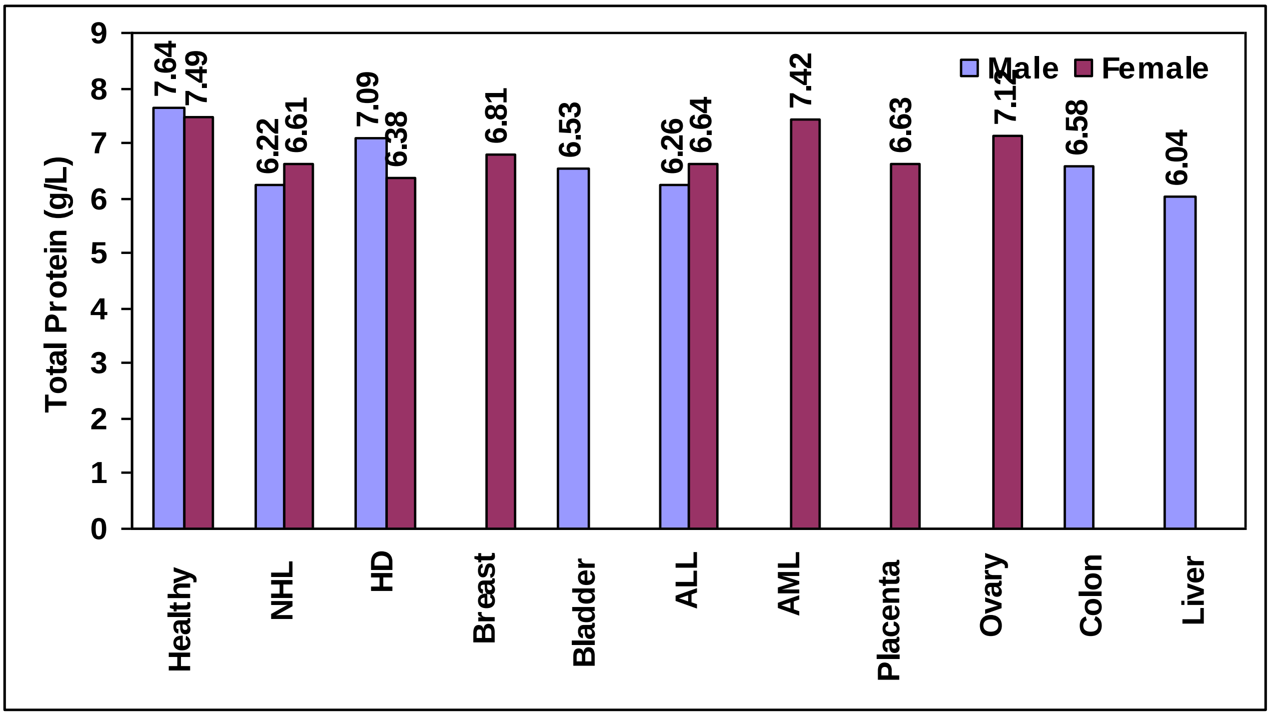


Figure 3-12 Total protein Concentrations in Sera of Patients and Healthy Controls.

The results of the present study do not agree with those obtained by Al-Sultani,[200] where she found a significant increase in total serum proteins in sera of patients with kidney, prostate, ureter, and bladder cancers. She attributed her finding to the obstruction of excessively synthesized protein to excretion by these organs.

3.6 Effect on Albumin Concentration

Albumin, with a molecular weight of about 65-66 kDa, is synthesized by the liver. It has a normal plasma biological half-life of about 20days.[15]

Among several important functions of albumin, we restrict our interest to its ability to scavenge ROS, where it is found to be an important antioxidant in plasma, by binding free fatty acid, divalent cations, HOCl and bilirubin and scavenging of $O_2^{\cdot -}$ and $ONOO^-$ by replace a highly free radical by a less reactive one.[11,150,158,164]

In the present study, albumin concentration in sera of patients with deferent types of cancers was found to significantly decrease, when compared with healthy controls as shown in Table 3-7and Figure 3-13.

Table 3-7 Albumin Concentrations (gm/dl) in Sera of Patients and Healthy Controls. (M = males, F = females, S = significant (when P < 0.05), NS =not significant).

Groups	Sex	Albumin gm/dl	± SD	Upper value	Lower value	P	Sig.	Reference Range
Healthy Control	M	4.82	0.58	5.8	4.32			3.5-4.4
Healthy Control	F	4.56	0.35	5.08	3.88			3.8-4.4
NHL	M	3.57	0.65	4.52	1.8	0.000	S	
NHL	F	3.86	0.7	5.21	2.16	0.01	S	
HD	M	4.02	0.44	4.52	3.33	0.000	S	
HD	F	3.59	0.706	4.9	2.6	0.008	S	
Breast Cancer	F	3.71	0.551	4.57	2.70	0.000	S	
Bladder Cancer	M	3.69	0.38	4.14	2.82	0.000	S	
ALL	M	3.57	0.41	4.35	3.1	0.001	S	
ALL	F	4.04	0.56	4.55	3.22	0.013	S	
AML	F	3.68	0.36	4.32	3.42	0.017	S	
Placenta Cancer	F	3.91	0.24	4.21	3.62	0.039	S	
Ovary Cancer	F	4.19	0.47	4.54	3.52	0.039	S	
Colon Cancer	M	3.27	0.713	4.27	2.64	0.054	S	
Liver Cancer	M	2.98	0.025	3.01	2.96	0.000	S	

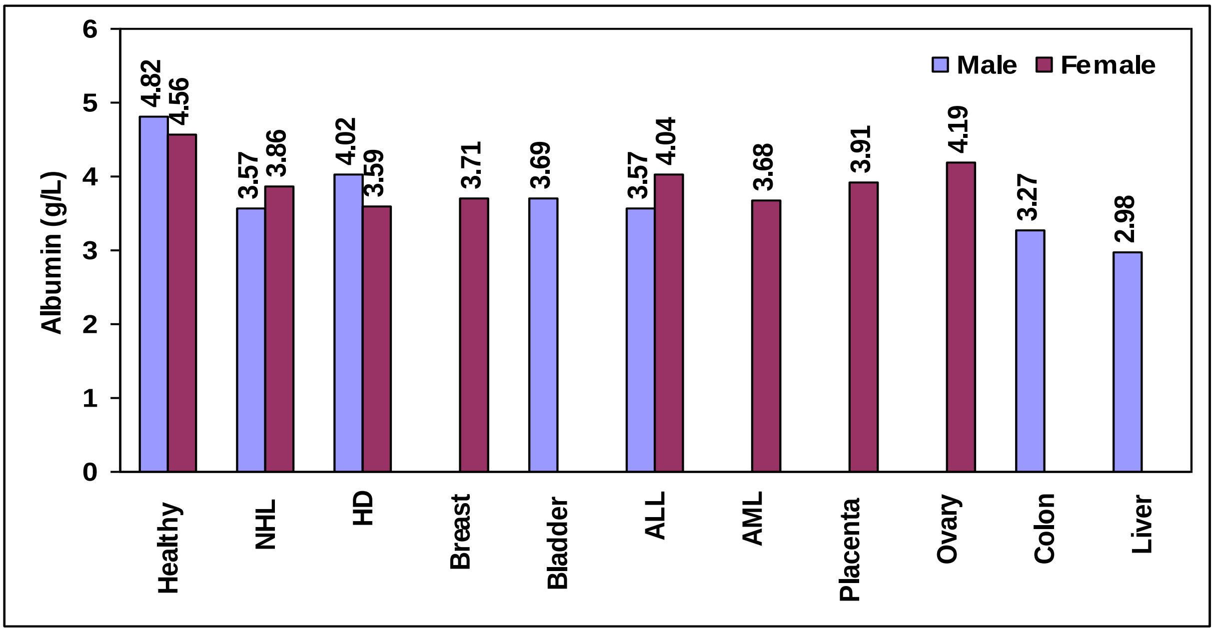


Figure 3-13 Albumin Concentrations in Sera of Patients and Healthy Controls.

Abnormal levels of albumin are not diagnostic of any particular disease. High levels (hyperalbuminaemia) are only found in hydrated patients. Low albumin levels (hypoalbuminaemia) are found in many conditions, where there are: (1) impaired synthesis as a result of liver disease, [2,5,16] (2) increased breakdown of protein due to tissue damage or inflammation, (3) reduced absorption of amino acids, (4) and increased protein loss associated with renal disease.[2,15] Very low albumin levels result in a loss of plasma osmotic pressure and cause movement of fluid from the circulatory system into tissue, causing oedema.[2,11,15]

Also, low levels of albumin have been reported in premature newborns with intrauterine growth retardation. These results were attributed to the scavenging property of albumin against oxidative stress.[164]

The results of the present study agree with those obtained by Al-Sultani in patients with kidney, prostate, ureter, and bladder cancers, as well as in patients with diabetes mellitus [200].

3.7 Effect on Globulins Concentration

Globulins are group of plasma proteins that consist of five major subgroup α_1, α_2, β_1, β_2, and γ. [2] The concentration of the main globulin factions in health is: α_1 0.3-0.5, α_2 0.5-0.7, β_1 and β_2 0.6 -1.2, γ 0.9-1.5 gm/dl.[13] Globulins play an important role in the very veried function.[11]

In the present study, globulins concentrations were estimated by subtracting albumin from total serum protein, therefore, do not provide full details about their fractions.

Compared with healthy controls, serum globulins concentration in the present study, was found to significantly increase in females with AML, and increase but not significantly in males with HD, females with breast cancer, males with bladder cancer, females with AML, females with ovary cancer, and males with colon and liver cancers. Serum globulins concentration was found to decrease in patients with NHL, females with HD, patients with ALL, and females with placenta cancer, when compared with healthy controls, as shown in Table 3-8 and Figure 3-14.

Table 3-8 Globulins Concentrations (gm/dl) in Sera of Patients and Healthy Controls. (M = males, F = females, S = significant (when P < 0.05), NS =not significant).

Groups	Sex	Globulin gm/dl	±SD	Upper value	Lower value	P	Sig.	Reference Range
Healthy Control	M	2.81	0.5	3.52	1.91			2.8-4.3
Healthy Control	F	2.92	0.39	3.58	1.68			2.8-4.3
NHL	M	2.64	0.52	3.38	1.64	0.34	NS	
NHL	F	2.74	0.55	3.69	1.56	0.37	NS	
HD	M	3.07	0.64	4.73	2.36	0.23	NS	
HD	F	2.79	0.677	3.77	1.72	0.632	NS	
Breast Cancer	F	3.09	0.701	4.17	1.7	0.226	NS	
Bladder Cancer	M	2.83	0.47	3.21	2.31	0.919	NS	
ALL	M	2.68	0.6	3.84	2.11	0.691	NS	
ALL	F	2.59	0.77	4.08	1.98	0.449	NS	
AML	F	3.74	0.4	4.69	3.1	0.029	S	
Placenta Cancer	F	2.72	0.44	3.07	2.09	0.594	NS	
Ovary Cancer	F	2.93	0.22	3.1	2.61	0.993	NS	
Colon Cancer	M	3.31	0.339	3.63	2.84	0.154	NS	
Liver Cancer	M	3.05	0.06	3.12	2.99	0.076	NS	

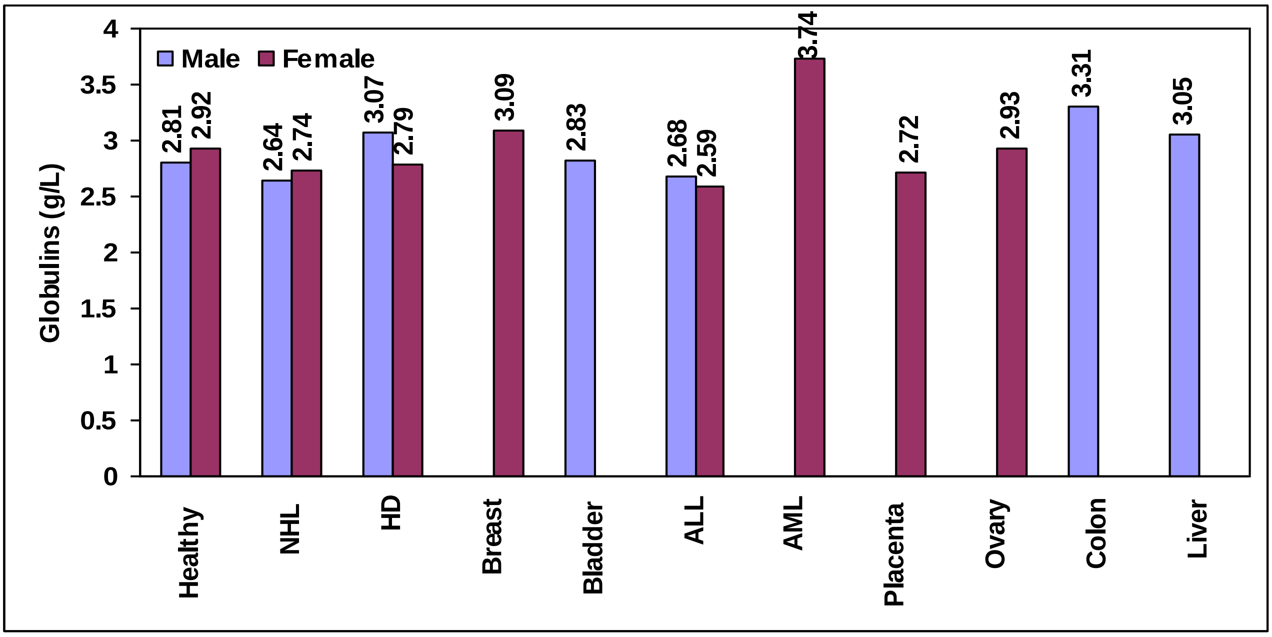


Figure 3-14 Globulins Concentrations in Sera of Patients and Healthy Controls.

Acute phase proteins are responsible for the increase of globulins in serum such as α_1-antitrypsin, α_1-acid glycoprotein, heptoglobin, anti hemophilicglobin, ceruloplamin and macoglobulin.[2,11,200] Al-Sultani has shown a significant increase in globulins in sera of patients with kidney, prostate, bladder and ureter cancer.[200]

3.8 Effect on Albumin/Globulin (A/G) Ratio

Useful diagnostic information can be obtained by determining the albumin/globulin ratio. [5,16]

Compared with healthy controls A/G ratio was found to significantly decrease in most patients with different types of cancers, in the present study. A/G ratio decreased but not significantly in females with placenta cancer. A/G ratio was found to increase in females with ALL, when compared with healthy controls, as shown in Table 3-9 and Figure 3-15.

Table 3-9 Albumin/Globulin Ratio in Sera of Patients and Healthy Controls. (M = males, F = females, S = significant (when P < 0.05), NS =not significant).

Groups	Sex	Alb/Glo	± SD	Upper value	Lower value	P	Sig.	Reference Range
Healthy Control	M	1.78	0.43	2.51	1.23			1.5-2.5
Healthy Control	F	1.59	0.32	2.8	1.19			1.5-2.5
NHL	M	1.39	0.365	2.23	0.87	0.008	S	
NHL	F	1.41	0.11	1.57	1.26	0.024	S	
HD	M	1.37	0.34	1.90	0.81	0.003	S	
HD	F	1.4	0.608	2.84	0.88	0.04	S	
Breast Cancer	F	1.28	0.45	2.29	0.84	0.002	S	
Bladder Cancer	M	1.35	0.32	1.79	1.14	0.01	S	
ALL	M	1.4	0.37	2.06	0.90	0.01	S	
ALL	F	1.7	0.549	2.26	0.85	0.72	NS	
AML	F	1.007	0.22	1.39	0.85	0.009	S	
Placenta Cancer	F	1.46	0.19	1.73	1.29	0.45	NS	
Ovary Cancer	F	1.42	0.055	1.47	1.34	0.041	S	
Colon Cancer	M	0.98	0.17	1.17	0.76	0.005	S	
Liver Cancer	M	0.97	0.028	1.0	0.94	0.000	S	

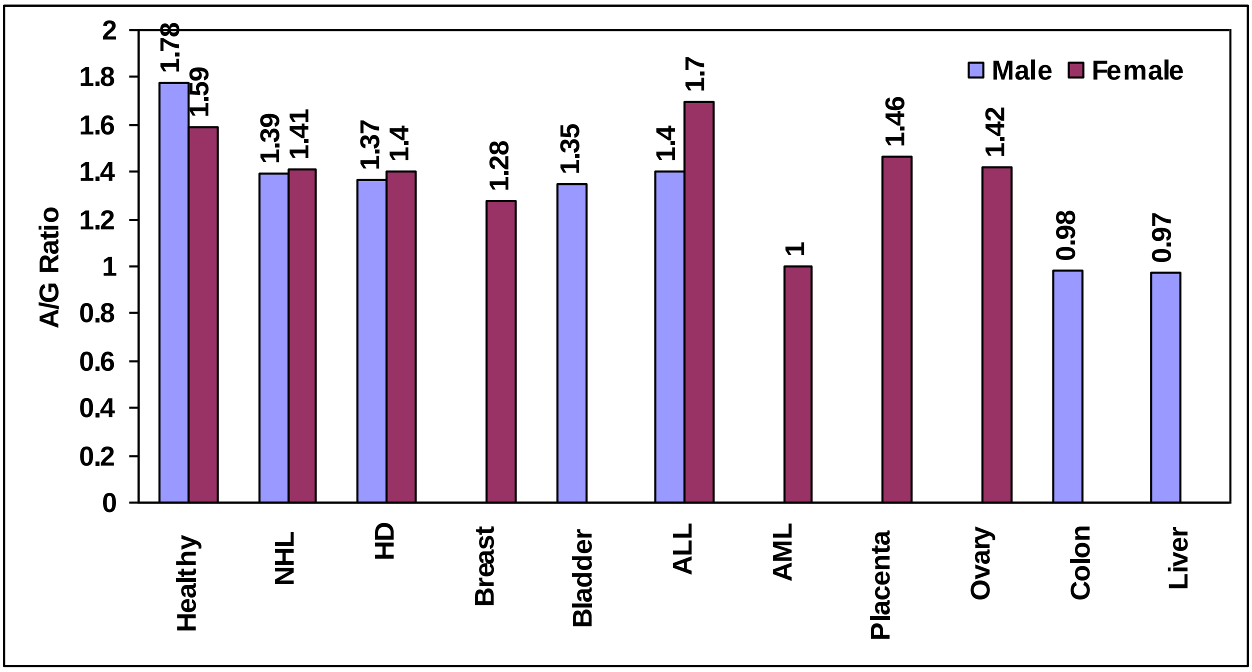


Figure 3-15 Albumin/Globulin Ratio in Sera of Patients and Healthy Controls.

Total protein concentration in serum is around 6.6-8.7 gm/dl in normal subjects[11]. The albumin / globulin ratio is close to 1.5-2.5 .[15] Increase in total serum proteins may occur in dehydration with the ratio remaining unaltered.[13] There are several conditions where increase in total protein is mainly due to the increase of one of the globulins. In many of these conditions, albumin levels remain the same or are reduced slightly such as in patients with multiple myeloma. [184]

A decrease in total proteins is invariably due to a fall in albumin level, which may be accompanied by an increase in globulin concentration. The ratio decreases in these cases.[15,184]

In the present study, albumin concentration was fall, and this event was accompanied by an increase in globulin concentration. Therefore A/G ratio was found to decrease in most patients with different types of cancers.

These results agree with those obtained by Al-Sultani in patients with kidney, prostate, bladder, and ureter cancers, as well as in patients with diabetes millitus.[200]

3.9 Determination of Abnormal Protein by Use PAGE Pattern

The electrophoretogram of serum proteins is helpful in detecting changes in the individual protein fractions and as well as abnormal bonds in certain disease conditions.[184]

As shown in Figures 3-16 and 3-17, sera of patients with different types of cancers and healthy control were assayed by the use of PAGE.

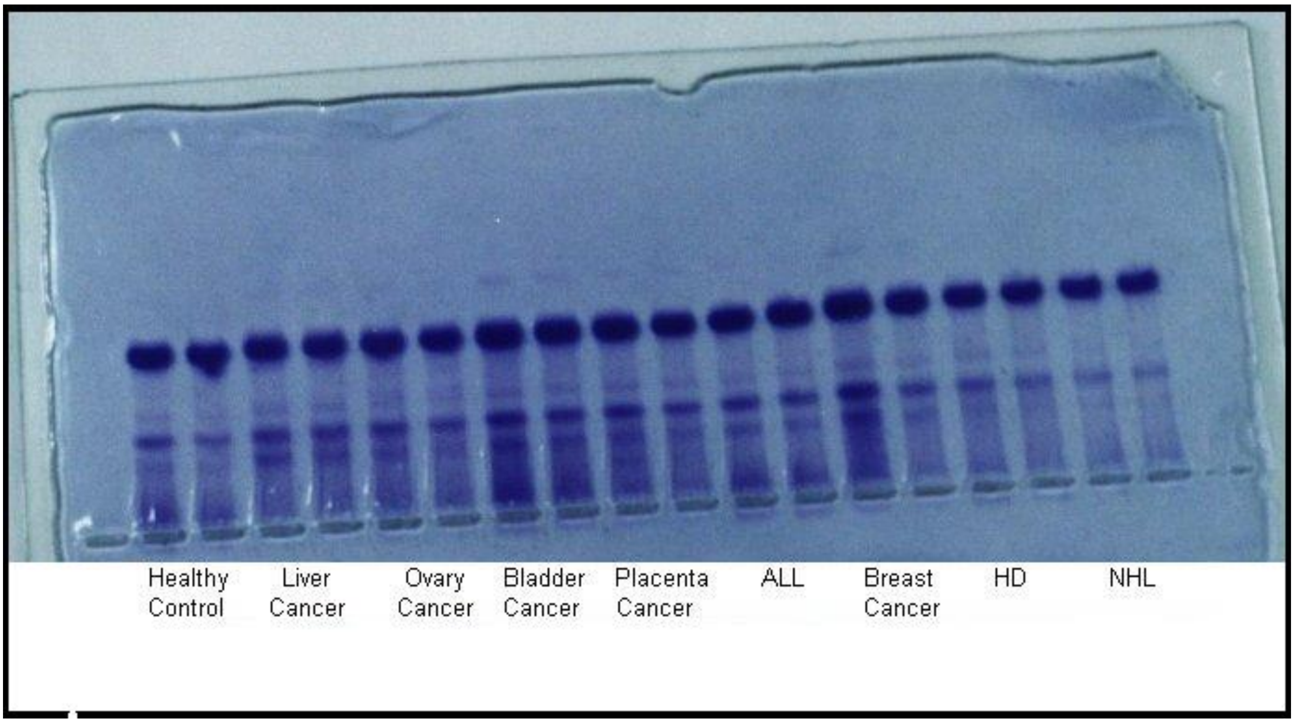


Figure 3-16 Electrophoretogram of Serum Proteins of Patients and Healthy Controls, (First assay).

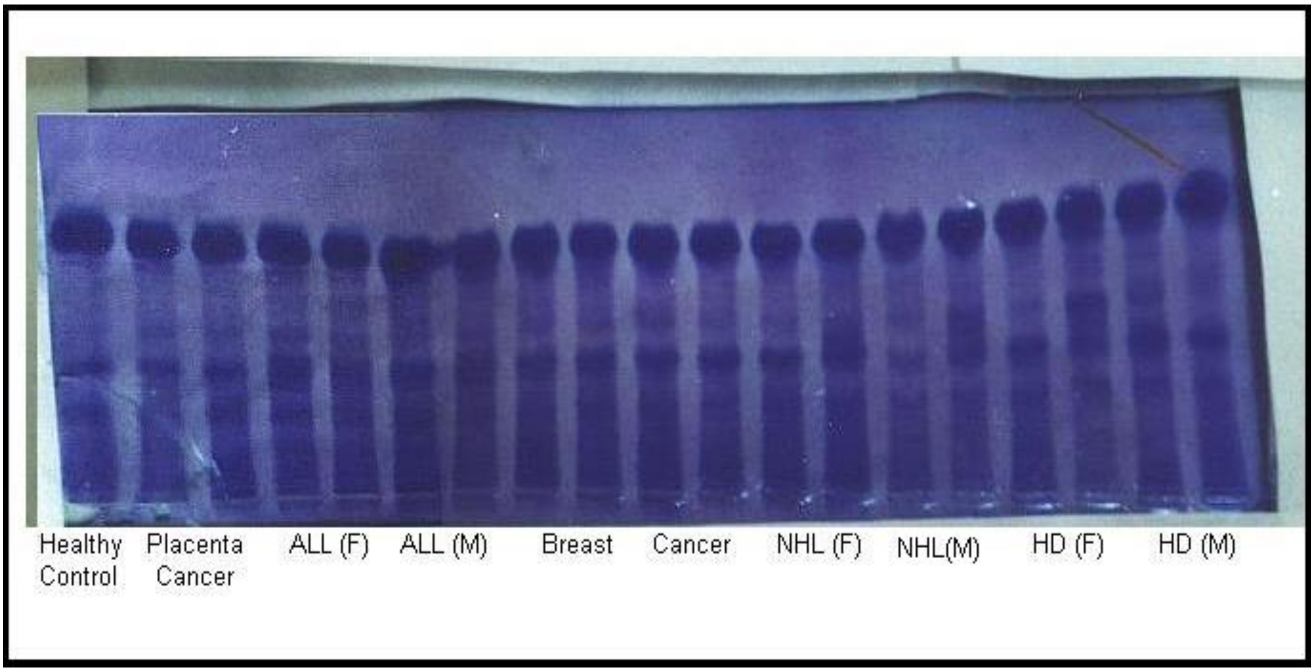


Figure 3-17 Electrophoretogram of Serum Proteins of Patients and Healthy Controls, (Second assay). (M = males, F = females).

The decrease in albumin is not clearly observed, except in these bands of patients with NHL and HD.

α_1 globulins bands in sera of patients with different types of cancer were found to slightly increase when compared with healthy control, due to increase in acute phase proteins including α_1–antitrypsin and α_1–acid glycoprotein. These results agreed with those obtained by Al-Sultani.[200]

Compared with healthy control, α_2-globulin bonds of sera of patients in the present study were found to clearly increase. This result may be due to increase in acute phase proteins which rise in malignancy, including heptoglobin, antihemophilicglobin, ceruloplasmin and macroglobulin.[11,15] Also, this result agrees with the previous studies[200].

β-globulin bonds were found to apparently increase in sera of all types of patients subject to present study, when compared with healthy controls, due to the rise in transferrin or high levels of estrogen, and increase in immune complex. These results are similar to that observed previously.[200]

A visible increase in γ-globulin bonds was observed in sera of patients with different types of cancer in the present study, when compared with these bands of healthy control. Such increase was noted in patients with chronic inflammatory disease and liver disease, or disseminated neoplasms, suggesting a polyclonal γ-globulin increase is associated with immune reaction.[11] Also, this result agrees with the previous studies.[200]

An additional band was observed in sera of patients with ALL between albumin and α_1-globulin bands, which may be due to increase in α_1-acid glycoprotein or α_1-fetoprotein.[11]

3.10 Effect of Oxidative Stress of Trace elements Concentration

3.10.1 Effect on Zinc Concentration

Previous studies estimated serum zinc in a number of diseases and found none in which it increased[13]. In the present study, zinc concentrations in sera of patient with different types of cancer were found to significantly decrease, when compared with healthy controls and slight excess of zinc concentrations in males over females was observed. Table 3-10, Figure 3-18.

Malabsorption, chronic disease of liver and kidney, alcoholism, and some types of animias, leukemia and other blood disease are associated with zinc deficiency.[5,13]

Other studies showed low level of zinc in patients with acute myocardial infarction (AMI) and angina pectoris (AP), and attributed this finding to oxidative stress associated with these diseases.[166]

Zinc concentration was determined in sera of patient with esophagus carcinoma, larynx and breast cancers by using HPLC technique, and they showed significant decrease, when compared with healthy control. This study suggests that zinc deficiency may due to reduced levels of alkaline phosphates [201].

The results of the present study agree with those results obtained by HPLC technique. [201]

In the present study, serum zinc concentrations show slight increase in patients who have high CDA activity than those who have low CDA activity, and this result may be due to the presence of zinc atom in the active site of CDA. [51,62,88,89]

Table 3-10 Zinc Concentrations (ppm) in Sera of Patients and Healthy Controls. (M = males, F = females, S = significant (when P < 0.05), NS =not significant).

Groups	Sex	Zinc (ppm)	± SD	Upper value	Lower value	P	Sig.	Reference Range
Healthy Control	M	0.971	0.147	1.23	0.76			0.7-1.2
Healthy Control	F	0.864	0.105	1.11	0.65			0.7-1.2
NHL	M	0.577	0.112	0.72	0.39	0.000	S	
NHL	F	0.528	0.085	0.67	0.39	0.000	S	
HD	M	0.455	0.109	0.57	0.31	0.000	S	
HD	F	0.50	0.097	0.71	0.38	0.000	S	
Breast Cancer	F	0.554	0.11	0.73	0.32	0.000	S	
Bladder Cancer	M	0.535	0.097	0.63	0.39	0.000	S	
ALL	M	0.553	0.06	0.61	0.47	0.0012	S	
ALL	F	0.43	0.038	0.47	0.38	0.000	S	
AML	F	0.54	0.08	0.62	0.46	0.016	S	
Placenta Cancer	F	0.596	0.033	0.36	0.55	0.000	S	
Ovary Cancer	F	0.623	0.065	0.17	0.55	0.018	S	
Colon Cancer	M	0.56	0.050	0.61	0.94	0.000	S	
Liver Cancer	M	0.61	0.02	0.63	0.59	0.000	S	

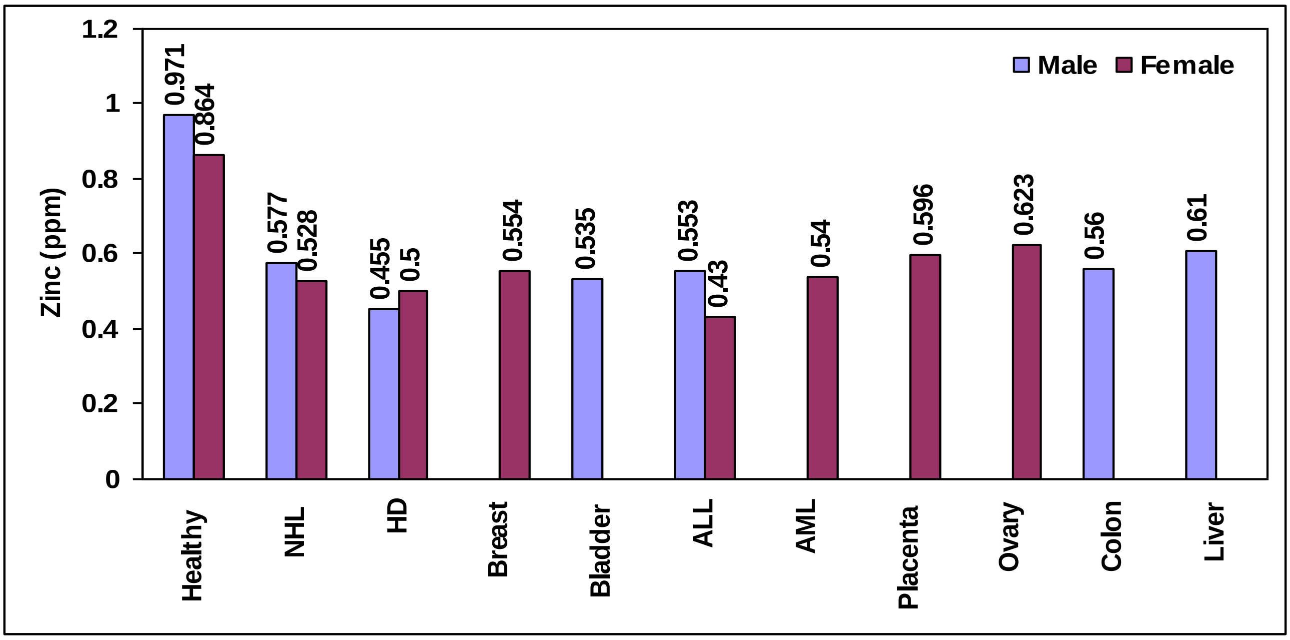


Figure 3-18 Zinc Concentrations in Sera of Patients and Healthy Controls.

The antioxidant properties of zinc have been demonstrated in vitro. Zinc may exert its antioxidant effect by decreasing the susceptibility of essential sulfhydryl groups of proteins to oxidation and by competing with prooxidant metals such as iron and copper for biological binding sites.[202]

The results of the present study suggest that zinc concentration may be considered a biochemical marker of oxidative stress associated with cancer.

3.10.2 Effect on Selenium Concentration

Non-metals such as selenium have strong tendency to form covalent bond with carbon and oxygen and may be incorporated as part of organic matrix of a structural protein or enzyme.[166]

Selenium has many biological functions mediated through an array of selenoproteins. These proteins can influence a range of biochemical systems in the body including those involved in antioxidant mechanisms, thyroid hormone metabolism and redox control.[11]

Selenium concentration in sera of patients with different types of cancer was determined and found to significantly decrease, when compared with healthy controls in the present study. Also, all results showed slight excess of selenium concentration in males over females, and they are still within reference intervals. Table 3-11, Figure 3-19.

Table 3-11 Selenium Concentrations (ppm) in Sera of Patients and Healthy Controls. (M = males, F = females, S = significant (when P < 0.05), NS =not significant).

Groups	Sex	Selenium (ppm)	± SD	Upper value	Lower value	P	Sig.	Reference Range
Healthy Control	M	0.1015	0.019	0.130	0.076			0.046-0.143
Healthy Control	F	0.087	0.018	0.110	0.076			0.046-0.143
NHL	M	0.061	0.008	0.077	0.039	0.000	S	
NHL	F	0.059	0.006	0.072	0.051	0.000	S	
HD	M	0.057	0.057	0.085	0.32	0.000	S	
HD	F	0.055	0.014	0.081	0.040	0.000	S	
Breast Cancer	F	0.056	0.011	0.089	0.039	0.000	S	
Bladder Cancer	M	0.049	0.004	0.055	0.039	0.000	S	
ALL	M	0.046	0.002	0.050	0.043	0.000	S	
ALL	F	0.0406	0.002	0.044	0.039	0.000	S	
AML	F	0.056	0.011	0.067	0.045	0.022	NS	
Placenta Cancer	F	0.065	0.019	0.091	0.045	0.025	S	
Ovary Cancer	F	0.0486	0.017	0.072	0.031	0.000	S	
Colon Cancer	M	0.058	0.002	0.061	0.055	0.000	S	
Liver Cancer	M	0.051	0.008	0.056	0.043	0.012	S	

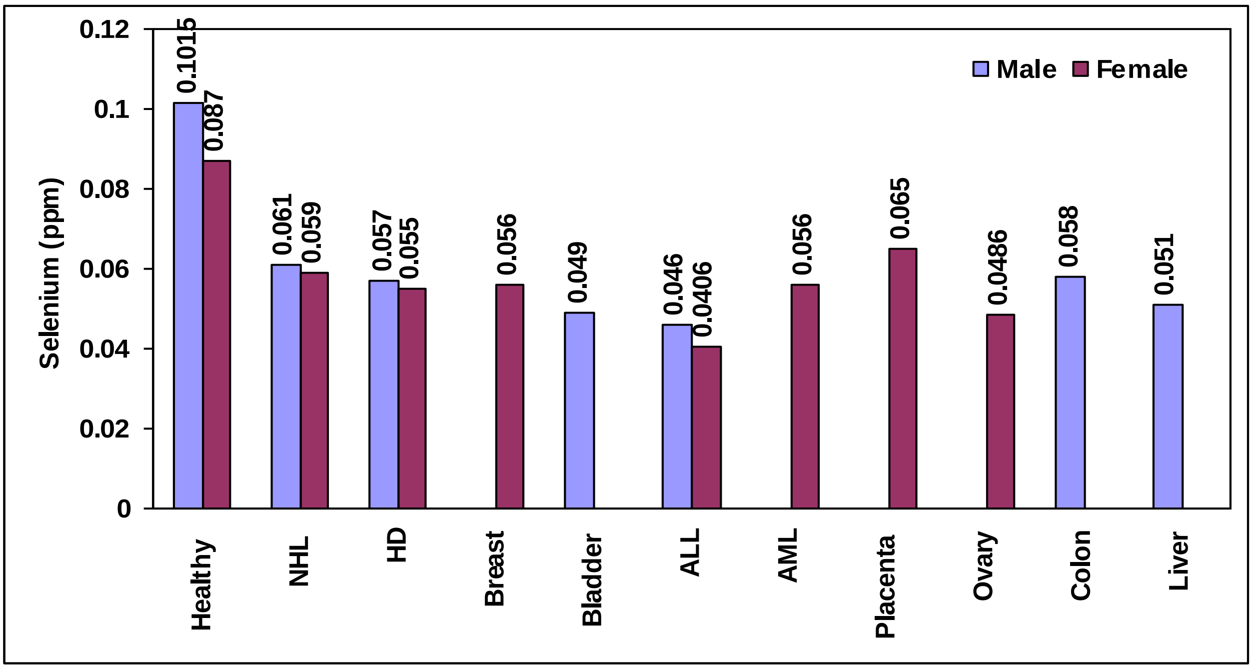


Figure 3-19 Selenium Concentrations in Sera of Patients and Healthy Controls.

Epidemiological studies have related low selenium status and intake to increased incidences of cancer and cardiomyopathies.[11]Chronic selenosis, or selenium toxicity, is characterized by loss of hair and nails, skin lesions, tooth decay, and nervous system abnormalities.[11]

Selenium is an essential element for humans, it is a constituent of GSH peroxidase; the key enzyme in the defense against oxidative stress. [5] Several cases of low plasma or blood selenium concentration and low GSH peroxidase activity were reported, and suggested that GSH peroxidase might reflect selenium status.[5]

Other studies showed low selenium concentration in sera of patients with AMI and AP, and attributed their finding to the acting of selenium as antioxidant by binding with vitamin E and as a constituent of GSH peroxidase to scavenge free radical which acting to detoxify tissue peroxidation.[166]

The results of the present study show slight increase in selenium concentrations in patients who have relatively high levels of GSH.

3.10.3 Effect on Copper Concentration

An important role of copper is as a component of enzymes or proteins involved in redox reactions, such as ceruloplasmin, SOD contain Zn and Cu, dopamin-β-hydroxylase, ascorbate oxidase, lysyl oxidase, tyrosinase, and cytochrome c oxidase.[5,188]

Compared with the healthy controls, copper concentration in sera of patients with different types of cancer was found to significantly increase, except those of males with colon cancer in which copper concentration increased but not significantly, in the present study. Table 3-12, Figure 3-20.

Table 3-12 Copper Concentrations (ppm) in Sera of Patients and Healthy Controls. (M = males, F = females, S = significant (when P < 0.05), NS =not significant).

Groups	Sex	Copper (ppm)	± SD	Upper value	Lower value	P	Sig.	Reference Range
Healthy Control	M	1.24	0.367	1.97	0.88			0.7-1.40
Healthy Control	F	1.342	0.179	1.66	1.01			0.8-1.55
NHL	M	1.585	0.275	1.92	1.12	0.003	S	
NHL	F	1.63	0.14	1.83	1.39	0.003	S	
HD	M	1.577	0.245	1.84	1.02	0.002	S	
HD	F	1.491	0.202	1.92	1.23	0.011	S	
Breast Cancer	F	1.53	0.202	1.92	1.21	0.000	S	
Bladder Cancer	M	1.416	0.268	1.82	1.02	0.02	S	
ALL	M	1.583	0.116	1.68	1.42	0.021	S	
ALL	F	1.686	0.028	1.72	1.65	0.000	S	
AML	F	1.77	0.05	1.82	1.72	0.01	S	
Placenta Cancer	F	1.423	0.267	1.80	1.21	0.016	S	
Ovary Cancer	F	1.493	0.32	1.93	1.17	0.05	S	
Colon Cancer	M	1.383	0.089	1.51	1.32	0.176	NS	
Liver Cancer	M	1.64	0.02	1.66	1.62	0.000	S	

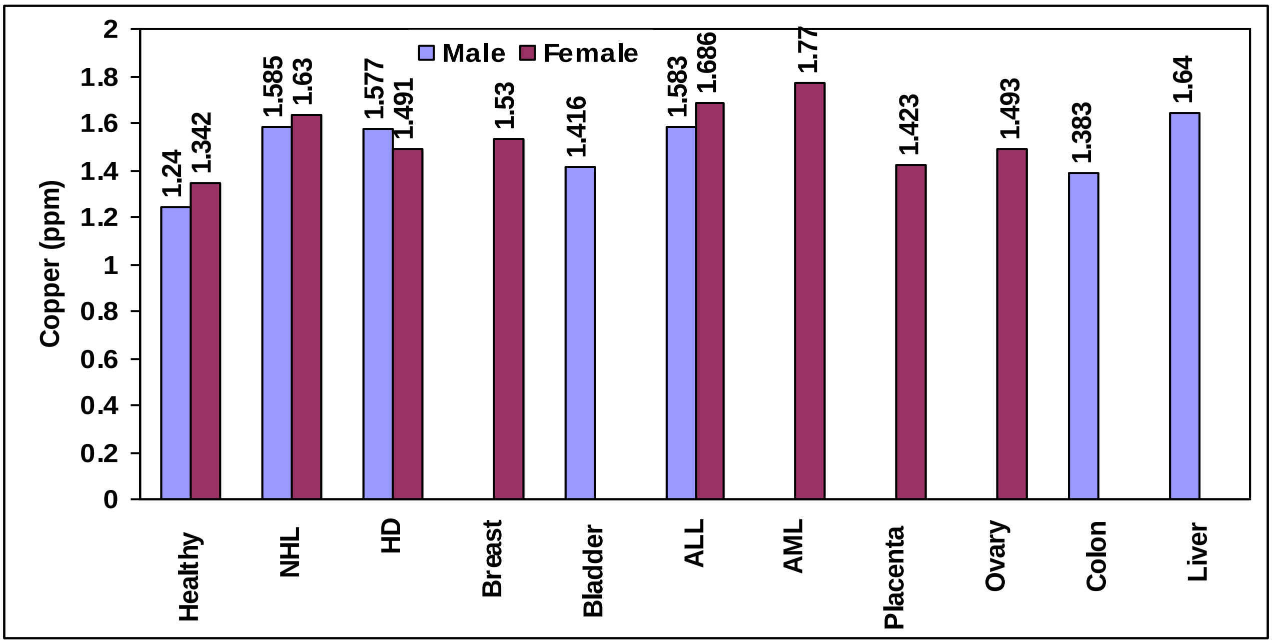


Figure 3-20 Copper Concentrations in Sera of Patients and Healthy Controls.

Previous studies indicate that Cu^{2+} may be specific markers for oxidative DNA damage. DNA-associated copper ions in cell might also react with phenolic compounds to produce ROS and electrophilic phenolic intermediates. This interaction could cause a range of DNA lesions including base modification, strand breaks and phenol adducts to the DNA bases, all of which might contribute to the carcinogenicity of certain phenolic compounds.[146]

Several studies have reported high levels of copper in patients with AMI and AP.[166,203] These studies attributed the redox activity of copper to its electronic configuration of d orbital which helped it to scavenge the free radical as in Cu-Zn-SOD reaction.[145,166] On the other hand, copper has an oxidant property, by generating free radical, such as $OH^{\cdot}$, $O_2^{\cdot -}$ in Fenton and Harber-Weiss reactions.[154,160]

Other studies included copper concentration analysis by using HPLC technique in sera of patients with esophagus carcinoma, larynx and breast cancers, where high levels were shown when compared with healthy controls. [201]

3.10.4 Effect on Iron Concentration

Iron can oxidize many biomolecules, and it can also generate ROS or other oxidants that can damage these molecules. The correlation between iron and oxidative stress is well established previousely.[157-160,203]

In the present study, iron concentrations in sera of patients with different types of cancer were determined, and the results showed wide variety, when compared with healthy controls. Iron concentration in sera of females with ovary cancer and males with liver cancer was found significantly increase, and increase but not significantly in sera of females with NHL and males with bladder cancer whereas iron concentration was found to significantly decrease in sera of females with HD, ALL, and placenta cancer, and decrease but not significantly in sera of males with HD, females with breast cancer, males with ALL, females with AML and males with colon cancer. Table 3-13, Figure 3-21.

This result may reflect the difference in the iron binding capacity, or reflect the effect of age, where serum iron was found to rise with age.[166]

Previous studies showed low levels of serum iron in patients with AMI and AP, whereas Al-Zamely found iron concentration to increase, in a similar study. [204]

Other study was found iron concentration to significantly decrease in sera of patients with esophagus carcinoma, larynx, and breast cancer using HPLC technique.[201]

Table 3-13 Iron Concentrations (ppm) in Sera of Patients and Healthy Controls. (M = males, F = females, S = significant (when $P < 0.05$), NS =not significant).

Groups	Sex	Iron (ppm)	± SD	Upper value	Lower value	P	Sig.	Reference Range
Healthy Control	M	0.856	0.077	1.22	0.75			0.65-1.75
Healthy Control	F	0.887	0.095	1.3	0.71			0.5-1.70
NHL	M	0.853	0.087	0.95	0.65	0.932	NS	
NHL	F	0.91	0.33	1.81	0.63	0.857	NS	
HD	M	0.839	0.201	1.39	0.52	0.779	NS	
HD	F	0.811	0.055	0.93	0.75	0.016	S	
Breast Cancer	F	0.84	0.134	1.25	0.63	0.133	NS	
Bladder Cancer	M	0.888	0.051	0.93	0.77	0.213	NS	
ALL	M	0.693	0.075	0.80	0.63	0.100	NS	
ALL	F	0.813	0.028	0.85	0.78	0.035	S	
AML	F	0.75	0.10	0.85	0.65	0.04	S	
Placenta Cancer	F	0.793	0.012	0.81	0.78	0.0003	S	
Ovary Cancer	F	0.93	0.063	1.02	0.88	0.04	S	
Colon Cancer	M	0.746	0.11	0.83	0.95	0.12	NS	
Liver Cancer	M	0.975	0.045	1.02	0.93	0.024	S	

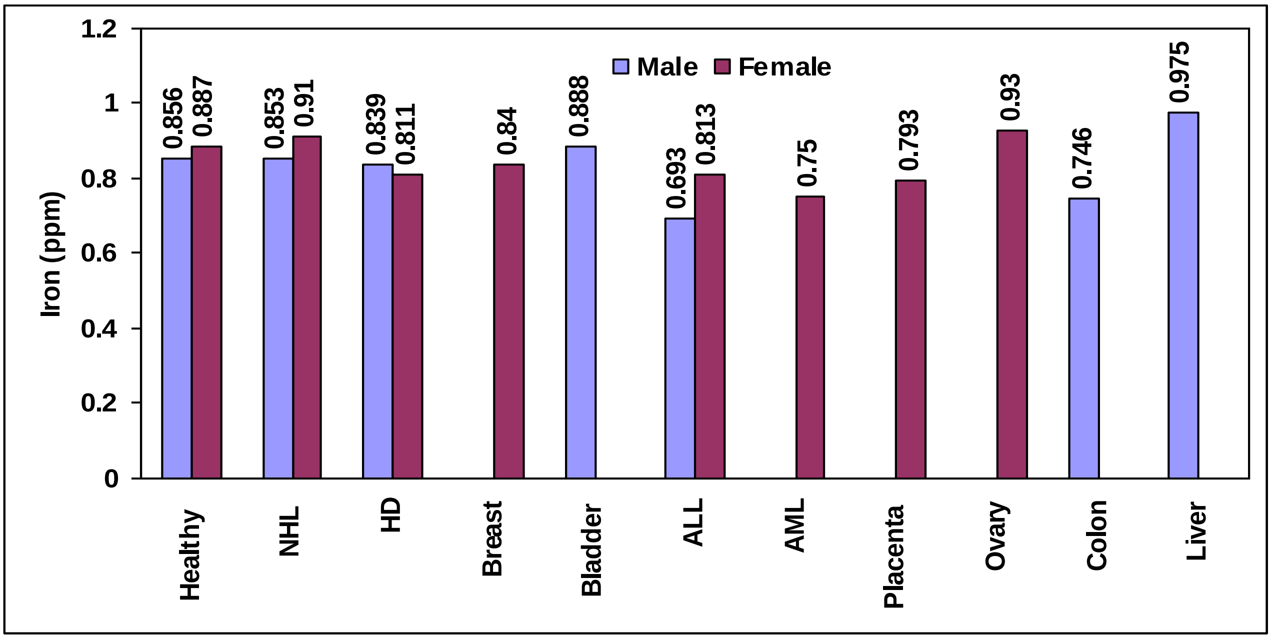


Figure 3-21 Iron Concentrations in Sera of Patients and Healthy Controls.

Conclusions

1- The optimum conditions used to determine serum CTD activity are 400µL of (0.002 M) cytidine as a substrate, 500 µL of (0.05 M Tris-HCL buffer, pH=7.5) and, 100 µL of serum which contain the enzyme, in a final volume of 1 ml, incubated for 5 minutes at 25 ^oC.

2- CTD activity significantly increased in sera of patients with NHL, breast, placenta and, liver cancer.

3- CTD activity decreased in sera of patients with HD, male with bladder cancer, female with ALL, and male with colon cancer.

4- Vitamin C (total and reduced) and glutathione (GSH) significantly decreased in sera of patients with different types of cancer, as a response to oxidative stress.

5- Total protein was found to decreased in sera of patients when compared with healthy controls due to decrease in albumin. Globulin was elevated but not sufficient to substitute the depletion in albumin. Also A/G ratio decreased more than that of healthy controls.

6- Significant decrease in serum zinc and selenium concentrations was observed in patients when compared with healthy controls due to its involvement in the antioxidation process.

7- Serum zinc concentrations showed slight increase in patients with higher CDA activity than those with low CDA activity, and this result may be due to the presence of zinc atom in the active site of CDA.

8- Serum copper concentrations were elevated in sera of patients when compared with healthy individuals.

9- Iron concentrations were fluctuated between increase and decrease in patients with different types of cancer.

10 -α_1 globulins, α_2-globulins, β-globulins and γ-globulins bonds were found to apparently increase in sera of patients, when compared with healthy controls on the electrophoretogram of PAGE.

Recommendations

1- Isolation and purification of CTD from sera of healthy individuals and patients with different types of cancer; to differentiate among them, and to study its isoenzymes.

2- Study the activity of CTD in other types of diseases such as diabetes milletus and cardiovascular diseases.

3- CTD activity may be considered a novel diagnostic tool to differentiate between HD and NHL. It may be an aid to the diagnosis of breast, placenta and, liver cancers.

AIM OF THE STUDY

1. Investigate the relationship between CTD activities and cancer, as a tumor marker, in several types of cancer.
2. Investigate the relationship between CTD activities and oxidative stress.
3. Determination the oxidative stress associated with cancer, by measuring the concentration of substances that act as antioxidant such as vitamin C, glutathione, and albumin.
4. Measurement the trace elements concentrations as antioxidants such as zinc selenium, copper, and iron.
5. Investigate the damaged protein may exist in sera of patients with different type of cancer, as a response to oxidative stress, using polyacrylamide gel electrophoreses.

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